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Vasodilator and Vasoconstrictor Substances Produced by the Endothelium

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Contents

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1 Introduction

Up to the late twentieth century, the vascular endothelium was primarily of anatomic interest. The histological study of aortic endothelium by Langhans in 1866 laid the groundwork for subsequent research such as O'Neil's (1947) anatomic studies of vascular pathology mediated by altered blood flow. Ultimately, the study of the vascular response to acetylcholine was to play the essential role in delineating the mechanism underlying the endothelial control of vascular tone (Furchgott and Zawadzki 1980), a mechanism which is so important as to be expressed early in vertebrate phylogeny (Miller and Vanhoutte 1986).

Henry Dale (1914) originally described in intact animals the vasodilator properties of choline esters, in particular, acetylcholine. Despite the fact that his observations have been repeated in vivo many times, in different species including the human, isolated blood vessels usually do not relax, but rather contract when exposed to acetylcholine (e.g., Furchgott 1955). However, in rings of isolated arteries, acetylcholine can induce relaxation (Jelliffe 1962; Vanhoutte 1976; DeMey and Vanhoutte 1980). When studying the receptor-mediated release of compounds from cultured endothelial cells, Buonassisi and Venter (1978) suggested that endothelial cells could release factors which could locally and systemically modulate vascular tone. Studies of the effect of acetylcholine in the canine cerebral artery suggested that acetylcholine might release a vasodilator polypeptide (Toda 1979). It was the classic experiments of Furchgott and Zawadzki (1980) that firmly established the essential role of the endothelium as a mediator

of vascular relaxation, when these authors made the transition from the in vitro study of vascular smooth muscle strips to rings. In preparing vascular rings, the intima of the blood vessel segment is less susceptible to mechanical injury. Hence, acetylcholine was found to induce relaxation in intact contracted aortic rings, while the vascular strips exhibited little change in tension (Fig. 1). This pioneering work, defining the obligatory role of the endothelium in the vascular relaxation evoked by acetylcholine, also determined that a diffusible substance is involved (Furchgott and Zawadzki 1980). The term endothelium-derived relaxing factor (EDRF) was coined a few years later (Cherry et al. 1982). Over the years that followed, it became obvious that many endogenous mediators could trigger the release of EDRF (Fig. 2).

It soon also became apparent that the venous endothelium, when exposed to arachidonic acid, could also induce contractions of the underlying

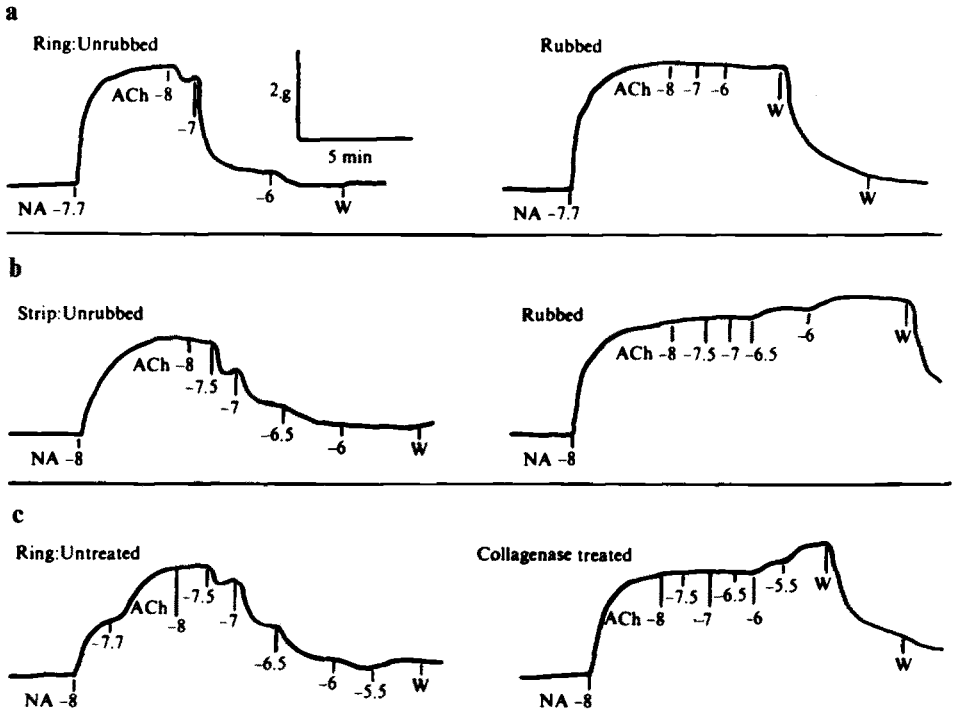


Fig. 1a–c. Loss of relaxation of the rabbit aorta to acetylcholine (*ACh*) after rubbing of the intimal surface or collagenase treatment to remove the endothelial cells. The recordings show the point at which each drug addition was made and the corresponding cumulative concentration of the drug (in log Molar) in the organ chamber. *NA*, norepinephrine; *w*, wash out of the chamber. Recordings were obtained from the same ring before and after rubbing of the intimal surface. (Modified from Furchgott and Zawadzki 1980)

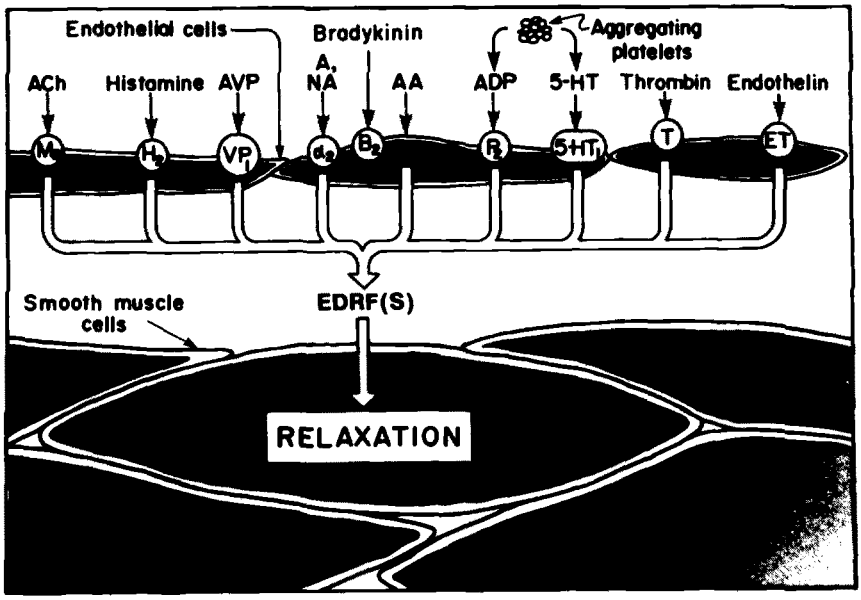


Fig. 2. Neurohumoral mediators which cause the release of endothelium-derived relaxing factor (*EDRF*) through activation of specific endothelial receptors (*circles*). In addition, EDRF can be released independently of receptor-operated mechanisms by the calcium ionophore A23187 (not shown). *A*, adrenaline (epinephrine); *AA*, arachidonic acid; *ACh*, acetylcholine; *ADP*, adenosine diphosphate; α, alpha-adrenergic receptor; *AVP*, arginine vasopressin; *B*, kinin receptor; *ET*, endothelin receptor; *H*, histaminergic receptor; *5-HT*, serotonin (5-hydroxytryptamine), serotonergic receptor; *M*, muscarinic receptor; *NA*, noradrenaline (norepinephrine); *P*, purinergic receptor; *T*, thrombin receptor; *VP*, vasopressinergic receptor. (Modified from Lüscher and Vanhoutte 1990)

vascular smooth muscle (Fig. 3) (DeMey and Vanhoutte 1982). Again, it became apparent that endothelium-dependent contractions can be evoked in a variety of blood vessels with a number of different stimuli (Fig. 4). This review, which by no means pretends to be exhaustive, will focus on the nature of the diverse vasoactive factors released by endothelial cells and their possible role in physiological hemodynamic responses. For their potential role in pathological situations and in pharmacological responses, the reader is referred to more specialized descriptions (e.g., Lüscher and Vanhoutte 1990).

Fig. 4. A number of physiochemical stimuli, neurohumoral mediators, and the calcium ionophore A23187 can evoke endothelium-dependent contractions in certain blood vessels, presumably because they evoke the release of endothelium-derived contracting factor(s) (*EDCF*). *AA*, arachidonic acid; *ACh*, acetylcholine; *ADP*, adenosine diphosphate; *5-HT*, serotonin (5-hydroxytryptamine), serotonergic receptor; *M*, muscarinic receptor; *P*, purinergic receptor. (From Lüscher and Vanhoutte 1990)

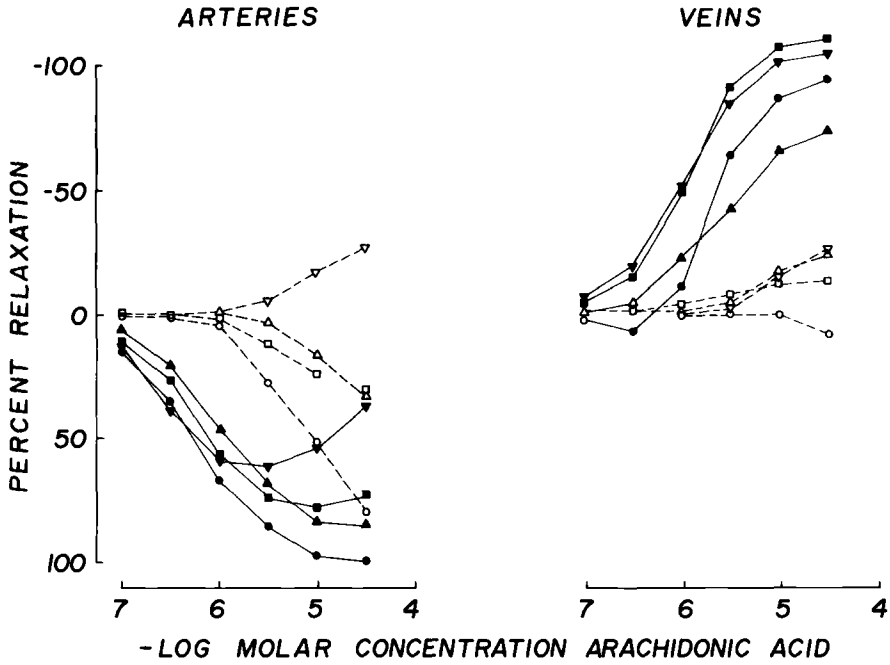
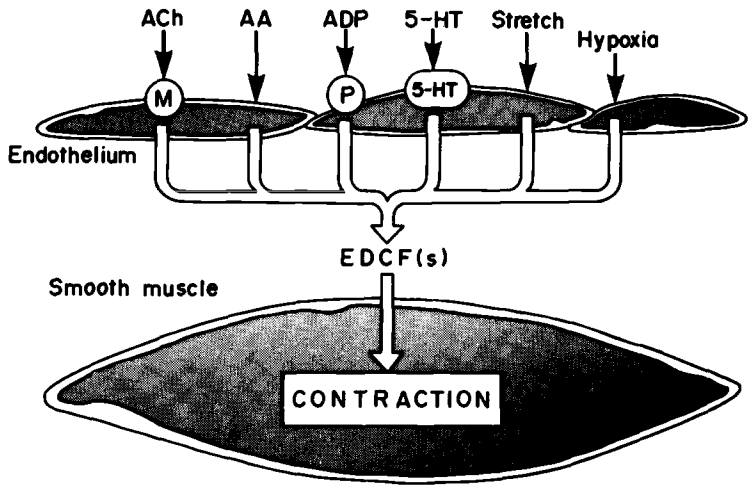


Fig. 3. Effect of increasing concentrations of arachidonic acid on the contractile responses of femoral (*square*), pulmonary (*circles*), saphenous (*triangles*), and splenic (*inverted triangles*) arteries (*left*) and veins (*right*) to the same dose of norepinephrine. Rings with (*solid symbols*) and without (*open symbols*) endothelium were studied in parallel. Note that in the arteries arachidonic acid causes endothelium-dependent relaxations, but endothelium-dependent contractions in the veins. (From De Mey and Vanhoutte 1982)



2 Endothelium-Dependent Relaxations

2.1 Characteristics of EDRF

Endothelium-derived relaxing factor is not a prostanoid as blockers of cyclooxygenase do not modify endothelium-dependent relaxation (Furchgott and Zawadzki 1980). Endothelium-dependent relaxation is not specific for particular contractile agonists, but can be elicited during stimulation with norepinephrine, histamine, serotonin, angiotensin II, prostaglandin $F_{2\alpha}$, potassium ions (Furchgott and Zawadzski 1980), or endothelin (Vanhoutte et al. 1989; V.M. Miller et al. 1989). While the acetylcholine-mediated relaxation is attenuated in high-potassium solutions, endothelium-dependent relaxations still occur, indicating that EDRF can induce vascular relaxation independent of membrane potential changes in the vascular smooth muscle (DeMey and Vanhoutte 1980; Furchgott and Zawadzki 1980).

2.1.1 Diffusibility

Endothelium-derived relaxing factor is a diffusible substance that does not require contact between the endothelium and vascular smooth muscle. Furchgott and Zawadzki (1980) and Furchgott (1981) demonstrated this using what they termed a "sandwich preparation" in which stimulation of an arterial segment with endothelium relaxed a contiguous arterial segment without endothelium. Indeed, the diffusibility of EDRF was confirmed when other investigators perfused vessels with endothelium and induced relaxation of vascular smooth muscle superfused with the effluent (Fostermann et al. 1984; Griffith et al. 1984; Rubanyi et al. 1985a). Cocks and associates were able to seed endothelial cells onto a donor ring without endothelium and restore endothelium-dependent relaxation (Cocks and Angus 1985). They were able also to culture endothelial cells or microcarrier beads and then install them into a perfusion circuit (Cocks et al. 1985), which enabled a large amount of EDRF to be produced for chemical manipulation and characterization. These different studies all concluded that the half-life of EDRF was short (50–60 s, depending upon the experimental conditions). They also demonstrated that the release of EDRF occurred under basal conditions, but that it could be augmented greatly by a variety of agonists, in particular acetylcholine and bradykinin (see Furchgott 1983, 1984; Furchgott and Vanhoutte 1989; Lüscher and Vanhoutte 1990; Bassenge and Heusch 1990).

2.1.2 Nitric Oxide and EDRF

2.1.2.1 Initial Similarities. Shortly before Furchgott and Zawadzki (1980) found that the endothelium could mediate relaxation of the vascular smooth

muscle, Ignarro and coworkers, in their continued study of nitrovasodilator action, discovered that nitric oxide could relax vascular smooth muscle through stimulation of soluble guanylate cyclase (Gruetter et al. 1979). Determination of the chemical properties of EDRF were important in that they set the stage for the hypothesis that the active component of EDRF was nitric oxide (Furchgott 1988; Ignarro et al. 1988).

Initially it was found that endothelium-dependent relaxation was associated with a concomitant rise in cyclic GMP in vascular smooth muscle (Holzmann 1982; Rapoport and Murad 1983; Furchgott 1984; Ignarro and Kadowitz 1985; Murad et al. 1987; Mulsch et al. 1987) and could be inhibited by methylene blue, an inhibitor of soluble guanylate cyclase (Gruetter et al. 1981). This discovery was important because it suggested that EDRF can be regarded as an endogenous nitrate. Nitrates and nitrate esters which induce vascular relaxation do so via methylene blue-sensitive activation of soluble guanylate cyclase in the vascular smooth muscle (Murad et al. 1979; Kukovetz et al. 1981; Gruetter et al. 1979, 1981; Ignarro et al. 1984a). Indeed, certain nitrovasodilators generate nitric oxide enzymatically or decompose to liberate the radical (Murad et al. 1978; Ignarro et al. 1981; Feelisch and Noack 1987). As authentic nitric oxide stimulates soluble guanylate cyclase in vascular smooth muscle (Murad et al. 1979; Craven and DeRubertis 1978; Gruetter et al. 1979, 1981), it can account for the relaxant properties of the nitrovasodilators.

2.1.2.2 Susceptibility to Free Radicals. It was also determined that EDRF could be destroyed by superoxide anions and protected by scavengers of oxygen-derived free radicals such as superoxide dismutase (Rubanyi and Vanhoutte 1986; Gryglewski et al. 1986b). In vivo, free radicals cause profound inhibition of endothelium-dependent dilatations of coronary resistance vessels (Stewart et al. 1988). Such sensitivity to free radicals not only supported the concept that EDRF itself was a radical, but also called for caution when extrapolating results from studies involving nonselective pharmacological inhibitors. Indeed, EDRF has been hypothesized to be a product of a cytochrome P-450-dependent enzyme (Pinto et al. 1986) or a product of lipooxygenase (Singer and Peach 1983), based upon experiments utilizing inhibitors of those metabolic pathways. However, it was subsequently determined that compounds such as phenidone, 3-amino-1[*m*-(trifluoromethyl)phenyl]-2-pyrazoline (BW 755C), dithiothreitol, hydroquinone, and pyrogallol inhibit endothelium-dependent relaxation by nonspecific generation of superoxide anions which scavenge EDRF (Moncada et al. 1986).

2.1.2.3 Interactions with Hemoglobin. Martin et al. (1985a,b) discovered that endothelium-dependent relaxation could be inhibited by hemoglobin.

Prior to their discovery, Savitzky et al. (1978) reported that infusion of hemoglobin into healthy subjects caused an increase in arterial blood pressure. The inhibitory action of hemoglobin on endothelium-dependent relaxation was initially attributed to three possible mechanisms of action: (a) auto-oxidation of hemoglobin which generated superoxide anion that scavenged EDRF (Misra and Fridovich 1972) – however, superoxide dismutase did not protect against hemoglobin-mediated inactivation (Hutchinson et al. 1987); (b) direct inhibition of soluble guanylate cyclase in vascular smooth muscle (Murad et al. 1978); or (c) direct binding of EDRF to hemoglobin (Martin et al. 1986b). The latter interpretation appears to be the correct one since EDRF can activate extracted guanylate cyclase in the presence of hemoglobin, but not intracellular guanylate cyclase (Forstermann et al. 1986; Ignarro et al. 1986). This inferred that the hemoglobin-bound EDRF still had the potential to activate guanylate cyclase, but its action on intracellular guanylate cyclase was prevented by the inability of the large hemoglobin molecule from gaining entrance into the cell. This finding was supported further by the observation that hemoglobin-agarose columns bind EDRF (Martin et al. 1986b). The discovery of hemoglobin–EDRF interaction is particularly important in light of the fact that it has been known for over 100 years that nitric oxide binds to the iron contained in the heme moiety of hemoglobin (see Gibson and Roughton 1957; Ignarro 1989).

2.1.2.4 EDRF Is Nitric Oxide. At the fourth symposium on the "Mechanisms of Vasodilatation" held in Rochester, Minnesota, in 1986, the similarities in the chemical properties and biological responses of EDRF and nitric oxide lead Furchgott (1988) and Ignarro et al. (1988) to, independently, propose that EDRF may be nothing else but nitric oxide (Table 1). One year later, Ignarro, Moncada, and their coworkers reported that the biologically active component of EDRF is indeed nitric oxide (Fig. 5) (Ignarro et al. 1987a,b; Palmer et al. 1987; Radomski et al. 1987a).

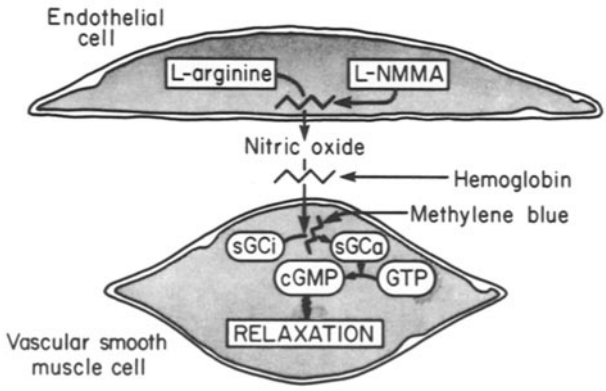


Table 1. Similarities between EDRF and nitric oxide

	NO	EDRF
Induces vasodilatation of arterial and venous smooth muscle	⊕	⊕
Induces heme-dependent activation of soluble guanylate cyclase	⊕	⊕
Increase cyclic GMP in vascular smooth muscle	⊕	⊕
Action on vascular smooth muscle inhibited by methylene blue	⊕	⊕
Biological effect reversed by hemoglobin	⊕	⊕
Binds to hemoglobin-agarose columns	⊕	⊕
Has high affinity for heme iron in hemoglobin, met-hemoglobin, and soluble guanylate cyclase	⊕	⊕
Increases cyclic GMP in platelets	⊕	⊕
Inhibits platelet aggregation	⊕	⊕
Induces platelet disaggregation	⊕	⊕
Inhibits platelet adhesion	⊕	⊕
Diffusible through biological membranes	⊕	⊕
Unstable with a T 1/2 of 3-5 s in physiological salt solution	⊕	⊕
Stabilized by superoxide dismutase or acidic pH	⊕	⊕
Spontaneous inactivation by O_2 or O_2^-	⊕	⊕
Released by endothelial cells	⊕	⊕

2.2 Excitation-Secretion Coupling Calcium and Endothelium-Dependent Relaxations

The vital role of calcium in endothelium-dependent relaxation was suggested first by the demonstration that the calcium ionophore A23187 induces the release of EDRF in isolated blood vessels (Furchgott and Zawadzki 1980; Furchgott 1983) from perfused vascular segments (Griffith et al. 1984) and from endothelial cells in culture (Cocks et al. 1985).

2.2.1 Extracellular Calcium

The importance of extracellular calcium was highlighted by the finding that removal of calcium from the perfusion medium impairs endothelium-dependent relaxations in isolated blood vessels (Singer and Peach 1982) and in perfused vascular segments (Long and Stone 1985; Griffith et al. 1986). This effect was due to an impaired production of EDRF, as restoration of

Fig. 5. Production of endothelium-derived nitric oxide from L-arginine and the inhibitory effect of L-N^G-monomethyl arginine (*L-NMMA*). The nitric oxide derived from L-arginine activities the soluble guanylate cyclase (*sGC*) in smooth muscle leading to accumulation of cyclic guanosine monophosphate (*cGMP*). Hemoglobin inactivates nitric oxide, while methylene blue prevents the activation of soluble guanylyl cyclase. *B*, kinin receptor; *i*, inactive; *a*, active; *GTP*, guanosine triphosphate. (From Vanhoutte 1991)

calcium to the effluent from the perfused arteries with endothelium failed to restore relaxation of the bioassay tissue without endothelium (Long and Stone 1985; Griffith et al. 1986).

In biochemical experiments, the importance of calcium in the production of EDRF can be estimated by measuring the production of cyclic GMP in blood vessels with endothelium in the presence or absence of calcium in the bathing medium. EDRF factor stimulates soluble guanylate cyclase in vascular smooth muscle at a steady-state level (basal release) and following receptor-mediated stimulation of EDRF production (stimulated release) (Holzmann 1982; Rapoport and Murad 1983; Furchgott 1984; Angus and Cocks 1989; Lüscher and Vanhoutte 1990). However, following the removal of calcium in the perfusion medium, both the basal release and stimulated release of EDRF was inhibited (as evidenced by low cyclic GMP levels), while the production of cyclic GMP in response to nitrovasodilators was unaffected (Holzmann 1982; Rapoport and Murad 1983; R.C. Miller et al. 1985). These studies highlight the fact that, as in many other systems, an increase in intracellular Ca^{2+} concentration plays an important role in the activation-secretion coupling in endothelial cells.

2.2.2 Role of Intracellular Calcium Stores

Both extracellular and intracellular Ca^{2+} participate in the release of EDRF. For example, endothelium-dependent relaxations are impaired in the absence of external Ca^{2+}, but the release of EDRF can still be stimulated in an attenuated, albeit transient manner (Peach et al. 1987). Part of the initial increase of intracellular Ca^{2+} in endothelial cells in response to bradykinin and adenosine triphosphate are preserved even in the absence of external Ca^{2+} (Colden-Stanfield et al. 1987; Morgan-Boyde et al. 1987; Luckhoff and Busse 1986). However, repeated challenges with the agonist are followed by decreasing responses. This suggests that the increase in Ca^{2+} secondary to calcium release from intracellular stores into the endothelial cell cytosol is not recycled, but requires extracellular Ca^{2+} for refilling. This hypothesis is consistent with the finding that bradykinin-stimulated $^{45}Ca^{2+}$ efflux from $^{45}Ca^{2+}$-loaded endothelial cells into calcium-free medium occurs only once (Freay et al. 1989). To judge from studies with fluorescent calcium indicator dyes such as Quin-2, Fura-2, and Indo-1, endothelium-dependent vasodilators induce a bi-phasic increase in cytosolic Ca^{2+} in the endothelial cell; a rapidly rising peak insensitive to external Ca^{2+} or the membrane potential, and a lower-level "plateau phase" which is dependent on external Ca^{2+} and membrane potential (Schilling et al. 1988; Hallam and Pearson 1986; Bregestovski et al. 1988; Luckhoff and Busse 1986). The plateau can be abolished by removal of extracellular Ca^{2+} in the

bathing medium (Peach et al. 1987; Hallam and Pearson 1986), endothelial cell depolarization, and by extracellular La^{3+} or Ni^{2+} (Colden-Stanfield et al. 1987; Hallam et al. 1988). Thus, apparently the initial increase in intracellular Ca^{2+} which accompanies endothelium-dependent relaxations is due to release from intracellular stores, while the sustained release depends upon external Ca^{2+}.

2.2.3 Calcium Entry in Endothelial Cells

While the importance of extracellular calcium in the full expression of endothelium-dependent relaxation is well defined, the route of its incorporation into the endothelial cell remains an enigma. There are five *possible* routes for calcium entry into endothelial cells: receptor-operated and voltage-gated calcium channels, Ca^{2+} leak channels, stretch-activated calcium channels, and sodium–calcium exchange.

2.2.3.1 Receptor-Operated Calcium Channels. The existence of endothelial receptor-mediated calcium channels is supported by the finding that agonists inducing the release of EDRF, such as bradykinin, histamine, and thrombin, increase $^{45}Ca^{2+}$ influx into endothelial cells (Whorton et al. 1984; A. Johns et al. 1987; D'Amore and Shepro 1977). In cultured endothelial cells, such agonists induce a nonselective ionic current (A. Johns et al. 1987; Lodge et al. 1988) which is independent of intracellular calcium (Schilling et al. 1988; Hallan et al. 1988). This suggests that receptor-mediated increases in intracellular Ca^{2+} are due to activation of nonselective ionic channels [by a second messenger(s) and by Ca^{2+}-dependent Ca^{2+} entry] through which extracellular Ca^{2+} can travel down its electrochemical gradient. Agonists that induce the release of EDRF increase the probability of channel opening in a concentration-dependent manner (A. Johns et al. 1987; Lodge et al. 1988).

2.2.3.2 Voltage-Gated Calcium Channels. Interpretation of the available evidence for and against the existence of voltage-gated calcium channels in endothelial cells is complicated by the fact that various calcium channel agonists and antagonists have been used in many different concentrations and tissues. This dilemma is further confounded by the fact that electrophysiological studies are typically performed in cultured cells, while most of the pharmacological experiments have been done on isolated vascular tissue. It probably would be presumptuous to infer that cells in culture have the same compliment of receptors and channels that endothelial cells express in vivo when exposed to pulsatile blood flow.

A first evidence against a role for voltage-gated calcium channels came from earlier work demonstrating that relaxations of isolated arteries evoked by acetylcholine are *impaired* by depolarizing solutions (DeMey

and Vanhoutte 1980; Furchgott and Zawadzki 1980). These findings are supported by studies of endothelial cells in culture in which depolarization by potassium ions leads to a decrease in both $^{45}Ca^{2+}$ uptake (A. Johns et al. 1987) and Ca^{2+} influx (Laskey et al. 1990; Morgan-Boyde et al. 1987) during the plateau phase, after exposure to agonists. The channels mediating bradykinin-induced Ca^{2+} influx in cultured endothelial cells are insensitive to verapamil (Whorton et al. 1984), and no net inward movement of current can be detected in response to membrane depolarization using isotonic solutions, or to the calcium channel agonist Bay K8644 (A. Johns et al. 1987; Takeda et al. 1987; Colden-Stanfield et al. 1987). These observations are supported by the findings that endothelium-dependent relaxations are not significantly altered by verapamil in the canine femoral artery (DeMey and Vanhoutte 1980) or by nifedipine, verapamil, and flunarizine in the rat aorta (Winquist et al. 1985; R.C. Miller et al. 1985; Jayakody et al. 1987). The opposing findings by Singer and Peach (1982) that endothelium-dependent relaxations could be attenuated by nifedipine and verapamil should be interpreted with caution as high concentrations of the antagonists were used (5×10^{-7} *M* and 10^{-5} *M*, respectively), and the effect was likely nonspecific as relaxation to the calcium ionophore A23187 was impaired also.

In conventional organ chambers, the calcium channel activator Bay K8644 failed to induce endothelium-dependent relaxation in either the canine femoral artery (Rubanyi et al. 1985b) or the rat aorta (Spedding et al. 1986). However, endothelium-dependent relaxations could be obtained in the perfused femoral artery (Rubanyi et al. 1985b). Likewise, the calcium channel agonist (+)–202,791 (Hof et al. 1985), which is more selective than Bay K8644 [a racemic mixture of (+) and (−) enantiomers which can be considered to be a partial agonist (Hof et al. 1985)], evokes endothelium-dependent relaxation in the perfused canine femoral artery at concentrations significantly below those that induce contractions of the smooth muscle (Rubanyi et al. 1985b). Thus, voltage-gated Ca^{2+} channels may be expressed in endothelial cells only when they are exposed to flowing solutions.

2.2.3.3 Ca^{2+} Leak Channels. The existence and role of a calcium influx through "Ca^{2+} leak" channels is suggested by the demonstration of a basal level of $^{45}Ca^{2+}$ influx into unstimulated endothelial cells (A. Johns et al. 1987). The influx, which occurs at a rate of 16 pmol/10^6 cells per second, could account for the basal production of EDRF by the endothelium and play a role in maintaining the Ca^{2+} plateau and replenishing intracellular Ca^{2+} stores after agonist stimulation.

2.2.3.4 Stretch-Activated Calcium Channels. A stretch- and/or pressure-activated cation channel has been described in cultured porcine aortic cells

(Lansman et al. 1987). By applying negative pressure to the inside of a membrane patch mounted on a pipet, an increased frequency of opening of a relatively nonselective cation channel was demonstrated. This prompts the suggestion that the endothelium may act as a mechanoreceptor and that pressure or stretch may activate the release of EDRF. This suggestion is in line with the observation that increases in shear stress promote the release of EDRF and the dilatation of blood vessels with endothelium (see Rubanyi et al. 1986; Lüscher and Vanhoutte 1990; Bassenge and Heusch 1990).

2.2.3.5 Sodium–Calcium Exchange. Na^+/Ca^{2+} exchanges may play a role in the endothelial calcium homeostasis by facilitating Ca^{2+} entry through the plasma membrane. This hypothesis is supported by the finding that replacement of Na^{2+} by Li^+ or sucrose inhibits relaxations to acetylcholine in canine femoral arteries (De Mey and Vanhoutte 1980). Dichlorobenzamil, an amiloride analog and a potent inhibitor of Na^+-dependent Ca^{2+} influx (Siegl et al. 1984), reduces endothelium-dependent relaxations (Winquist et al. 1985; Schoeffler and Miller 1986). The finding that amiloride analogs per se induce endothelium-dependent relaxations (Cocks et al. 1988) does not argue against the role for plasma membrane Na^+/Ca^{2+} exchange, as these drugs inhibit Na^+/Ca^{2+} exchanges in mitochondria (Jurkowitz et al. 1983), which could in turn secondarily increase cytoplasmic Ca^{2+} (Winquist et al. 1985). Na^+-dependent Ca^{2+} influx can be demonstrated in Fura-2-loaded endothelial cells after Na^+ loading with the sodium ionophore monensin (Sage et al. 1991).

2.2.3.6 Role of Potassium Ions. Irrespective of the avenue of calcium influx, the rate at which this occurs is modified by the membrane potential which affects the electrochemical gradient providing the driving force for the Ca^{2+} flux. Potassium is the predominant ion that modulates the potential of the endothelial cell membrane via its distribution across the plasma membrane through potassium channels (A. Johns et al. 1987; Laskey et al. 1990; Olesen et al. 1988). Currently, there are three well-characterized potassium channels in endothelial cells: (a) a voltage-gated or shear stress-gated potassium channel (Olesen et al. 1988) which may function to hyperpolarize the endothelial cell in response to increases in shear stress – this channel may enhance the electrical gradient to facilitate Ca^{2+} entry, which would promote the synthesis and release of EDRF; (b) a Ca^{2+}-activated potassium channel (Gordon and Martin 1983; Sauve et al. 1988; Fichtner et al. 1987) which may serve to repolarize the endothelial cell after agonist-induced cation influx and thus maintain the electrical gradient necessary for the continued influx of Ca^{2+} for the sustained release of EDRF; and (c) muscarine-gated

potassium channels (Olesen et al. 1988; Busse et al. 1988) which might facilitate agonist-induced Ca^{2+} influx and thus the synthesis of EDRF.

2.2.4 Calcium and Synthesis of EDRF

While it has been firmly established that an elevation of intracellular Ca^{2+} is an absolute requirement for the occurrence of endothelium-dependent relaxations, the role(s) played by the ion is not defined completely. However, the discovery that nitric oxide synthetase requires calmodulin for activity (Bredt and Snyder 1990; Busse and Mulsch 1990) highlights one essential role for Ca^{2+} in the synthesis of nitric oxide, the active component of EDRF.

2.3 Phosphatidylinositol Pathway

The initial increase in endothelial cell calcium may be mediated by the phosphatidylinositol pathway. Following stimulation by endothelium-dependent vasodilators, PIP_2 phosphodiesterase (phospholipase C) acts upon phosphatidylinositol 4,5-biphosphate (PIP_2) to generate diacylglycerol (DG) and inositol triphosphate (IP_3)(Fig. 6). IP_3 then mobilizes intracellular calcium

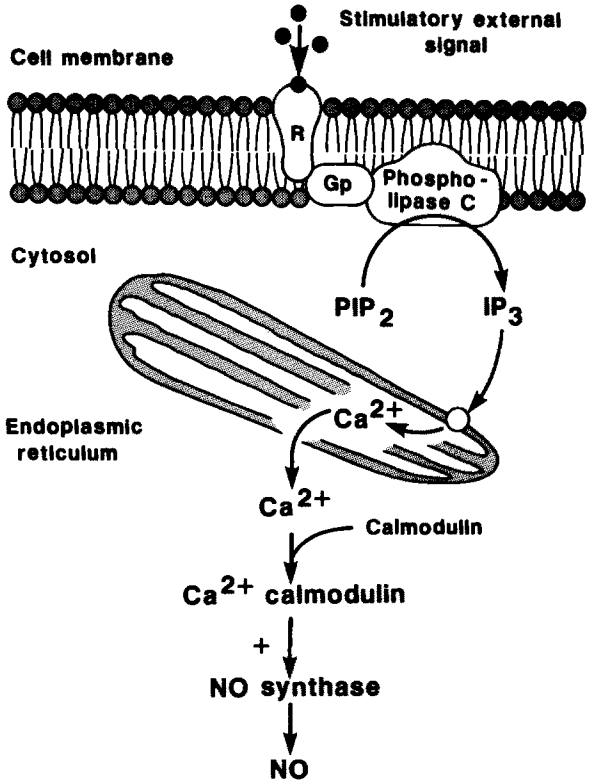


Fig. 6. Phosphatidylinositol turnover and synthesized nitric oxide (*NO*). *R*, membrane receptor; *Gp*, G-protein; PIP_2, phosphatidylinositol 4,5-diphosphate; IP_3, inositol triphosphate

while a DG-activated protein kinase C allows plasma membrane influx of extracellular calcium. This hypothesis is consistent with the observations that in cultured endothelial cells, bradykinin or adenosine diphosphate stimulate phospholipase C to release IP_3 (Derian and Moskowitz 1986; Whorton et al. 1984; Lambert et al. 1986; Pirotton et al. 1987) and that exogenous phospholipase C induces endothelium-dependent relaxations (Forsterman and Neufang 1985) while phospholipase B and phospholipase D do not (DeNucci et al. 1988a). However, endothelium-dependent relaxations are also potentiated by inhibiting DG kinase (DeNucci et al. 1988a) and inhibited by phorbol esters, which mimic activation of phospholipase C (Lewis and Henderson 1987; Weinheimer et al. 1986). These observations argue against the assumption that the phosphatidylinositol pathway is involved in the production of EDRF. Alternatively, activation of protein kinase C by DG may involve at least two phosphorylations which occur at different rates (Angus and Cocks 1989; Brock and Capasso 1988).

2.4 G-Proteins and Endothelium-Dependent Relaxations

The link between an agonist binding to an endothelial cell receptor and the release of EDRF most likely involves several regulatory G-proteins (Fig. 7) (Flavahan et al. 1989; Voyno-Yasenetskaya et al. 1989; Flavahan and Vanhoutte 1990). Regulatory G-proteins are associated with most membrane-bound receptors (Rodbell 1985; Dohlman et al. 1987; Dolphin 1987). Following receptor activation, the binding of guanine triphosphate to the α-subunit of the associated G-protein is facilitated, and the activated G-protein then dissociates from the receptor, releasing its α-subunit. The α-subunits associated with different G-proteins activate or inhibit various intracellular processes (Dolphin 1987). The nonhydrolyzable GTP analog GTPγS stimulates basal IP_3 and increases cytosolic Ca^{2+} levels in permeabilized endothelial cells (Brock et al. 1988). In the porcine coronary artery, pertussis toxin [an inactivator of certain G-proteins, in particular G_i-protein (Dolphin 1987)] inhibits, to varying degrees, endothelium-dependent relaxations evoked by α_2-adrenergic agonist UK 14304 5-hydroxytryptamine, thrombin, or aggregating platelets (Flavahan et al. 1989). In addition, in the canine coronary artery, fluoride ions, which can activate G-proteins directly (Sternweis and Gilman 1982; Bigay et al. 1987), induce pertussis toxin-sensitive endothelium-dependent relaxations (Flavahan and Vanhoutte 1990; Cushing et al. 1990). The fluoride ions most likely activate a G-protein linked to a calcium channel in the endothelial cell membrane (Graier et al. 1990). Other receptor-mediated releases of EDRF (e.g., by bradykinin or adenosine diphosphate, ADP) occur via phospholipase C and the phosphatidylinositol pathway that activate G_β-proteins. It is the dysfunc-

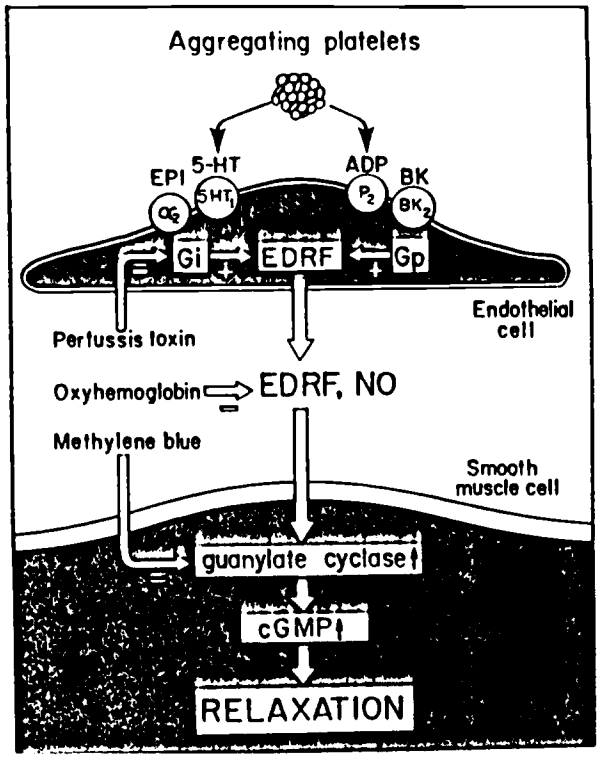


Fig. 7. Release of endothelium-derived relaxing factor (*EDRF*) by endothelial cells involves at least two types of signal-transducing G-proteins, one of which is sensitive to pertussis toxin. Agonists at the endothelial cell membrane can use one and/or the other pathway. α_2, α_2-adrenoceptor; *ADP*, adenosine diphosphate; *BK*, bradykinin, kinin receptor; *cGMP*, cyclic GMP; *Gi*, *Gp*, G_i-, G_p-proteins; *5-HT*, serotonin, serotonin receptor; *NO*, nitric oxide; P_2, P_{2Y}-purinoceptor. (From Vanhoutte 1991)

tion of the pertussis toxin-sensitive pathway in endothelial cells which best explains the reduced endothelium-dependent relaxation observed in arteries with regenerated endothelium, early atherosclerosis, or after reperfusion injury (see Vanhoutte and Shimokawa 1989; see Lüscher and Vanhoutte 1990; Pearson et al. 1990a,b).

2.5 Synthesis of EDRF

Following identification of nitric oxide as the active component of EDRF (Palmer et al. 1987; Ignarro et al. 1987a,b), the search was on to determine how the endothelium synthesized the radical. Basic information used to elucidate the endothelial pathway of nitric oxide production came from previous experiments in macrophages which produce the radical. In these cells, L-arginine is a precursor for NO_2^- and NO_3^- (Stuehr and Marletta

1985; Hibbs et al. 1987), nitric oxide being an intermediate in the pathway (Marletta et al. 1988; Tayeh and Marletta 1989).

2.5.1 L-Arginine as the Precursor of Nitric Oxide Synthesis

Using a bioassay cascade from porcine aortic endothelial cells in culture, Moncada and associates demonstrated that L-arginine enhances the release of nitric oxide stimulated by bradykinin, particularly in cells cultured in the absence of this semi-essential amino acid (Palmer et al. 1988a,b). The specificity of this pathway was highlighted by the fact that D-arginine was without effect. In addition, the potentiation of nitric oxide production by L-arginine could be inhibited by the methylated form of the amino acid, N^G-monomethyl-L-arginine (L-NMMA). These findings demonstrated that L-arginine is the substrate for the endothelial synthesis of nitric oxide.

To determine which nitrogen atom of L-arginine served as the precursor for nitric oxide production, endothelial cells were treated with 15N-labeled L-arginine. These studies revealed that endothelial cells synthesized nitric oxide from the terminal guanidino nitrogen atom(s) of L-arginine (Fig. 8) (Palmer et al. 1988a,b).

The production of nitric oxide from L-arginine is a discriminatory pathway with strict structural and isomeric substrate specificity. L-arginine and L-citrulline enhance the release of nitric oxide from stimulated porcine endothelial cells in culture, while L-ornithine, L-α-amino μ-guanidino butyric acid, L-homoarginine, guanidine hydrochloride, canavanine, and the dipeptide L-arginine-aspartate are without effect (Palmer et al. 1988a). The enhanced nitric oxide production mediated by L-citrulline is due to the conversion of L-citrulline to L-arginine and subsequent metabolism to nitric oxide

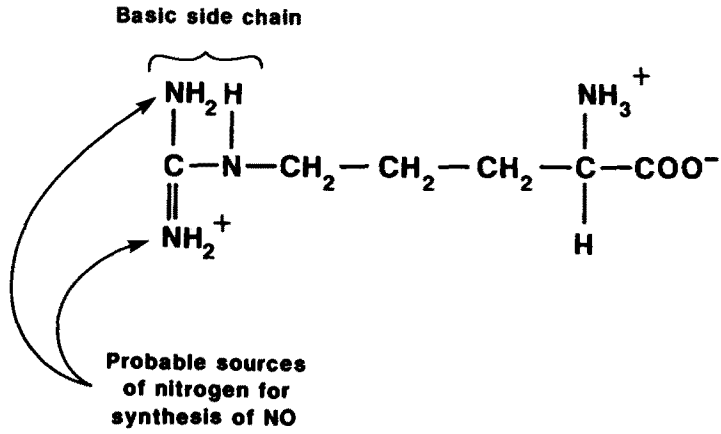


Fig. 8. Sources of nitrogen for the synthesis of nitric oxide (*NO*)

(Palmer et al. 1988a; Hecker et al. 1990). This conversion might constitute an important source of substrate to maintain nitric oxide production (Hecker et al. 1990).

2.5.2 *Inhibition of Nitric Oxide Synthesis*

The important discovery of the role of L-arginine in endothelium-dependent relaxation led the way for the use of specific inhibitors of EDRF production. L-NMMA was the first drug used to inhibit the synthesis by endothelial cells of nitric oxide (Palmer et al. 1988a,b; Rees et al. 1989a). L-NMMA had been used previously to inhibit NO, NO_2^-, and NO_3^- synthesis from L-arginine in macrophage (Hibbs et al. 1987; Marletta et al. 1988). L-NMMA acts as a competitive inhibitor of nitric oxide production by competing with L-arginine for the enzyme that metabolizes the amino acid. This conclusion is supported by the fact that inhibition by L-NMMA can be overcome by adding high concentrations of L-arginine (Palmer et al. 1988a,b; Rees et al. 1989a; Moore et al. 1990). Moore et al. (1990) have done much to characterize the structural and chemical requirements of a competitive inhibitor of nitric oxide production from L-arginine. The most essential component is modification of the guanidino (but not the N_α) terminal of L-arginine. Substitution of a methyl group (as in L-NMMA) or a nitro group (as in L-N^G-nitro-arginine) on the guanidino nitrogen atoms results in a potent inhibitor, while substitution on the N_α (as in L-N_α-monocarbobenzoxy arginine) is without effect (Moore et al. 1990). However, the position of substitution is not the only factor producting inhibition as substitution of an N^G-tosyl moiety on L-arginine is without effect. This indicates that the chemical constitution of the modified group is also important. In addition, the size and location of side chains substituted on L-arginine can also effect inhibition. Bulky substitutes on the N_α region result in weak, nonspecific inhibitors of endothelium-dependent relaxations, while addition of an α-methyl group to the carboxyl moiety of L-arginine does not impair the metabolism of L-arginine (Moore et al. 1990).

2.5.3 *Pathway of Nitric Oxide Production*

There are two possible pathways for the production of nitric oxide from L-arginine. The first pathway involves a deamination of L-arginine to generate NH_3 which is subsequently oxidized to nitric oxide. This pathway is not probable as NH_3 is apparently not an intermediate in the production of nitric oxide. The second, and most probable pathway, involves a stepwise N-oxidation of the guanidino atoms of L-arginine to nitric oxide (Fig. 9). Although the pathway has not been characterized in endothelial cells, utilizing information gained in other cell lines, the following pathway is reasonable

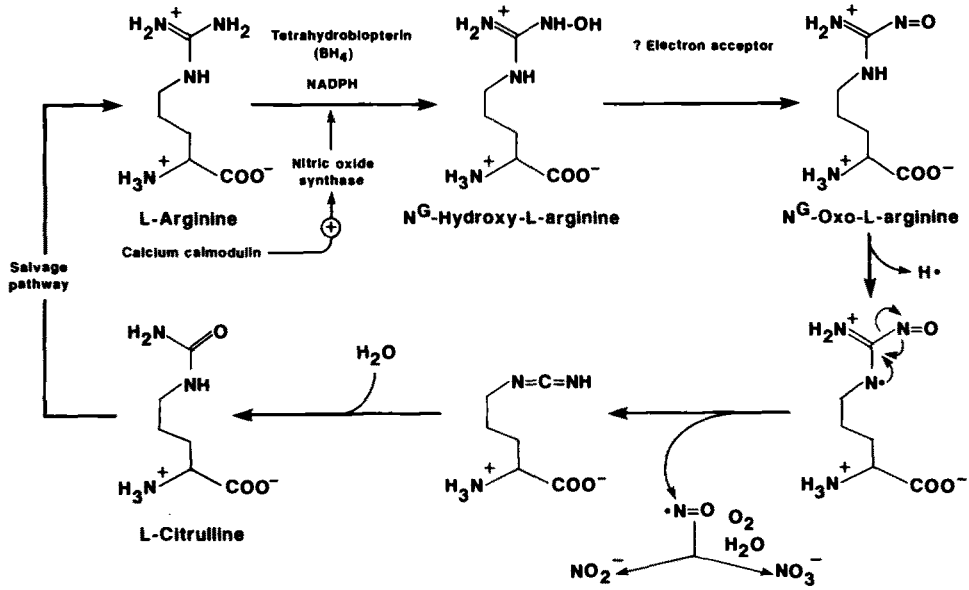


Fig. 9. Chemical pathways for the generation of nitric oxide (*NO*)

(Marletta 1989). The first step of the pathway involves a monooxygenase-type hydroxylation reaction occurring at one of the guanido nitrogens to generate N^G-hydroxyl-L-arginine. This step would have the absolute requirement of reducing equivalents. Thus, in the macrophage, NADPH and 6-methyltetrahydrobiopterin (BH_4) are required for this conversion (Tayeh and Marletta 1989). This is consistent with the finding that the enzyme in the vascular endothelium which converts L-arginine to L-citrulline is NADPH dependent (Palmer and Moncada 1989; Mulsch et al. 1989a; Mayer et al. 1989). In addition, in the macrophage, N^G-hydroxy-L-arginine serves as a precursor for NO_2^- and NO_3^- production (Stuehr et al. 1991). The second step involves a two-electron oxidation of the unstable hydroxylamine moiety to produce the nitrosoamidine-like intermediate N^G-oxo-L-arginine. However, this product could also be formed by a second hydroxylation upon N^G-hydroxyl-L-arginine followed by a dehydration. The attractive feature of the third step is that, by a simple loss of a hydrogen atom, the amino acid radical generated would be very unstable and would fragment as indicated in step four to yield nitric oxide and an amino acid carbodiimide, the diimide of ornithine (Marletta 1989). Subsequent reaction of the diimide, *N*-[(2-amino)-valeryl]-carbodiimide, with H_2O (typical for carbodiimides), would then yield citrulline.

2.5.4 Nitric Oxide Synthase

The enzyme which initiates the conversion of L-arginine to nitric oxide by the endothelium, termed nitric oxide synthase, has been characterized partially in endothelial cells (Bredt and Snyder 1990) and other tissue (Knowles et al. 1990; see also Moncada et al. 1991), and cloned in brain tissues (Wang et al. 1992). Apparently, the activation of the nitric oxide synthase in endothelial cells is initiated by an increase in cytosolic Ca^{2+} (Mulsch et al. 1989a; Mayer et al. 1989) and requires calmodulin (Busse and Mulsch 1990). Two types of enzyme are present in cultured and native endothelial cells: a cytosolic isoform and a particulate one. The latter is responsible for 95% of the EDRF-nitric oxide-synthesizing activity (Forstermann et al. 1991). The membrane-associated enzyme possesses a molecular mass of 135 kDa, is NADPH and 5,6,7,8-tetrahydrobiopterin sensitive, and Ca^{2+}/calmodulin dependent. This is in agreement with the calcium dependency of endothelium-dependent relaxations (see above). The characteristics of the soluble endothelial enzyme are similar.

Besides the constitute nitric oxide synthase, vascular endothelial cells may express an inducible form in response to endotoxin and cytokines (Kilbourn and Belloni 1990), which will evoke a long-lasting release of large amounts of nitric oxide resulting in cytotoxic activities (Li et al. 1991). Activated endothelial cells express a cytosolic enzyme (of 150-kDA molecular mass) independent of Ca^{2+}/calmodulin with a rank order of potency similar to that of cytokine-activated macrophages (Forstermann et al. 1991; Gross et al. 1991). This form of nitric oxide synthase can also be induced in vascular smooth muscle cells and can produce high concentrations of nitric oxide for prolonged periods of time (Busse and Mulsch 1990; Beasley et al. 1991; Schini et al. 1991) during immunological and inflammatory reactions. Such events may play a role in the irreversible phase of septic shock, which is characterized by peripheral vascular paralysis.

2.6 Nitric Oxide and Vascular Smooth Muscle

Nitric oxide is a very lipophilic compound. As such, it does not require a cell surface receptor to mediate its action on vascular smooth muscle. Indeed, nitric oxide produced by the endothelium probably moves by diffusion into the contiguous vascular smooth muscle to mediate vascular relaxation by a cyclic-GMP dependent process(es) (Fig. 5) (Furchgott 1984; Ignarro and Kadowitz 1985; Murad et al. 1987).

2.6.1 Activation of Guanylate Cyclase

The mechanism by which nitric oxide activates soluble guanylate cyclase in the vascular smooth muscle is well defined (see Ignarro 1989). Activation

of guanylate cyclase by nitric oxide has an absolute requirement for ferroprotoporphyrin IX (heme) (Craven and De Rubertsis 1978). Without heme, nitric oxide has little, if any, activity on soluble guanylate cyclase (Craven and De Rubertsis 1978; Ignarro et al. 1982). Heme itself has little ability to stimulate guanylate cyclase and may even be inhibitory to activation (Ignarro and Wood 1987). However, protoporphyrin IX (heme with the iron molecule removed) is a potent activator of the enzyme (Wolin et al. 1982). Thus, the iron atom of heme must provide some type of stearic hindrance to the activation of guanylate cyclase which can be overcome by removing the iron atom (as with protoporphyrin IX) or by the binding of nitric oxide. This interaction is supported by the findings in studies using electron-paramagnetic resonance which demonstrate that nitric oxide binding to heme causes the iron atom of the ferroprotoporphyrin IX moiety to change its orientation and protrude from the plane of the heme (see Ignarro et al. 1984b). This then either allows the heme moiety to interact with guanylate cyclase and promote its activation, or it removes stearic hindrance of Mg-GTP to the catalytic site of the enzyme and promotes the production of cyclic GMP. It thus appears that the nitric oxide receptor in vascular smooth muscle is the heme component of soluble guanylate cyclase.

In cerebral arteries exposed to chronic hemorrhage and exhibiting chronic vasospasm, endothelium-dependent relaxations are inhibited (Nakagomi et al. 1987a,b; Kim et al. 1988a,b; Hongo et al. 1988a,b). This impairment is seen despite a normal release of EDRF (Kim et al. 1989) and can be attributed to an abnormal sensitivity of the soluble guanylate cyclase to the endothelial mediator (Kim et al. 1991). In most other models of vascular disease, the sensitivity of vascular smooth muscle to EDRF appears to be normal (see Lüscher and Vanhoutte 1990).

2.6.2 Cyclic GMP and Relaxation of Vascular Smooth Muscle

The activation of guanylate cyclase and subsequent elevation of cyclic GMP by nitric oxide in vascular smooth muscle is associated with relaxation (Kukovetz et al. 1979; Gruetter et al. 1981; Rapoport and Murad 1983; see Ignarro and Kadowitz 1985; Murad 1986; Waldman and Murad 1987). Several possibilities have been put forward to explain cyclic GMP-mediated, endothelium-dependent relaxations of vascular smooth muscle (Angus and Cocks 1989; Martin and Sanchez-Ferrer 1990). Possible calcium-specific effects include the inhibition of a sarcolemmal calcium channel to inhibit Ca^{2+} influx (Collins et al. 1986; Ratz et al. 1987) or the activation of a sarcolemmal calcium extrusion pump (Popescu et al. 1985), both secondary to activation of cyclic GMP-dependent protein kinases (Rapoport et al. 1983; Fiscus et al. 1984; Popescu et al. 1985). The intracellular liberation of calcium

may be impaired following inhibition of phosphatidylinositol hydrolysis (Rapoport 1986), the intracellular sequestration or the binding of calcium may be increased (Lincoln 1983), or the dephosphorylation of the myosin light chain may be accelerated (Rapoport et al. 1983). A cyclic GMP-dependent, calcium-independent mechanism involves the activation of a sarcolemmal potassium channel such that potassium conductance increases and the vascular smooth muscle hyperpolarizes (Komori and Suzuki 1987).

2.7 Evidence for the Existence of More than One EDRF

Several bioassay studies demonstrate the existence of more than one mediator of endothelium-dependent relaxations (Rubanyi and Vanhoutte 1987; Boulanger et al. 1989; Hoeffner et al. 1989). Initially an inhibitor of lipoxygenase (nordihydroguaiaretic acid) was demonstrated to block the basal release of EDRF from a perfused artery (detected by relaxation of a bioassay ring) while inhibitors of cytochrome P-450 (metyrapone) and phospholipase A_2 (quinacrine) had no effect (Rubanyi and Vanhoutte 1987). In addition, all three blockers attenuated or inhibited the initial large transient relaxation produced by acetylcholine infusion, but did not affect the smaller secondary sustained relaxation. However, these results might have been due to different cellular mechanisms leading to the production of the same factor (Rubanyi and Vanhoutte 1987). In subsequent studies, it was found that treatment of cultured endothelial cells with the Na^+/K^+ ATPase inhibitor ouabain could impair the stimulated release of EDRF by the calcium ionophore A23187 and bradykinin, while the basal and ADP-stimulated release of EDRF was unaffected (Boulanger et al. 1989). They also demonstrated that treatment of the bioassay ring with ouabain could impair relaxation to basal EDRF and ADP-stimulated release, but had no effect on relaxation induced by exogenously added nitric oxide or release of EDRF stimulated by the calcium ionophore A23187 or bradykinin. This strongly suggested that cultured porcine aortic endothelial cells release two endothelium-derived relaxing factors; one is released under basal conditions and upon stimulation with adenosine diphosphate and the other (which presumably is nitric oxide) upon stimulation with bradykinin and A23187 (Fig. 10) (Boulanger et al. 1989). Similar conclusions have been reached in perfused canine coronary arteries (Hoeffner et al. 1989). In the perfused rat lung, L-NMMA N^G inhibits the vasodilatation to bradykinin, but does not impair that to acetylcholine (Archer et al. 1989). Indeed, acetylcholine-induced relaxation is not associated with the generation of nitric oxide in that artery (Menon et al. 1989). In addition, endothelium-dependent relaxations to bradykinin in the porcine coronary artery are largely independent of nitric oxide, as L-NMMA, hemoglobin, and methylene blue only weakly inhibit the relaxations to that agonist (Flavahan et al. 1989; Richard et al. 1990). The most

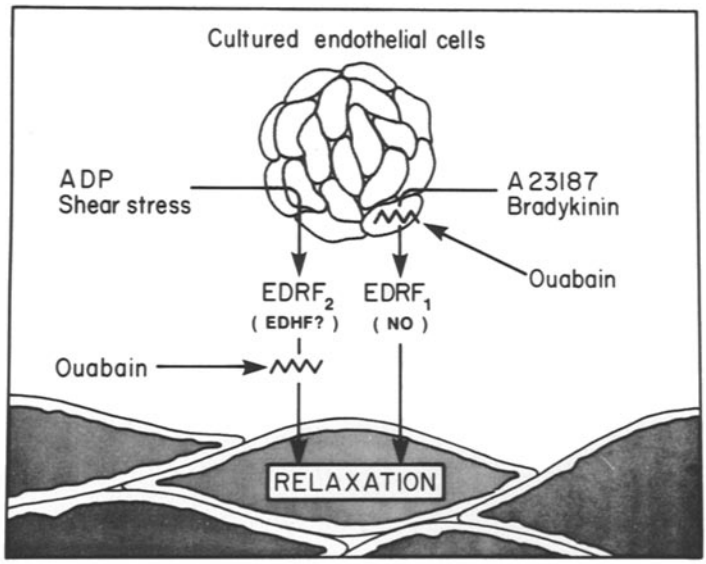


Fig. 10. To judge from bioassay studies with cultured porcine endothelial cells, these cells release at least two EDRFs. One is most likely NO; its release, stimulated by bradykinin or the Ca^{2+} ionophore A23187, but not its action, can be prevented (*zig-zag line*) by ouabain. The other factor is of unknown nature; it is released under basal conditions or when the endothelial cells are exposed to adenosine diphosphate (*ADP*). Its action, but not its release, can be prevented by ouabain (*zig-zag line*). It possibly could be endothelium-derived hyperpolarizing factor (*EDHF*). (From Lüscher and Vanhoutte 1990)

likely candidate as mediator of these nitric oxide-independent relaxations is endothelium-derived hyperpolarizing factor. However, in certain arteries, prostacyclin may contribute as well.

2.7.1 Endothelium-Derived Hyperpolarizing Factor

Many endothelium-dependent relaxations are accompanied by hyperpolarization of the vascular smooth muscle (Bolton et al. 1984; Komori and Suzuki 1987a,b; Feletou and Vanhoutte 1988; Komori et al. 1988). Bioassay studies in canine arteries demonstrate that the endothelium-dependent hyperpolarization is due to a diffusible substance called endothelium-derived hyperpolarizing factor (EDHF) (Feletou and Vanhoutte 1988) (Fig. 11). With one exception (Tare et al. 1990), the data available suggest that EDRF (nitric oxide) and EDHF are separate compounds (Bolton and Clapp 1986; Komori and Suzuki 1987a,b; Feletou and Vanhoutte 1988; Komori et al. 1988; Chen and Suzuki 1989). For example, in the guinea pig mesenteric artery, hyperpolarization is induced by carbachol but not by substance P, while both agonists induce endothelium-dependent relaxation (Bolton and

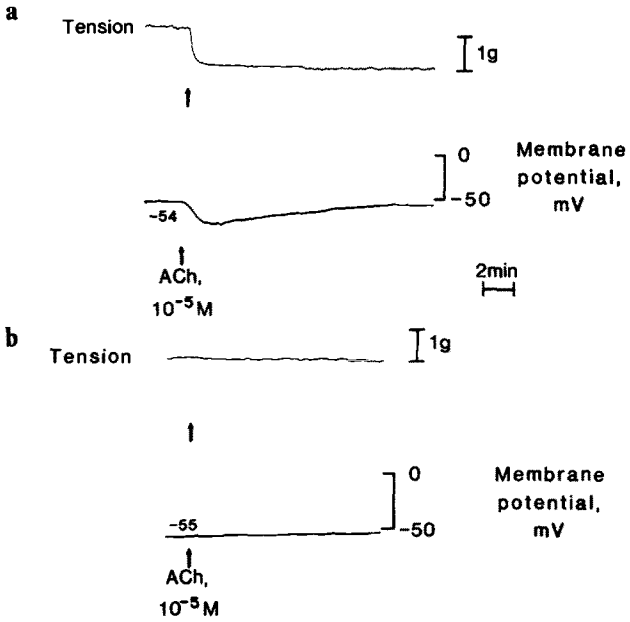


Fig. 11a,b. Bioassay, with canine coronary arteries without endothelium, of the hyperpolarizing factor released from femoral arteries of the same donor animal. If the organ chamber contains femoral artery with endothelium **a** the injection of acetylcholine (*ACh, arrows*) causes a sustained relaxation, but a transient hyperpolarization of the bioassay tissues. These responses are not observed if the chamber contains a femoral artery without endothelium. **b** This experiment demonstrates that the endothelium-dependent hyperpolarization evoked by ACh is due to a diffusible factor (EDHF) (Indomethacin, 10^{-5}M). (From Feletou and Vanhoutte 1988)

Clapp 1986). Endothelium-dependent hyperpolarization is also induced by acetylcholine but not by oxotremorine in the rabbit saphenous artery, while both agonists induce comparable endothelium-dependent relaxation (Komori and Suzuki 1987a). The endothelium-dependent hyperpolarization of the smooth muscle of the canine coronary artery evoked by acetylcholine is inhibited by ouabain or potassium-free solution, while endothelium-dependent relaxation is not (Feletou and Vanhoutte 1988). Pharmacological analysis demonstrates that, in the canine femoral artery, activation of the M_2 subtype of muscarinic receptor leads to the production of EDRF while activation of the M_1 receptor subtype releases hyperpolarizing factor (Fig. 12) (Komori and Suzuki 1987b). In addition, both hemoglobin (which inactivates nitric oxide) and methylene blue (which prevents activation of guanylate cyclase) block endothelium-dependent relaxations but do not affect endothelium-induced hyperpolarizations (Komori et al. 1988; Chen et al. 1988; Chen and Suzuki 1989). Exogenous nitric oxide does not affect the membrane potential of the canine mesenteric artery (Komori et al.

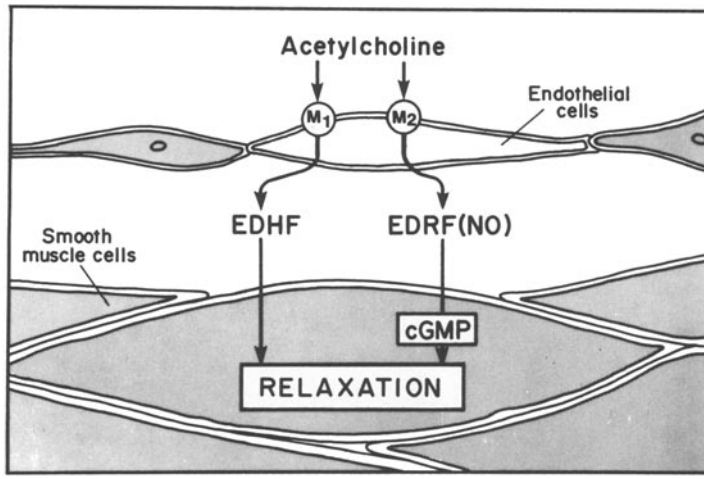


Fig. 12. The endothelial cells, when exposed to acetylcholine, release two vasoactive factors. EDHR hyperpolarizes the cell membrane, thus initiating the relaxation and/or making the vascular smooth muscle more sensitive to the action of EDRF which presumably is NO. The latter sustains the relaxation by entering the cell and activating soluble guanylate cyclase which leads to an accumulation of cyclic GMP (*cGMP*). The muscarinic receptors (*M*) on the endothelial cell membrane triggering the release of the two factors do not belong to the same subtype. (From Lüscher and Vanhoutte 1990)

1988). Likewise, in cerebral arteries, acetylcholine-mediated endothelium-dependent vasodilatation can be attributed solely to nitric oxide-independent hyperpolarization of vascular smooth muscle (Braydon 1990).

2.7.2 Prostacyclin and Platelet/Blood Vessel Interactions

It has long been recognized that platelets do not adhere to the vascular wall (Stemerman and Spaet 1972) and that the metabolism of arachidonic acid through cyclooxygenase contributes to the antithrombogenic property of the endothelium (e.g., Kloeze 1969). Moncada, Vane, and their associates demonstrated that arachidonic acid is metabolized to generate prostacyclin, a potent inhibitor of platelet aggregation which possesses vasodilator properties (Moncada et al. 1976; Gryglewski et al. 1976; Johnson et al. 1976).

The synthesis of prostacyclin occurs predominantly in the endothelium (MacIntyre et al. 1978; Weksler et al. 1978; Baenziger et al. 1977; Herman et al. 1977; Moncada et al. 1977). While the vascular smooth muscle has the capability to generate PGI_2, endothelial cells are capable of synthesizing 10–20 times more of the prostanoid (Eldor et al. 1981). This enhanced synthetic capability is due to the ability of the endothelium to generate 20 times the amount of PGH_2 (the precursor of PGI_2) because of a higher concentration of PGH synthase (Eldor et al. 1981), while the concentrations of

PGI_2 synthase in the endothelium and vascular smooth muscle are identical (DeWitt et al. 1983). In the vasculature, there are also regional differences in prostacyclin production with arterial endothelium capable of synthesizing three to ten times that of veins (Skidgel and Printz 1978).

PGI_2 has a short half-life (2–3 min) and is hydrolyzed rapidly to 6-keto-$PGF_{1\alpha}$ (Vane 1985). PGI_2 is not only a potent inhibitor of platelet aggregation (Moncada et al. 1976) but it can promote platelet disaggregation as well (Gryglewski et al. 1978). PGI_2 also relaxes vascular smooth muscle (Dusting et al. 1977; Shimokawa et al. 1988a). These effects are mediated by increased levels of cyclic AMP (Gorman et al. 1977; Tateson et al. 1977; Nakahata and Suzuki 1981). PGI_2 also increases cyclic AMP in endothelial cells (Hopkins and Gorman 1981; Karnushina et al. 1983; Leitman et al. 1986); the physiological implications of this are unknown (Whorton et al. 1985) although in the porcine coronary artery prostacyclin stimulates the release of EDRF (Shimokawa et al. 1988a).

2.8 Hemodynamic Factors and Release of EDRF

2.8.1 Basal Release of EDRF

Endothelial cells in an unstimulated state spontaneously release EDRF (Martin 1988). It is likely that the tonic, basal release of EDRF plays a major role in the modulation of blood pressure and systemic vascular resistance (Rees et al. 1989b; Vallance et al. 1989).

When EDRF was bioassayed from perfused vascular segments, it became obvious that it was released by the endothelium even in the absence of vasodilator agents. Thus, if a blood vessel with endothelium (the donor segment) was perfused with physiological salt solution, the effluent induced relaxation in a vessel without endothelium (the bioassay segment) (Fig. 13). Such basal release of EDRF was demonstrated in the rabbit aorta (Long and Stone 1985), the rat aorta (Griffith et al. 1984; Martin et al. 1985a,b), and the canine femoral artery (Rubanyi et al. 1985a). The basal release of EDRF from perfused vessels has subsequently been demonstrated in virtually every blood vessel tested and in superfused cultured endothelial cells (Gryglewski et al. 1986a; Boulanger et al. 1989). In perfusate from unstimulated cultured endothelial cells, nitric oxide can be chemically detected (Palmer et al. 1987; Ignarro et al. 1987a,b; Meyers et al. 1989).

In conventional organ chambers using rings of isolated blood vessels, the basal release of EDRF is demonstrated by the reduced contraction to vasoconstrictor substances in the presence of the endothelium. Thus, when arteries or veins are contracted with various agonists, the preparations with endothelium typically exhibit an attenuated maximal contraction and/or a shift in the concentration–response curve of the agonist to the right when

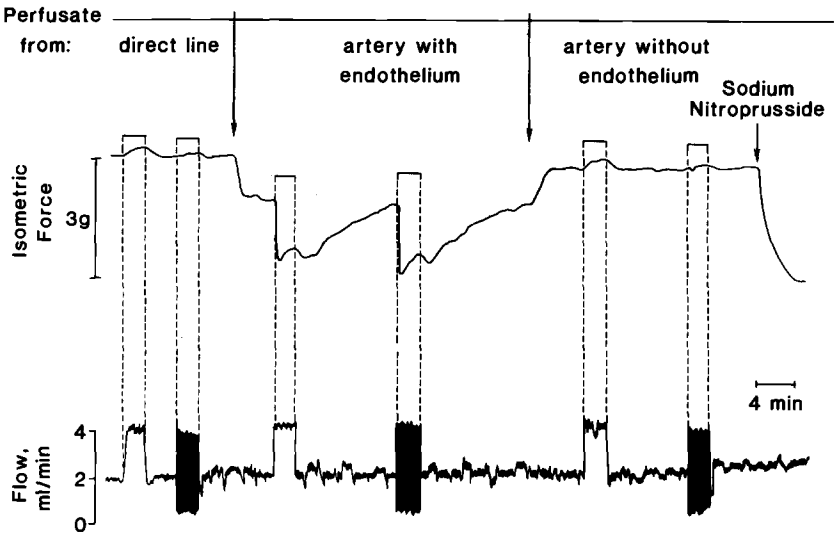


Fig. 13. Effects of variations in flow (*lower trace*) on the contraction (*upper trace*) to prostaglandin $F_{2\alpha}$ in a bioassay ring of a canine coronary artery. In a first step of the experiments, the bioassay ring is superfused directly with Krebs-Ringer bicarbonate solution; increasing the flow from 2 to 4 ml/min or maintaining the same average flow (2 ml/min), but applying it in a pulsatile manner (*black parts of the lower trace*) does not cause relaxation of the bioassay ring. At the *first arrow*, the bioassay ring is exposed to solution flowing through an artery with endothelium, which causes an immediate relaxation due to the basal release of EDRF. Increasing the flow, or making it pulsatile, causes a marked further relaxation of the bioassay ring. These responses are not seen if the flow pattern is altered during superfusion of the bioassay ring with solution flowing through an artery without endothelium (*second arrow*), although under these conditions the bioassay tissue relaxes when exposed to sodium nitroprusside. The experiments demonstrate that increases in shear force evoke the release of EDRF. (From Rubanyi et al. 1986)

compared to vessels without endothelium (e.g., Cocks and Angus 1983; Cohen et al. 1983a,b; Miller and Vanhoutte 1985b). That this is due to EDRF and not to a "barrier" function of the endothelium is evidenced by the fact that endothelium-dependent inhibition of contractile responses is reversed by methylene blue (R. C. Miller et al. 1984) and hemoglobin (Martin et al. 1986a). If the blood vessels exhibit intrinsic tone, hemoglobin, added to the organ chamber, induces an increase in tension of quiescent (noncontracted) vascular segments with (but not without) endothelium (Martin et al. 1985a,b). Thus, even in the absence of stimulation by shear stress or Chemical agents, EDRF is released tonically from the endothelium and inhibits the underlying smooth muscle. This conclusion is strengthened by the observation that L-NMMA (Rees et al. 1989a) and L-N^G-nitro arginine (Moore et al. 1990), two inhibitors of nitric oxide synthesis from L-arginine, cause gradual increases in tension in noncontracted isolated vascular rings

with endothelium, while inducing no significant changes in rings without endothelium. In the isolated perfused guinea pig heart, a continuous basal production of nitric oxide can be measured spectrophotometrically, the inhibition of which, by methylene blue, hemoglobin, or N^G-monomethyl-L-arginine, causes a significant increase in coronary vascular resistance (Kelm and Schrader 1990).

Biochemically, the basal release of EDRF is mirrored by an enhanced production of cyclic GMP exhibited by arteries with endothelium versus those without endothelium (Holzmann 1982; Rapoport and Murad 1983; Ignarro et al. 1984a; Furchgott et al. 1984; Martin et al. 1985a). This enhanced production of cyclic GMP can be inhibited by hemoglobin (Holzmann 1982; Furchgott et al. 1984; Martin et al. 1985a, 1986a).

2.8.2 EDRF and Geometry of Blood Vessel

Murray (1926a,b) determined that when flow rate and diameter of a blood vessel are related by the cubic expression $Q = \alpha D^3$, there is minimal power loss and volume retention in a vascular segment. Thus, Murray extrapolated that there is an "optimal" relationship between diameter (D) and flow (Q) in a blood vessel. Griffith and Edwards (1990), when measuring optimal branching of arterioles from in vivo measurements of blood vessel diameter, determined that the release of EDRF is necessary for the calculated and measured optimal relationship between flow and diameter to be expressed, particularly when vascular smooth muscle tone is high. This is related to the finding that initial stretch or agonist-induced tone of vascular smooth muscle has a modulatory effect on either basal or stimulated EDRF release (Dainty et al. 1990).

2.8.3 Endothelium-Dependent Vasodilatation

The fact that increases in arterial blood flow induce concomitant increases in vascular diameter was observed first by Schretzenmayr (1933). Flow-induced dilatation serves to reduce the shear stress or viscous drag inside the blood vessel which accompanies an increase in fluid flow through the artery. The phenomenon, confirmed by other investigators, was termed *ascending dilatation*, as it was hypothesized that a wave of dilatation was induced in the arterioles and was propagated in a retrograde way to involve the larger arteries (Fleisch 1935; Hilton 1959; Duling and Berne 1970; Ingebrigtsen and Leraand 1970; Gerova et al. 1981). However, the theory of ascending dilatation was disproved with the demonstration that flow-induced dilatation could occur even if the artery was transsected to prevent the retrograde propagation of stimuli (Lie et al. 1970; Smiesko et al. 1985).

2.8.3.1 Endothelium Dependency. The importance of the endothelium in mediating the vascular response to acute changes in blood flow was highlighted by Holtz and associates when they demonstrated that flow-induced dilatation in vitro and in vivo is dependent on an intact endothelium (Holtz et al. 1983a,b; Kuo et al. 1990; see Bassenge and Heusch 1990) and is not modified by indomethacin (Holtz et al. 1984). These observations were confirmed by other investigators in vivo and in vitro using a variety of arteries from different sources (Rubanyi et al. 1986; Smiesko et al. 1985; Pohl et al. 1986a; Hull et al. 1986; Young and Vatner 1987) (Fig. 7). Flow-mediated vasodilatation is localized to segments of blood vessels with endothelium and does not spread to contiguous de-endothelialized areas (Bassenge and Pohl 1986).

2.8.3.2 Role of EDRF. Pharmacological analysis confirmed that flow-mediated vasodilatation is due to EDRF and not to prostacyclin or some other autacoid released by the endothelium (Fig. 13). Indeed, the response (a) is unaffected by indomethacin (Rubanyi et al. 1986); (b) is blocked by hemoglobin (Griffith et al. 1987); (c) is prevented by treatment of the vascular segment with methylene blue (Kaiser et al. 1986); and (d) is potentiated by the free radical scavenger superoxide dismutase (Rubanyi et al. 1986).

Pulsatile flow also stimulates the release of EDRF from perfused arterial segments (Rubanyi et al. 1986; Pohl et al. 1986b) (Fig. 13). While there is also a concomitant release of prostacyclin during pulsatile perfusion (Busse et al. 1984; Rubanyi et al. 1986), the level of prostacyclin released is below the threshold to induce relaxation, and the increase in flow cannot be blocked with indomethacin (Rubanyi et al. 1986). The identity of EDRF as the mediator of pulsatile-induced dilatation is consistent with the findings that the effect can be blocked by hemoglobin (Pohl et al. 1986b) and potentiated by superoxide dismutase (Rubanyi et al. 1986).

The exact transduction of the signal for EDRF release by an increased blood flow remains an enigma. However, Lansman et al. (1987) have identified a nonselective cation channel in the endothelium which exhibits an increased frequency of opening induced by pressure and/or stretch. A shear stress-gated potassium channel has also been identified which may function to hyperpolarize the endothelial cell in response to shear stress and increase the electrical gradient to facilitate Ca^{2+} entry, which would promote the synthesis and release of EDRF (Olesen et al. 1988). Indeed, in human endothelial cells shear stress produces prolonged elevations of IP_3 (Nollert et al. 1990), a compound associated with receptor-mediated production of EDRF (see section 3.2).

2.8.4 Endothelium-Dependent Adaptation to Subacute Changes in Blood Flow

While acute changes in perfusion flow enhance the release of EDRF, prolonged exposure to increases in shear stress in vitro reduces the ability of the endothelium to release endothelium-derived relaxing factor in response to muscarinic activation (Hoeffner and Vanhoutte 1989). This finding is consistent with the observation that, in the isolated resistance rat mesentery, constriction induced by contractile agonists is enhanced at high steady-state flow rates (Tesfamariam et al. 1985). It has been postulated that this phenomenon might be due to adaptation of endothelial cell receptors that are exposed to increased shear stress for prolonged periods of time (Hoeffner and Vanhoutte 1989). However, it could also be due to an enhanced depletion of arginine from the endothelium in vitro during augmented flow which would secondarily attenuate the production of EDRF.

2.8.5 EDRF and Chronic Changes in Blood Flow

Chronic increases in blood flow can also modulate the release of EDRF. Following 6 weeks of elevated blood flow (seven to ten times control flow) due to an arterial-venous fistula, both basal release and stimulated release of EDRF from the canine femoral artery are augmented (V. M. Miller et al. 1986; Miller and Vanhoutte 1988).

2.8.6 Temperature and Endothelium-Dependent Responses

The effect of cooling on endothelium-dependent relaxation depends upon the agonist and the blood vessel examined. For example, in the saphenous artery of the dog, hypothermia (15°C) tends to augment endothelium-dependent relaxation to acetylcholine, while hyperthermia (41°C) impairs the relaxation (De Moura and Vanhoutte 1988). These effects were attributed to a change in cholinergic receptor affinity rather than a direct effect on EDRF release. In contrast, in the rat jugular vein, cooling (10°C) attenuates endothelium-dependent relaxation to serotonin and acetylcholine, while the relaxation to sodium nitroprusside remains unaltered (Bodelsson et al. 1989). In this blood vessel, hypothermia appears not only to impair the stimulated release of EDRF, but also to inhibit its basal production. This could have important pathological consequences for vascular disease such as Raynaud's phenomenon, as cooling also augments the contractile response to aggregating platelets in peripheral arteries (Lindblad et al. 1984).

2.8.7 Endothelium-Derived Vasoactive Factors and the Platelet/Blood Vessel Interaction

The demonstration that the endothelium plays a key role in the immediate changes in vasomotor tone that follow platelet aggregation came from the observation that aggregating platelets caused relaxation of isolated canine coronary arteries if the intima is intact (Fig. 14) (Cohen et al. 1983a). If the endothelium is removed, however, platelets only induce contraction of the blood vessel. These observations also provided a physiological role for endothelium-mediated relaxation of vascular smooth muscle which, up to that point in time, was primarily only of pharmacological interest (Fig. 15) (Cohen et al. 1983a).

2.8.7.1 Platelet-Mediated Release of EDRF. In the canine coronary artery, the endothelium-dependent relaxation to aggregating platelets is due primarily to platelet-derived adenine nucleotides (ATP and ADP) acting on endothelial purinergic receptors to release EDRF, while the constrictor effect of platelets on arteries without endothelium are mediated by the unopposed action of platelet-derived 5-hydroxytryptamine (serotonin) acting on the vascular smooth muscle (Cohen et al. 1983b; Houston et al. 1985). When human platelets are used in canine coronary arteries, adenine nucleotides acting on the endothelium are again found to be the mediators of EDRF release while the constrictor effect of platelets on the vascular smooth muscle is mediated by platelet-derived serotonin and thromboxane A_2 (Houston et al. 1986). Subsequent experiments using human

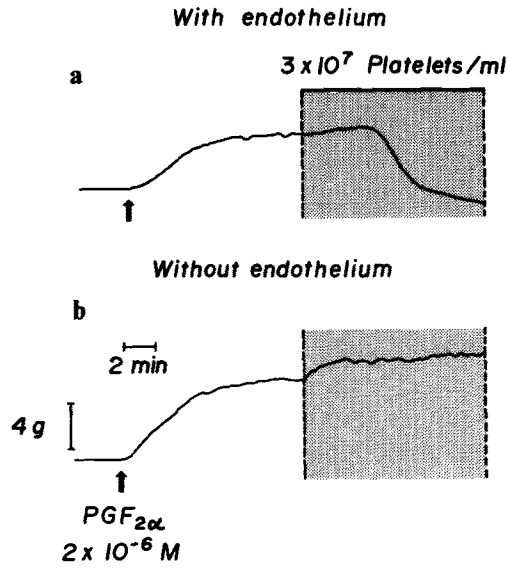


Fig. 14a,b. Effect of aggregating platelets on the responsiveness of isolated canine arteries with **a** and without **b** endothelium during contractions to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). When the platelet suspensions are added to the organ chambers, aggregation occurs immediately. This results in relaxation in the presence, but causes contraction in the absence of endothelium. These experiments demonstrate that aggregating platelets are a strong stimulus for the release of EDRF and that the presence of the endothelium considerably brakes the vasoconstrictor response to the platelet products. (From Cohen et al. 1983a)

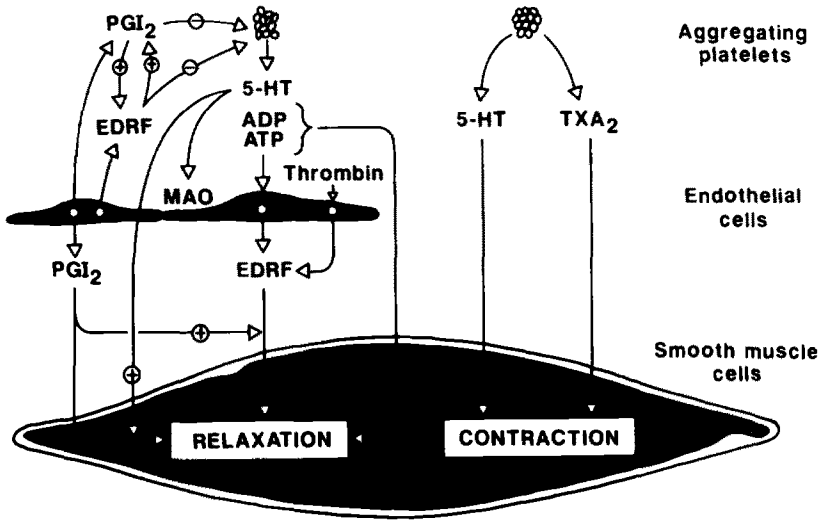


Fig. 15. Interactions between platelet products, thrombin, and the endothelium. If the endothelium is intact, several of the substances released from the platelets [in particular, the adenine nucleotides (*ADP*, *ATP*)] and serotonin (*5-HT*) cause the release of endothelium-derived relaxing factor (*EDRF*) and prostacyclin (PGI_2). The same is true for any thrombin formed. The released EDRF will relax the underlying vascular smooth muscle, opening up the blood vessel, and thus flushing the microaggregate away; it will also be released toward the lumen of the blood vessel to break platelet adhesion to the endothelium and, synergistically with prostacyclin, inhibit platelet aggregation. In addition, monoamine oxidase (*MAO*) and other enzymes will break down the vasoconstrictor serotonin, limiting the amount of the monoamine that can diffuse toward the smooth muscle. Finally, the endothelium acts as a physical barrier that prevents access to the smooth muscle of the vasoconstrictor platelet products serotonin and thromboxane A_2 (TBA_2). These different functions of the endothelium play a key role in preventing unwanted coagulation of blood and vasospastic episodes in blood vessels with a normal intima. If the endothelium cells are removed (e.g., by trauma), the protective role of the endothelium is lost locally, platelets can adhere and aggregate, and vasoconstriction follows; this contributes to the vascular phase hemostastis. (From Vanhoutte 1991)

platelets and human coronary arteries as a bioassay tissue demonstrated that adenine nucleotides were the primary mediators of EDRF release in the human coronary circulation, while serotonin and thromboxane A_2 were constrictors of the vascular smooth muscle (Forsterman et al. 1989). The importance of EDRF over prostacyclin in mediating endothelium-dependent relaxation to aggregating platelets is highlighted by the fact that the relaxation to the platelets is not altered by inhibitors of cyclooxygenase (Cohen et al. 1983a; Fosterman et al. 1989). By contrast, in the porcine coronary artery, endothelium-dependent relaxations to aggregating platelets are due mainly to serotonin-mediated release of EDRF (acting via $5HT\text{-}_{1-like}$-serotonergic receptors) while adenine nucleotides contribute to the balance

of the response by acting on P_{2y}-purinergic receptors (Houston et al. 1987; Shimokawa et al. 1987; Shimokawa and Vanhoutte 1989b) (Fig. 3). In addition, the contractile response to aggregating platelets in porcine coronary vessels without endothelium is primarily due to the action of platelet-derived serotonin acting on $5HT\text{-}_2$ serotonergic receptors of the vascular smooth muscle (Shimokawa and Vanhoutte 1989a). There is heterogeneity in platelet-mediated responses in the same organism in that adenine nucleotides are the primary mediators of endothelium-dependent relaxation in the porcine basilar artery, while constriction after removal of the endothelium is mediated by activation of $5HT\text{-}_{1-like}$ serotonergic receptors on the vascular smooth muscle (Shimokawa et al. 1988b). In addition, between porcine coronary, carotid, femoral, and renal arteries, there exists heterogeneity in the relative contribution of adenine nucleotides versus serotonin to the stimulated release of EDRF by aggregating platelets (Shimokawa and Vanhoutte 1989a). Interestingly, in porcine pulmonary arteries, constrictions induced by aggregating platelets are mediated in part by histamine acting on H_1-histaminergic receptors of the vascular smooth muscle, with minimal contribution of serotonin or thromboxanes (Zellers et al. 1991).

2.8.7.2 EDRF and Platelet Aggregation and Adhesion. Azuma et al. (1986) reported another important endothelium–platelet interaction when they demonstrated that the endothelium of the rabbit aorta inhibited platelet aggregation in vitro, an effect most likely mediated by EDRF. While their experiments were not conducted in such a way as to rule out contribution by prostacyclin, subsequent investigation using appropriate pharmacological blockers definitively demonstrated that EDRF released from the rabbit aorta inhibited human platelet aggregation in vitro (Furlong et al. 1987). These observations were also confirmed using cultured human umbilical vein endothelial cells and washed human platelets (Alheid et al. 1987). With the discovery that the active component of EDRF is nitric oxide (Palmer et al. 1987; Ignarro et al. 1987a,b), investigators confirmed that nitric oxide inhibited human platelet aggregation (Schafer et al. 1980; Mellion et al. 1981, 1983; Radomski et al. 1987a,b) and promoted platelet disaggregation in vitro (Radomski et al. 1987b). Unlike prostacyclin, the antiaggregatory effect of EDRF is potentiated by washing the platelets (Radomski et al. 1987a). This is due to the ability of nitric oxide to bind to (and thus be inactivated by) plasma proteins, in particular to ferrous hemoproteins (Keilin and Hartree 1937). It was this very property of plasma that prompted some investigators to assert that EDRF was only an autacoid with no systemic antithrombotic activity (Edwards et al. 1986). Indeed, human plasma can inhibit endothelium-dependent relaxation due to a haptoglobin–hemoglobin complex that avidly binds endothelium-derived

nitric oxide (Edwards et al. 1986). It has long been known that hemoglobin binds nitric oxide (Gibson and Roughton 1957), and oxyhemoglobin has been used with other hemoproteins as a blocker of endothelium-dependent relaxation even before the active component of EDRF was characterized as nitric oxide (Furchgott et al. 1984; Martin et al. 1985a,b, 1986a). However, the subsequent discovery that EDRF release alters platelet function in vivo establishes the fact that EDRF may play a vital systemic role in the prevention of thrombosis (Hogan et al. 1988).

In addition to inhibiting platelet aggregation, endogenous EDRF (Sneddon and Vane 1988; Radomski et al. 1987c) and nitric oxide (Radomski et al. 1987c) inhibit platelet adhesion to endothelial cells, another property not shared with prostacyclin. This must contribute to the key role of EDRF as mediator of platelet/endothelium interaction.

2.8.7.3 EDRF, Cyclic GMP, and Platelets. The action whereby EDRF mediates its platelet effects is thought to be via activation of the soluble guanylate cyclase. Nitroso compounds inhibit platelet function by stimulating the accumulation of cyclic GMP (Mellion et al. 1983; Haslam et al. 1979; Pareti et al. 1978; Best et al. 1980; Davidson and Haslam 1981; Nishikawa et al. 1982). Indeed, nitric oxide, carcinogenic nitrosamine, and *N*-methyl-*N'*-nitrosoguanidine all activate platelet guanylate cyclase, elevate intracellular concentrations of Cyclic GMP, and inhibit aggregation (Mellion et al. 1983). EDRF released from cultured bovine endothelial cells is associated with activation of guanylate cyclase and inhibition of aggregation of rabbit platelets (Hawkins et al. 1988). In addition, inhibition of guanylate cyclase by LY 83583 partially reverses the inhibitory effect of EDRF on platelet aggregation, adhesion, and secretion (Mulsch et al. 1989b). The lipophilic analog of cyclic GMP (8-bromo cyclic GMP) can inhibit platelet aggregation (Mellion et al. 1981; Pareti et al. 1978). *S*-nitrosothiols also inhibit platelet aggregation and promote platelet disaggregation via heme-dependent activation of soluble guanylate cyclase and stimulation of cyclic GMP accumulation (Mellion et al. 1983).

Selective cyclic GMP phosphodiesterase inhibitors (M&B 22948 and MY 5445) potentiate inhibition of platelet adhesion by both nitric oxide and prostacyclin while the specific cyclic AMP phosphodiesterase inhibitor HL 725 is without effect (Moncada et al. 1988). In addition, forskolin, which directly activates adenylate cyclase, inhibits platelet adhesion; M&B 22948 potentiates the forskolin-mediated inhibition, while HL 725 is without effect. These findings imply that cyclic GMP selectively controls the membrane properties related to platelet adhesion (Moncada et al. 1988).

The accumulation of cyclic GMP inhibits platelet activation apparently by inhibiting calcium influx and mobilization from intracellular stores,

thereby lowering cytosolic calcium which is associated with platelet aggregation (Henderson et al. 1987). This concept is supported by the fact that nitric oxide is less potent at inhibiting aggregation induced by the calcium ionophore A23187 (Radomski et al. 1987a) and can effectively block thrombin-stimulated and inositol 1,4,5-triphosphate mobilization of cytosolic-free calcium in Fura-2-loaded platelets (Rao et al. 1990). The exact mechanism whereby cyclic GMP exerts its effect is not defined. The nitric oxide generating compounds sodium nitroprusside and Sin-1 stimulate platelet cytosolic ribosyltransferase activity and induce ADP ribosylation of a low molecular-weight protein which is associated with a concomitant rise in cyclic GMP (Brune and Lapetina 1989). However, lipophilic cyclic GMP analogues (8-bromo cyclic GMP and dibutyryl cyclic GMP) do not enhance the enzyme's activity (Brune and Lapetina 1989). Thus, the low molecular-weight protein might not be the specific effector of cyclic GMP-mediated platelet deactivation. In addition, serotonin, prostaglandin endoperoxides, and ascorbic acid elevate platelet cyclic GMP without inhibiting platelet function (Schoepflin et al. 1977). These differences could be reconciled if compounds that elevate platelet cyclic GMP but do not inhibit platelet function, themselves promoted calcium entry and thereby negated might otherwise be expected the effect that they to produce.

The modulatory effect of EDRF on cyclic GMP content of washed human platelets can also be demonstrated in the crystalloid-perfused isolated rabbit heart. Platelet cyclic GMP levels increase after passing through the coronary circulation, reflecting a basal level of EDRF production (Pohl and Busse 1989). Indeed, inhibitors of EDRF decrease the recovery of platelets from the coronary perfusate and inhibit the accumulation of cyclic GMP, indicating enhanced aggregation and adhesion in the absence of basally released EDRF. Stimulated release of EDRF in the perfused heart can increase platelet cyclic GMP to almost 300% above control values (Pohl and Busse 1989).

2.8.7.4 Synergistic Action of EDRF and Prostacyclin on Platelets. A reciprocal potentiation of both the anti- and the disaggregating activity is observed between low concentrations of prostacyclin and authentic nitric oxide or EDRF released from endothelial cells (Radomski et al. 1987b; MacDonald et al. 1988; Busse et al. 1989). At subthreshold levels of detection, EDRF and prostacyclin act in concert to inhibit platelet aggregation and promote disaggregation. This synergism is blocked by inhibitors of cyclooxygenase or scavengers of EDRF. The maximal level of platelet disaggregation produced by either nitric oxide or prostacyclin are comparable (approximately 70%) and cannot be further enhanced by addition of the other compound (Radomski et al. 1987b). This suggests a common final

pathway of platelet disaggregation between EDRF and prostacyclin. Prostacyclin also potentiates the basal release of EDRF in the perfused porcine coronary artery (Shimokawa et al. 1988a). This could be due to ligand receptor-mediated activation of phospholipase C, which might be a common transduction mechanism for increasing cytosolic calcium and the generation of both EDRF and PGI_2 (DeNucci et al. 1988a). These findings highlight the fact that EDRF and prostacyclin can regulate platelet/blood vessel interaction at concentrations too low to be detected by chemical analysis (Radomski et al. 1987b).

3 Endothelium-Dependent Contractions

3.1 Endothelium-Dependent Contractions in Response to Hypoxia

Even before the importance of endothelium-dependent responses was recognized, it had been demonstrated that hypoxia augmented contraction to several vasoactive agents in isolated blood vessels (DeTar and Bohr 1972; Vanhoutte 1976; Van Nueten et al. 1980; DeMey and Vanhoutte 1980). In canine arteries and veins, hypoxic augmentation of contraction involves an endothelium-dependent component (DeMey and Vanhoutte 1982, 1983; Rubanyi and Vanhoutte 1985; Katusic and Vanhoutte 1986). In the canine caronary artery, a diffusible factor released by the endothelium contributes and has been termed endothelium-derived contracting factor (EDCF) (Fig. 16) (Rubanyi and Vanhoutte 1985; Vanhoutte et al. 1989). Endothelium-dependent contraction to hypoxia in these canine blood vessels is not modified by inhibitors of cyclooxygenase, lipoxygenase, phospholipase A_2, serotonergic or histaminergic blockers, or by the radical generators quinacrine or phenidone (Rubanyi and Vanhoutte 1985). While hypoxic augmentation of contraction could be attenuated by calcium channel blockers (Katusic and Vanhoutte 1986), the inhibitory effect of these compounds is on the vascular smooth muscle rather than on the endothelium (Iqbal and Vanhoutte 1988). The rapid onset and reversal of the contraction and the inability to bioassay the contracting factor in cascade superfusion experiments make it unlikely that the vasoconstrictor peptide endothelin mediates the response (Vanhoutte et al. 1989). Endothelium-dependent contraction to hypoxia in the canine coronary artery can be blocked by methylene blue and inhibitors of nitric oxide synthesis from L-arginine (Shepherd et al. 1990; Pearson et al. 1990c; Gräser and Vanhoutte 1991). Contractions to hypoxia can be induced in preparations without endothelium by moderate concentrations of donors of nitric oxide or a stable analog of cyclic GMP (Gräser and Vanhoutte 1991). These observations imply that when the level

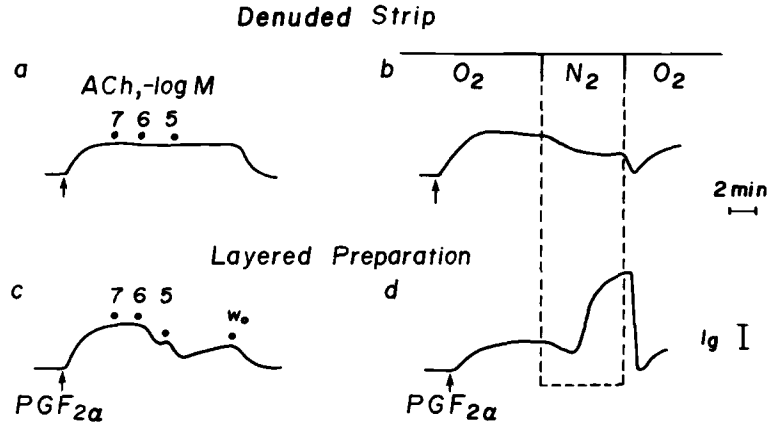


Fig. 16a–d. In a strip of canine coronary artery without endothelium, acetylcholine (*ACh*) causes no relaxation during a contraction to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$); sudden anoxia (N_2) causes a decrease in tension **a,b**. If the strip without endothelium is "sandwiched" (layered) with a strip of artery with endothelium (intimal side against intimal side), the sandwich relaxes to acetylcholine **c** and contracts when made anoxic. **d** This experiment demonstrates that a diffusible substance plays a key role in the endothelium-dependent contractions evoked by anoxia. W_o, washout. (From Rubanyi and Vanhoutte 1985)

of nitric oxide is lowered below a critical level in vascular smooth muscle, the latter becomes sensitive to a direct constrictor effect of hypoxia, which presumably results in the opening of voltage-operated Ca^{2+} channels. Alternatively, nitric oxide formed from L-arginine may combine with superoxide anion formed in the hypoxic milieu of the endothelial cell (Ratych et al. 1987) to form the peroxynitrite anion ($ONOO^-$) (Blough and Zafiriuo 1985). Peroxynitrite anion is toxic and unstable, and can decompose into highly reactive agents (Beckman et al. 1990) which subsequently may be metabolized to EDCF or may induce its synthesis. Such a mechanism would be consistent with the augmentation of endothelium-dependent hypoxic contractions following coronary reperfusion (Pearson et al. 1990c, 1991) which are tissues rich in superoxide anions (Werns et al. 1976; Korthuis and Granger 1986).

Endothelium-dependent hypoxic contractions are not observed in peripheral veins (DeMey and Vanhoutte 1982). However, this lack of response is due to a lack of sensitivity of the venous smooth muscle to the contracting factor, as coronary arterial smooth muscle "sandwiched" with venous endothelium exhibits hypoxic contraction (Rubanyi and Vanhoutte 1985).

In the pulmonary artery and vein, endothelium-dependent hypoxic contraction is particularly prominent (DeMey and Vanhoutte 1982). This response could play an important role in hypoxic pulmonary vasoconstriction (Vanhoutte and McGoon 1986). However, the proposed

mechanisms underlying the hypoxic vasoconstriction in pulmonary blood vessels are controversial. In the rat lung, L-NMMA enhances hypoxic pulmonary vasoconstriction both in perfused tissue and isolated vascular preparations (Archer et al. 1989). L-arginine reverses hypoxic contraction both in the presence and absence of L-NMMA, suggesting that during hypoxia, not only is EDCF produced, but EDRF is also produced (possibly in large amounts) to counteract the effects of the EDCF (Archer et al, 1989). Experiments in isolated pulmonary arteries of the same species support that intermetation (Yuan et al. 1990). However, in isolated segments of rabbit pulmonary artery, hypoxic vasoconstriction is not only inhibited by hemoglobin, methylene blue, and hydroquinone, but is also associated with a concomitant decrease in cyclic GMP production in the vascular smooth muscle (R.A. Johns et al. 1989). These findings suggest that in the rabbit pulmonary artery hypoxic pulmonary vasoconstriction is due in part to a down-regulation of the basal production of EDRF.

Endothelium-dependent contractions to hypoxia also occur in the human internal mammary artery (Lin et al. 1990a). However, in this blood vessel, the contraction can be attenuated by indomethacin or L-NMMA and abolished by a combination of both compounds (Lin et al. 1990b). This suggests that, in the human internal mammary artery, two contracting components are involved during hypoxia: one involves a product of cyclooxygenase (see below) and the other the metabolism of L-arginine into nitric oxide.

3.2 Cyclooxygenase-Dependent, Endothelium-Dependent Contractions

Arachidonic acid induces endothelium-dependent contraction in systemic and pulmonary veins (De Mey and Vanhoutte 1982; Miller and Vanhoutte 1985a), as well as in the basilar artery of the dog (Shirahase et al. 1986; Katusic et al. 1988). The endothelium-dependent contraction to arachidonic acid can be inhibited by blockers of cyclooxygenase, but is not prevented by blockers of thromboxane or prostacyclin synthesis (Miller and Vanhoutte 1985a; Katusic et al. 1988) (Fig. 9). Cyclooxygenase-dependent endothelium-dependent contraction can also be induced by acetylcholine and the calcium ionophore A23187 in the canine basilar artery (Katusic et al. 1988), by acetylcholine in the aorta of spontaneously hypertensive or diabetic rats (Lüscher and Vanhoutte 1986; Tesfamariam et al. 1989, 1990), and by stretch in the canine basilar artery (Katusic et al. 1987).

So far, two mediators of cyclooxygenase-dependent endothelium-dependent contraction have been identified. In the canine basilar artery, superoxide anions, generated by hydroperoxidase activity of cyclooxygenase, are the likely mediator of endothelium-dependent contractions to the calcium ionophore A23187 (Katusic and Vanhoutte 1989). However, in the aorta

of spontaneously hypertensive and diabetic rats, cyclooxygenase-dependent endothelium-dependent contractions are most likely mediated by prostaglandin H_2 (Auch–Schwelk et al. 1990; Kato et al. 1990; Tesfamarian et al. 1990). Interestingly, superoxide anion induces hyperconstriction of hypertensive rat aortae, possibly by acting on prostaglandin H_2 receptors (Auch-Schwelk et al. 1989, 1990).

3.3 Cyclooxygenase-Independent, Oxygen-Derived Free Radicals

Superoxide anion can constrict vascular smooth muscle (Auch-Schwelk et al. 1989, 1990; Katusic and Vanhoutte 1989; Lawson et al. 1990). In various models of endothelial cell dysfunction such as atherosclerosis or during regeneration after intimal injury, serotonin induces endothelium-dependent contractions which are not impaired by blockers of cyclooxygenase (Shimokawa et al. 1987; Shimokawa and Vanhoutte 1989b; cartier et al. 1991). In the rat aorta following intimal regeneration, endothelium-dependent contraction to serotonin is mediated by superoxide anions and is not blocked by indomethacin (Cartier et al. 1991; Lin et al. 1991). Whether superoxide anions mediate the cyclooxygenase-independent endothelium-dependent contractions to serotonin in atherosclerotic blood vessels remains to be determined.

4 Endothelin

Cultured endothelial cells produce a vasoconstrictor peptide(s) (Hickey et al. 1985; Gillespie et al. 1986; O'Brien et al. 1987). The peptide, subsequently isolated and called endothelin by Yanagisawa et al. (1988a,b) is the most potent vasoconstrictor known (EC_{50} of 0.4 nM) in various vascular preparations. Interestingly, it has striking homology with the sarafotoxins produced by the asp (Fig. 17) (Lee and Chiappinelli 1988; Takasaki et al. 1988) and is present in the blood of humans (Peter and Duncan 1989; Ando et al. 1989; Koyama et al. 1989; Cernacek and Stewart 1989).

4.1 Production

4.1.1 Molecular Biology

The endothelin family consists of three vasoconstrictor peptides (Fig. 17) (Inoue et al. 1989a). Endothelin-1 (ET-1) is the only member of the family to be synthesized by endothelial cells (detected either at the messenger RNA or peptide level). Release of the peptide is regulated by synthesis and

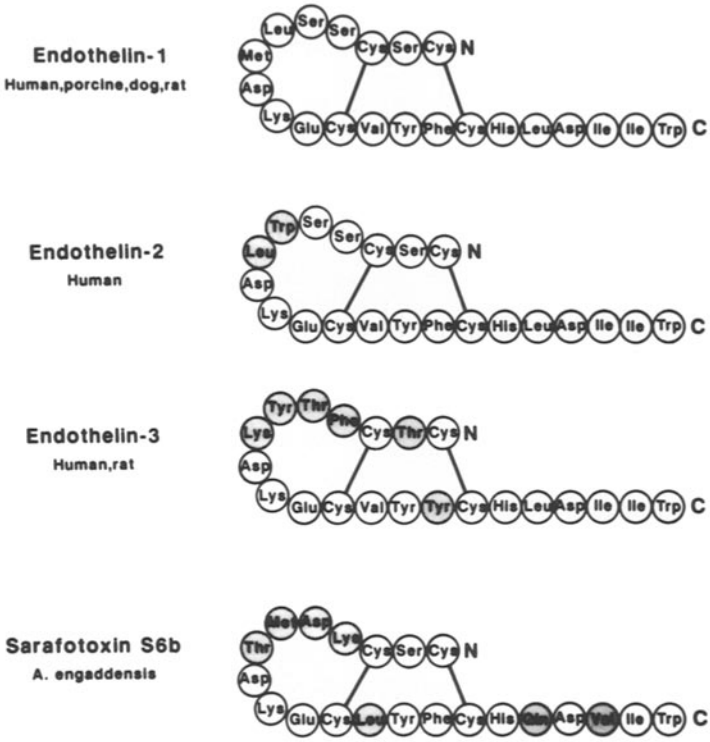


Fig. 17. Structure of the endothelins and sarafotoxin

by post-translational regulation of messenger RNA degradation (Yanagisawa and Masaki 1989). Indeed, the messenger RNA for endothelin has a half-life of 15 min attributed to several 'AUUUA' sequences in messenger RNA's 3' nontranslated regions (Yanagisawa et al. 1989). Since ultrastructural studies demonstrate no dense secretory granules in which synthesized endothelin can be stored, the peptide is apparently produced on demand with the messenger RNA for its production being rapidly inactivated. Factors that induce messenger RNA production and release of endothelin include thrombin (Yanagisawa et al. 1988a; Schini et al. 1989), [arg]-vasopressin (Resink et al. 1988; Emori et al. 1989), angiotensin II (Emori et al. 1989; Yanagisawa et al. 1988a), epinephrine (Yanagisawa et al. 1988a), hypoxia [after 12- (Highsmith et al. 1988) or 24-h (Hieda and Gomez-Sanchez 1990) exposure], the calcium ionophore A23187 (Emori et al. 1989; Yanagisawa et al. 1988a), phorbol esters (Emori et al. 1989; Yanagisawa et al. 1989), and shear stress (Yoshizumi et al. 1989). Aggregating platelets can include the production of endothelin messenger RNA (Kurihara et al. 1989) and endothelin synthesis by releasing platelet-transforming growth factor B-1 (TGF-β) which is stored in platelet α-granules (Emori et al. 1989). This

is consistent with the fact that the endothelin genome contains nuclear factor-1-binding elements (Inoue et al. 1989b) which are involved in gene regulation in response to TGF-β.

4.1.2 Cellular Signalling

The 5′ promotor region of the human endothelin-1 gene contains several components responsive to 12-*O*-tetra-decanoylphorbol 13-acetate which are found in other genes that can be activated by phorbol esters (Inoue et al. 1989b). The production of endothelin-1 by human endothelial cells is probably induced by activation of the phosphatidyl inositol system and increased intracellular calcium. This is supported by the findings that the calcium ionophore A23187 and phorbol esters can stimulate endothelin production in vitro (Emori et al. 1989; Yanagisawa et al. 1989).

4.1.3 Synthesis of Endothelin

Endothelin is sythesized by endothelial cells initially as a 203 residue peptide named pre-pro endothelin. After subsequent processing, it becomes the 39 amino acid termed "big endothelin" (Emori et al. 1989). Big endothelin, which is secreted by cultured human and bovine endothelial cells, has a binding affinity two orders of magnitude less than endothelin and is two orders of magnitude less potent in its vasoconstrictor effects (Hirata et al. 1990). Big endothelin is transformed by enzymatic cleavage (endothelin-converting enzyme) between $TRYP^{21}$ and VAL^{22} to produce the very potent 21 amino acid endothelin.

4.2 Interactions with Endothelial Cells

Under basal conditions, an intact endothelium attenuates the contractile response to endothelin (Saito et al. 1989; Warner et al. 1988; De Nucci et al. 1988b; U.M. Miller et al. 1989), apparently in a similar manner to endothelium-dependent inhibition of contraction observed to agonists such as 5-hydroxytryptamine (Cohen et al. 1983b), ergonovine (Shimokawa et al. 1989), and norepinephrine (Miller and Vanhoutte 1985b). This could be due both to basally released EDRF and stimulated release by endothelin. Endothelium-derived nitric oxide factor inhibits the stimulated release of endothelin from cultured cells (Boulanger and Lüscher 1990). In addition, EDRF released by the endothelium profoundly inhibits the constrictor response to the peptide (Fig. 18) (Vanhoutte et al. 1989; U.M. Miller et al. 1989).

Endothelin also stimulates the release of EDRF (Fig. 18) (Warner et al. 1989; De Nucci et al. 1988b; Schini et al. 1990). In the perfused rat mesen-

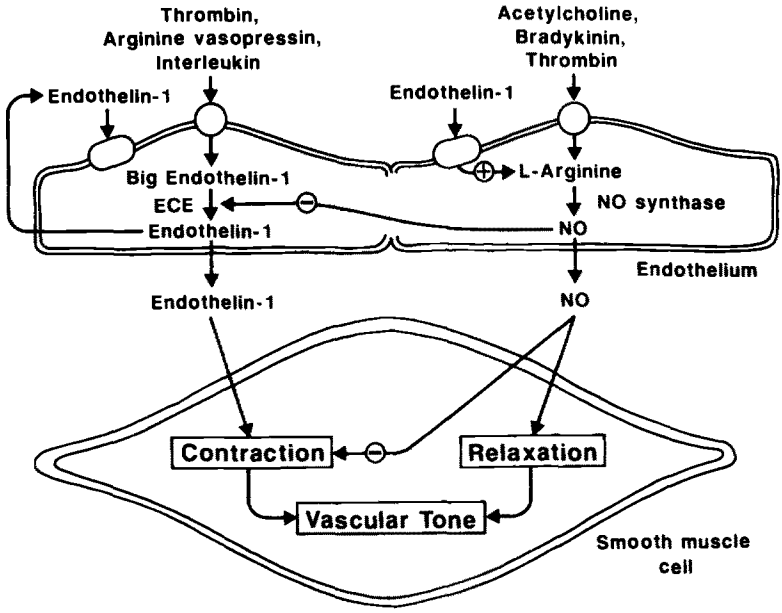


Fig. 18. The activation of endothelial cells by several vasoactive hormones and neurotransmitters enhances the production of the potent contractile peptide endothelin-1 and the potent relaxing factor nitric oxide (*NO*). The production of endothelin-1 results from the procession of its precursor big endothelin-1 by the endothelin converting enzyme (*ECE*), while the production of nitric oxide results from the conversion of the amino acid L-arginine into L-citrulline by nitric oxide synthase. Nitric oxide curtails both the production and the action of endothelin-1. Endothelin-1, if it appears in the blood stream, can stimulate the production of endothelium-derived nitric oxide, which then counteracts its potent contractile activity. The interaction between endothelin-1 and nitric oxide produced by endothelial cells may be of key importance in the local regulation of vascular tone. (From Schini and Vanhoutte 1991)

teric artery, endothelium evokes an initial dilation which is inhibited by methylene blue or hemoglobin, but is unaffected by indomethacin (Warner et al. 1989). By contrast, in the perfused rat heart, hemoglobin or methylene blue do not modify the initial vasodilation to endothelin (Baydoun et al. 1990). This can be explained by the peptide's stimulation of the production of prostacyclin (De Nucci et al. 1988b; Karwatowska-Prokopczuk and Wennmalm 1990).

4.3 Effects on Vascular Smooth Muscle

Endothelin-1 (ET) binds to a single class of sites (ET_A receptors) on the cell membrane of vascular smooth muscle (Marsden et al. 1989; Hirata et al. 1988a; Power et al. 1989; Stasch et al. 1989). Reversible binding of endothelin has been demonstrated in the porcine aorta (Kanse et al.

1988). Endothelin-1 is not an agonist for the dihydropyridine-binding site that constitutes the voltage-dependent, L-type calcium channel (Hirata et al. 1988b; Auguet et al. 1988; Gu et al. 1989), since dihydropyridines do not interfere with endothelin binding (Van Renterghem et al. 1988).

The actions of endothelin on vascular smooth muscle eventually result from a rise in intracellular calcium (Fig. 19) (Marsden et al. 1989; Hirata et al. 1988b). Endothelin increases the calcium peak in Fura-2-loaded vascular smooth muscle cells (Marsden et al. 1989; Hagiwara et al, 1988) even in the absence of external calcium (Marsden et al. 1989). When applied outside the patch in single cell patch-clamp experiments, the peptide induces a rapid increase in the calcium currents inside the patch (Silberberg et al. 1989). This indicates that the peptide acts by a readily diffusible second messenger. The available evidence suggests that endothelin acts via the metabolism of phosphatidylinositol 4,5-biphosphate (PIP_2) in vascular smooth muscle cells. It is likely that endothelin stimulates the phosphoinositide cascade by activating phospholipase C to generate IP_3 (Resink et al. 1988; Marsden et al. 1989; Van Renterghen et al. 1988; Sigura et al. 1989). This interpretation is supported by the finding that inhibitors of phospholipase C abolish the actions of endothelin on vascular smooth muscle (Sugiura et al. 1989).

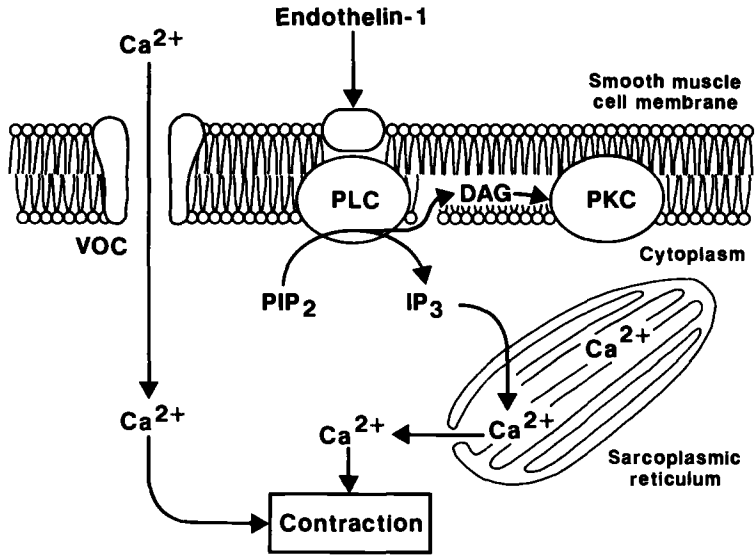


Fig. 19. Endothelin-1 is a potent and long-lasting vasoconstrictor peptide. The contractions are due to an influx of extracellular calcium by activation of L-type calcium channels (voltage-operated channels, *VOC*) and also to the mobilization of intracellularly stored calcium following the activation of phospholipase C (*PLC*) with the resulting production of inositol trisphosphate (IP_3) and diacylglycerol (*DAG*). PIP_2, phosphadidylinositol 4,5-bisphosphate; *PKC*, protein kinase C. (From Schini and Vanhoutte 1991)

The relative role of extracellular calcium and contribution of voltage-dependent L-type channels in smooth muscle contraction elicited by endothelin most likely reflects heterogeneity in vascular smooth muscle. Increases in the calcium signal in Fura-2-loaded rat aortic smooth muscle cells is partially inhibited by ethylene glycol tetra-acetic acid (EGTA) and the absence of extracellular calcium (Auguet et al. 1988) or by dihydropyridine calcium channel blockers (Hirata et al. 1988b; Hagiwara et al. 1988; Van Renterghem et al. 1988). In vivo, manganese and verapamil impair the pressor response to infused endothelin, implying a role for calcium entry (Cao and Banks 1990). Nifedipine antagonizes contraction, while diltiazem, another blocker of the same calcium channel, does not (Topouzis et al. 1989). However, in the porcine coronary artery and in canine veins, contractions to endothelin are not antagonized by Ca^{2+} channel blockers (Hagiwara et al. 1988; Vanhoutte et al. 1989). Overall, contractions of smooth muscle to endothelin are independent of the entry of extracellular calcium in some systems (Auguet et al. 1988), while they depend on it in others (Goto et al. 1989). In addition, the requirement for extracellular calcium can be concentration dependent, since in the porcine coronary artery the response to endothelin requires external calcium at peptide concentrations below 3–5 nmol, while higher concentrations of the peptide induce contraction in its absence (Goto et al. 1989). In preparations that require external calcium for contraction, endothelin may activate nonselective cation channels which permit the entry of extracellular calcium and only secondarily activate L-type calcium channels (Van Renterghem et al. 1988).

Endothelin is more potent in arterioles than in large arteries (Antonio and Tippins 1989) and more potent in veins than arteries (Vanhoutte et al. 1989; V.M. Miller et al. 1989). For example, the threshold for endothelin-induced contraction and smooth muscle depolarization in veins is 100 times lower than in arteries (Vanhoutte et al. 1989; V.M. Miller et al. 1989).

4.4 Vascular Effects In Vivo

In vivo, infusion of endothelin induces a transient vasodilation (Lippton et al. 1988; Wright and Fozard 1988; De Nucci et al. 1988b) which can be attributed to the release of EDRF (Warner et al. 1989; De Nucci et al. 1988b) or prostacyclin (Rae et al. 1989) or to an unrelated effect of the peptide on the vasculature (Baydoun et al. 1990). The pressor response to endothelin results from an increase in peripheral vascular resistance and has been demonstrated in conscious rats (Fink et al. 1989; Han et al. 1989), conscious dogs (Goetz et al. 1988), anesthetized rats (Yanagisawa et al. 1988a,b), and anesthetized dogs (W.M. Miller et al. 1989). The half-life of parenterally administered endothelin is approximately 2 min (Pernow et al.

1989; Shiba et al. 1989), while the pressor response is typically sustained for more than 1 h. This could be associated with a slow dissociation of endothelin from its receptors on smooth muscle (Hirata et al. 1988a). In addition, endothelin is stable in heparinized whole blood in vitro and is not significantly altered in the bloodstream (Pernow et al. 1989; Shiba et al. 1989). The compound is most likely cleared by trapping in parenchymal organs such as the kidney, liver and spleen (Koseki et al. 1989; Shiba et al. 1989), or the lung. However, the possible role of peptidases should not be discounted (Maggi et al. 1990; Hay 1989).

4.5 Effect on Coronary Perfusion

In the isolated heart, endothelin initially produces dose-dependent vasodilatation and has a positive chronotropic effect (Baydoun et al. 1989) followed by a depressor response (Karwatowska-Prokopczuk and Wennmalm 1990). The depressor response (characterized by decreased oxygen uptake, myocardial acidosis, and release of purines) can be mimicked by inducing comparable vasoconstriction alone (Karwatowska-Prokopczuk and Wennmalm 1990). Endothelin may increase the release of prostacyclin in the perfused heart (Karwatowska-Prokopczuk and Wennmalm 1990) or have no prostanoid-stimulatory effect on prostanoid production (Baydoun et al. 1990). To judge from studies with methylene blue and hemoglobin, endothelin probably does not induce the release of EDRF in the perfused rabbit heart (Baydoun et al. 1990).

4.6 Platelets

In vitro, endothelin does not modify the aggregation of platelets (De Nucci et al. 1988b; Thiemermann et al. 1990; Patel et al. 1989; Edlund and Wennmalm 1990) or their content of cyclic AMP or cyclic GMP (Thiemermann et al. 1988). In human platelets, endothelin does not modify the response to collagen, adenosine diphosphate, thrombin, epinephrine, arachidonic acid, prostaglandin H_2, or thromboxane analogs (Ohlstein et al. 1990). In addition, endothelin does not induce granular release or calcium mobilization in human platelets (Ohlstein et al. 1990). However, the peptide may potentiate the action of subthreshold concentrations of norepinephrine on platelet aggregation in certain subjects (Matsumoto et al. 1990).

In vivo, endothelin can inhibit platelet aggregation (Thiemermann et al. 1988, 1989, 1990). This platelet-inhibiting effect is transient (15-30 min), is associated with an increase in platelet cyclic AMP, and can be abolished by treatment with indomethacin (Thiemermann et al. 1990). It can thus be attributed to the release of prostacyclin. Endothelin-1 and endothelin-3 have

comparable inhibitory influences on platelet function (Lidbury et al. 1989), while endothelin-3 does not possess pressor activity (Walder et al. 1989; Lidbury 1989). Thus, the endothelin-induced prostacyclin release must be an endothelin receptor-coupled event rather than a response to the elevated pressure or increased vascular resistance.

4.7 Growth

Endothelin may promote the proliferation of vascular smooth muscle and fibroblasts (Komuro et al. 1988; Takuwa et al. 1989).

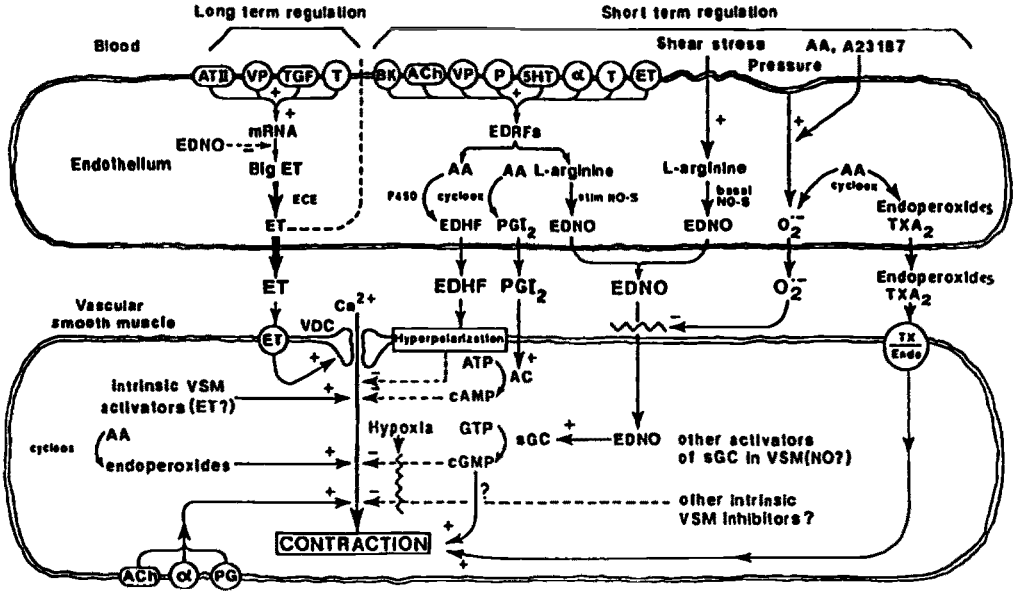


Fig. 20. Neurohumoral mediators which cause the release of endothelium-derived relaxing factors (*EDRFs*) through activation of specific endothelial receptors (*circles*). In addition, EDRF can be released independently of receptor-operated mechanisms by shear stress. The endothelial cells also release substances that can evoke endothelium-dependent contractions. *AA*, arachidonic acid; *AC*, adenylate cyclase; *ATII*, angiotensin receptor; *ATP*, adenosine triphosphate; *α*, alpha$_2$-adrenergic receptor; *BK*, kinin receptor; *cAMP*, cyclic adenosine monophosphate; *cGMP*, cyclic guanosine monophosphate; *cycloox*, cyclooxygenase; *5HT*, serotonergic receptor; *ACh*, muscarinic receptor; *ECE*, endothelin converting enzyme; *EDHF*, endothelium-derived hyperpolarizing factor; *EDNO*, endothelium-derived nitric oxide; *ET*, endothelin, endothelin receptor; *GTP*, guanosine triphosphate; *NO*, nitric oxide; *NO-S*, NO synthase; *P*, purinergic receptor; *PG*, prostaglandin receptor; *PGI$_2$*, prostacyclin; *sGC*, soluble guanylate cyclase; *T*, thrombin receptor; *TGF*, transforming growth factor B receptor; *TX/Endo*, thromboxane endoperoxide receptor; +, activation; −, inhibition; *TXA$_2$*, thromboxane A$_2$; *VOC*, voltage-operated C^{2+} channel; *VP*, arginine vasopressin receptor; *VSM*, vascular smooth muscle

5 Conclusions

The endothelium controls the tone of the underlying smooth muscle by secreting a variety of factors (Fig. 20) which can either cause relaxation or contraction. In addition, particularly through the production of nitric oxide and prostacyclin, it helps to control the adhesion and the aggregation of platelets. The different mediators and their relative importance vary from blood vessel to blood vessel. Under pathological conditions, the release (or the action) of relaxing (and antiplatelet) mediator is usually curtailed, while that of the constrictor factors is maintained or even augmented; this favors the occurrence of hyperconstriction (vasospasm) and thrombosis (see Vanhoutte and Shimokawa 1989; Lüscher and Vanhoutte 1990; Vanhoutte 1991).

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Rev. Physiol. Biochem. Pharmacol., Vol. 122

Muscle Relaxation and Sarcoplasmic Reticulum Function in Different Muscle Types

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Contents

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1 Introduction and Historical Overview

The early classification of the skeletal muscle types was based on their morphological, physiological, and biochemical heterogeneity (Padykula and Herman 1955; Dubowitz and Pearse 1960; Burke et al. 1971, 1973; Buchthal and Schmalbruch 1980; Eisenberg 1983; Pette 1985). An explosive increase in information regarding the molecular basis of this heterogeneity, as well as its role in the functional adaptation of the muscles, has taken place during the past decade.

The distinction of the individual muscle (fiber) types now seems to require a more flexible approach, since several characteristics of the muscle follow a continuous distribution within a certain range, instead of falling into well-separated categories (Ogata 1988; Pette and Staron 1990). The technique of single fiber isolation, together with microdetection methods, revealed the coexistence of several components previously believed to be characteristic for only one or the other fiber type (Staron et al. 1983, 1987).

Our understanding of the mechanism of the Ca^{2+} regulation in the muscle and its role in the relaxation process has come a long way since the concept of the "relaxing factor" was formulated (Marsh 1951, 1952; Bendall 1952, 1953). The emerging high-resolution structural data on the main proteins involved in the process, and the biochemical, physiological characterization of their site-directed mutants, created a new dimension in the research on muscle heterogeneity (Wagenknecht et al. 1989; Stokes and Green 1990a; Tanabe et al. 1990a,b; MacLennan 1990). At the same time, the diversity of the molecular systems responsible for the Ca^{2+} regulation and relaxation may help to generate a comprehensive view of the adaptation of the muscle to altered environmental conditions and functional demands – in a broader sense, of the adaptation of biological systems in general.

In 1949 Hill defined relaxation as the process "whereby after the contraction the muscle returns to its initial length or tension" (Hill 1949). The identification of the contractile proteins and their interactions helped to create the now generally accepted sliding filament contraction mechanism theory (Szent-Györgyi 1951, 1953; Huxley and Niedergerke 1958; Huxley and Taylor 1958). According to this theory, the molecular background of relaxation, is the separation of the actin and myosin filaments, or the breakup of the cross-bridges. It was first attributed to the elimination of ATP from the muscle cytoplasm (Engelhardt 1946). Marsh and Bendall isolated a so-called relaxing factor (Marsh factor), a protein with ATPase activity (Marsh 1951, 1952; Bendall 1952, 1953). Kumagai et al. described this relaxing factor as a lipid-containing protein fraction (Kumagai et al. 1955), identical with the fraction responsible for the lipid-dependent ATPase activity detected earlier in the actin- and myosin-free high-speed pellet of muscle homogenates by Kielley and Meyerhof (1948, 1950).

In nearly parallel studies, Ebashi and Hasselbach recognized independently the role of the relaxing factor (Marsh factor, Erschlaffungsfaktor) in the binding of calcium (Ebashi 1960, 1961; Hasselbach and Makinose 1961). The high-speed pellet fraction of the muscle was identified as the endoplasmic reticulum (sarcoplasmic reticulum) of the muscle, which forms sealed vesicles capable of accumulating calcium at the expense of ATP hydrolysis (Nagai et al. 1960; Hasselbach and Makinose 1961; Ebashi and Lipmann 1962). The enzyme responsible for the ATP-dependent calcium transport was named sarcoplasmic reticulum (SR) Ca^{2+}-ATPase.

Other muscle components influencing the amount and distribution of Ca^{2+} in the muscle cytoplasm have major impacts on the contraction-relaxation cycle as well (Klein et al. 1991). Therefore, the relaxation of a muscle represents a set of different biophysical, biomechanical, and biochemical events. Any of these events alone or in combination with others may alter the complex physiological process we call relaxation. The interpretation of the relaxation properties of a muscle should consider the delicate network of these parameters.

The functional heterogeneity of skeletal muscles is reflected in their relaxation properties as well. The great amount of information collected during the past years on the factors influencing muscle relaxation, has helped to create a more comprehensive view. This article is an attempt at summarizing those data, with a focus on the factors possibly responsible for the differences in relaxation of different skeletal muscle types. The relaxation mechanism of the cardiac muscle shares many features with that of the skeletal muscles, particularly the slow type. A detailed review on cardiac muscle relaxation has been published recently by Brutsaert and Sys (1989). Despite the similarities, the complexity and diversity of relaxation

processes in smooth muscles and in invertebrate muscles does not allow their inclusion in this work.

Chapter 2 summarizes the muscle type-specific differences in muscle inactivation and relaxation detected by physiological methods, and the possible mechanisms responsible for these differences. Chapter 3 covers the molecular systems participating in the muscle activation and inactivation. The chapters that follow describe the main differences between the sarcoplasmic reticulum system and the Ca^{2+}-ATPase enzyme in different muscle types, and their involvement in muscle adaptation and disease. The final chapter outlines the challenging still-open questions and future perspectives of muscle relaxation and sarcoplasmic reticulum research.

Fundamental details on the physiology of muscle relaxation, as well as on the biochemistry and thermodynamics of sarcoplasmic reticulum function, can be found in the following reviews: Martonosi and Beeler (1983); Gillis (1985); Kodama (1985); Inesi et al. (1990).

2 Physiological Differences in the Inactivation and Relaxation of Skeletal Muscle Types

2.1 Relaxation Properties of the Different Skeletal Muscle Types

The relaxation characteristics of different muscle types are not identical. In the rat soleus muscle, Close detected two populations of motor units, one with an average isometric twitch contraction time of 38 ms, the other with one of 18 ms. In the extensor digitorum longus muscle, at 35°C, all the motor units had an isometric twitch contraction time of around 11 ms. The half relaxation time was about 6.6 times longer in rat soleus than in extensor digitorum longus (EDL) muscles (Close 1967). In the gastrocnemius muscle of the cat, Burke et al. detected three motor unit populations with average twitch contraction times of 34 ms, 40 ms, and 73 ms respectively (Burke et al. 1971). Histochemical methods identified the fastest, fatigue-sensitive unit as the IIB, or fast glycolytic muscle type, the slightly slower, fatigue resistant group as the IIA, fast oxidative glycolytic, and the slowest, extremely fatigue resistant population as the I, or slow oxidative fiber type (Buller et al. 1960; Pette and Vrbova 1985; Pette and Staron 1990) (Fig. 1).

The comparison of contraction-relaxation data between different studies or different laboratories is rather complicated. The experimental conditions, with a variety of preparations such as intact muscles, muscle fiber bundles, isolated individual fibers, different incubation conditions regarding temperature, concentration of ions, and other substances in the medium, different stimulation methods and protocols, may shift the absolute value of the measured parameters within a wide range. The broad variety of physiological

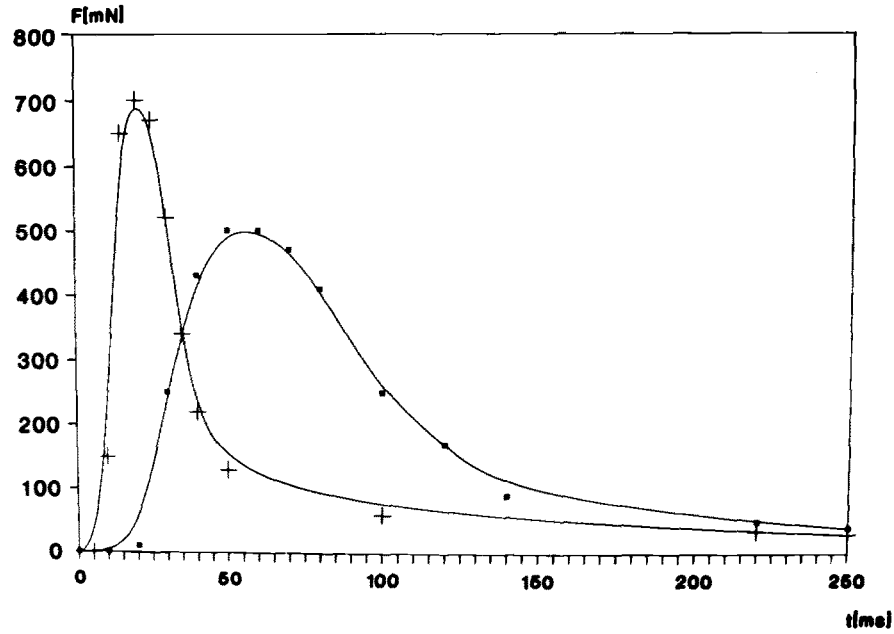

Fig. 1. Single-twitch contractions of rabbit fast and slow muscles: +, m. gastrocnemius, ⊡, m. soleus. (Unpublished results of I. Ocsovszky, reproduced courtesy of the author)

parameters used to characterize the relaxation process (i.e. half relaxation time, maximal slope of the tension decline, rate constant of the exponential decay phase, stimulation frequency needed for fused tetanus, etc.) generates further complications. Critical assessment of these different parameters can be found in the review by Gillis (1985).

Despite all the variability, there is a clear difference in the relaxation speed of the different skeletal muscle types. There are two fast populations, differing from each other only in their resistance to fatigue, and a third population that is two to three times slower.

An interesting overlap exists in the relaxation speed of the different muscle populations between animal species with different body sizes. Muscles of smaller animals, sometimes even the slow-type muscles, relax faster than the fast-twitch muscles of animals with larger body weight (Barnard et al. 1971; Luff 1981). This warrants further caution in interpreting data obtained on different species.

Fibers falling into the same histochemical-type categories in different rat muscles showed differences in their contraction speed (Thornell et al. 1987). The IIA-type fibers, (identified on the basis of SDH staining, myosin heavy-chain content, and M-band structure) in the anterior tibial muscle had 12–13 ms contraction time, in contrast to the 16–20 ms measured in

the same type fibers originating from the soleus. The contraction time of the type-I fibers from the anterior tibial muscle was 20 ms, in the soleus 30–37 ms (Thornell et al. 1987). Kugelberg and Thornell found larger ter-

sensitivity, selectivity, and spectral parameters were used in those studies. For technical details, advantages, and limitations of the methods see the following reviews: Jobsis and O Connor (1966); Ashley and Ridgway (1970); Blinks et al. (1978); Wier 1980; James-Kracke (1986); Cobbold and Rink (1987). The recently developed simultaneous use of high- and low-affinity indicators (i.e., fura-2 and arsenazo III) in the same experiment improved the resolution of the in situ calcium detection methods (Klein et al. 1991; Simon et al. 1991).

A comparative study of the calcium signal in slow and fast mammalian muscles was carried out by Eusebi et al. (1980, 1985). Using aequorin as the indicator, they detected much shorter Ca^{2+} signals in the EDL than in the soleus muscle of rats. At 25°C, the half rise time of the signal was 13.4±0.57 ms in EDL versus 17.7±0.5 ms in soleus. The decay time of the signal was 16±4 ms in EDL, 42.4±13 ms in soleus. The amplitude of the Ca^{2+} signal in the EDL was found to be two to three times higher as well. Despite the known uncertainties in the calibration of the fluorescent responses, this difference fits well with the morphometrically detected differences in the amount of sarcoplasmic reticulum in these two muscle types (Eisenberg 1983; Ferguson and Franzini-Armstrong 1988) (Fig. 2).

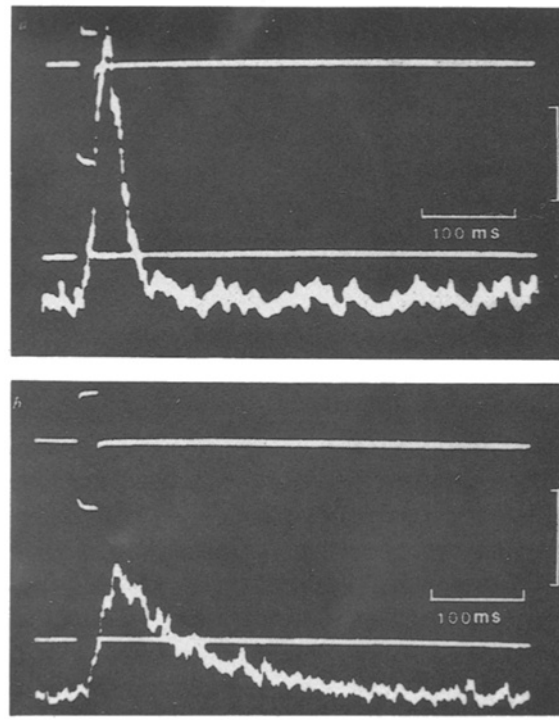

Fig. 2. Calcium transient in extensor digitorum longus (*upper panel*) and soleus (*lower panel*) muscle of rat. The lowest traces on both panels show the aequorin responses, the middle ones record membrane potentials, and the upper ones the clamp currents. Calibration *bars* are 2 nA for the light responses, 50 mV for the membrane potentials, and 1 μA for the clamp current; temperature, 25°C. A time constant of 5 ms was used for the light recordings. (From Eusebi et al. 1980)

The calcium transient remained unchanged in both muscle types when tested in Ca^{2+} -free media, indicating that the source of the activator is within the muscle, most likely the sarcoplasmic reticulum (Eusebi et al. 1980, 1985). Decreasing the incubation temperature slowed the Ca^{2+} signal, both in the soleus and in the EDL, giving a nearly linear temperature-dependence plot between 15° and 40°C. The studies of Eusebi et al. (1980, 1985) reflected important differences between the fast and slow skeletal muscles regarding their ways of handling Ca^{2+}.

2.3 Possible Mechanisms Responsible for Muscle-type-specific Differences in Relaxation

The conventional physiological methods have certain limitations in identifying subcellular or molecular structures responsible for the differences in the relaxation properties of muscle types. There are theoretically at least four levels at which the rate of muscle relaxation can be limited: (a) the Ca^{2+} affinity of the troponin complex, (b) the reuptake of calcium into the sarcoplasmic reticulum, (c) the rate of the breakup or rotation of the cross-bridges between the actin and myosin molecules, and (d) general viscoelastic parameters of the muscle (Stein et al. 1982). The amount of calcium liberated from the SR and/or flowing in from the extracellular space and the kinetic parameters of the activation system, together with cytoplasmic Ca^{2+} buffering elements, may contribute to the relaxation detectable by physiological methods.

Extensive studies on the thermal sensitivity of the relaxation process in both fast and slow mammalian muscles concluded that the rate-limiting step in relaxation is the Ca^{2+} uptake into the sarcoplasmic reticulum, in single twitches as well as in tetanus (Sandow and Zeman 1979; Briggs et al. 1977; Fryer and Neering 1986). Stein et al. found activation enthalpy values for the tension decay of 117 kJ/mol below 20°C and 70 kJ/mol above this temperature (Stein et al. 1982). These values are remarkably similar to the activation energy values for the SR Ca-ATPase found by Inesi et al.: 117 kJ/mol below and 70 kJ/mol above the breakpoint in the Arrhenius plots at 20°C (Inesi et al. 1973).

The slowing of relaxation at reduced temperatures, the so-called cold tension, was found to be more prominent in the slow soleus than in the fast EDL muscle of the rat and the cat (Hill 1972; Iaizzo and Poppele 1990). This supports the view that the less-developed SR system imposes more limits on relaxation under certain circumstances.

Repeated stimulation of mammalian muscles increases the tension two- to threefold, and causes a significant delay in the relaxation rate (Hartree and Hill 1921; Cooper and Eccles 1930; Ritchie and Wilkie 1955; Rosenblueth

and Rubio 1960; Norris 1961; Burke et al. 1970). This delayed relaxation is probably part of the fatigue process (Gollnick et al. 1991; Westerblad and Lennergren 1991; Westerblad et al. 1991).

Chua and Dulhunty, using diazepam to block the Ca^{2+} uptake into the sarcoplasmic reticulum, detected a 138% increase in the single-twitch relaxation time of rat soleus. The EDL muscle presented only a 16% increase (Chua and Dulhunty 1987). In repeated stimulation, ultimately in tetanus, this difference between the reaction of the two muscle types investigated largely diminished. They concluded that the SR plays a rate-limiting role in the soleus even in single twitches, while in fast muscles with a more abundant and active sarcotubular system, and with a larger amount of parvalbumin, the SR function becomes rate limiting only under the excess Ca^{2+} load of repeated stimulation. However, the interaction of diazepam with other muscle components cannot be ruled out in this experiment.

The load dependence of muscle relaxation (i.e., unloaded muscles relax slower than they do under loaded conditions) is considered to be related to the abundance and activity of the SR Ca^{2+} transport system as well. This load dependence was found in both fast and slow skeletal muscles, as well as in cardiac muscles of mammals (von Kries 1892; Jewell and Wilkie 1960; Bahler 1971; Brutsaert et al. 1978a, 1978b). In frog muscles, with a less developed sarcoplasmic reticulum system, load dependence of relaxation was not detected. The most likely explanation for the phenomenon is that a well-developed, active SR system eliminates the calcium fast enough so that the load-induced forced detachment of the cross-bridges determines the rate at which the muscle relaxes, while with the less-developed sarcotubular system the elimination of calcium remains the slowest step in the process, irrespective of the loading conditions (Bahler 1971; Brutsaert et al. 1978a; Huxley 1985).

Besides the undisputed function of the sarcoplasmic reticulum in relaxation, several other systems may also play a role in the chain of events which finally determines the speed at which a certain muscle can relax. These additional mechanisms may explain some of the functional heterogeneities of the muscle types with respect to their relaxation as well.

The number of actomyosin cross-bridges in a muscle depends on the overlap of the contractile filaments, i.e., the length of the muscle under study (Bremel and Weber 1972). Stein et al. (1982) observed a delayed relaxation in rat and mouse fast and slow muscles stretched beyond the length producing maximum twitch tension. The distortion of the structural elements, related to the activation of the muscle, may explain why stretching of a muscle leads first to an increased, later – under further stretching – to a decreased calcium signal (Blinks et al. 1978). The calcium affinity of the main regulatory protein troponin C depends on the actual sarcomere length

(Robertson et al. 1982; Stephenson and Williams 1983; Hoffmann and Fuchs 1987). These altered regulatory functions of the activation side may cause differences in relaxation as well (Endo 1972). No direct comparison of these functions in mammalian fast and slow skeletal muscles has been published.

Some studies detected a higher calcium sensitivity in skinned mammalian slow muscles than in fast ones (Kerrick et al. 1976; Takagi and Endo 1977). Stephenson and Wilkie, however, observed a 10%–90% increase in contraction force of skinned fast muscles after a threefold increase in the Ca^{2+} concentration. A similar increase in force required a tenfold increase in the calcium concentration in the slow soleus muscle (Stephenson and Wilkie 1981). Since the skinning procedure eliminated the differences at the sarcolemmal-T tubule level, and the addition of sodiumazide and caffeine eliminated the possible role of the mitochondrial and sarcoplasmic reticular calcium regulation, they concluded that the contractile apparatus was the key determining element in the relaxation speed of skeletal muscles. In these experiments the skinned fast muscles relaxed about six times faster than the slow ones with rapid decreases in the Ca^{2+} concentration of the incubating medium.

The troponin C in fast skeletal muscles has four calcium-binding sites while the slow/cardiac isoform has only three (Wilkinson 1980; Robertson et al. 1981; Pan et al. 1990). Two of the binding sites, in both the fast and the slow/cardiac forms, have high affinity. The calcium exchange rate at these sites does not support their possible involvement in the regulation of contraction-relaxation (Potter and Gergely 1975). The low-affinity sites, two in the fast, only one in the slow/cardiac isoforms, are the real Ca^{2+}-sensitive regulator of the contractile machinery. The millisecond time scale of binding and release is compatible with the on-off rate of the calcium signals (Robertson et al. 1981; Johnson et al. 1981). Detailed comparative studies on the fast and slow troponin C structure and function are needed to clarify the contradictory results published regarding the Ca^{2+} sensitivity of the fast and slow muscle types (Schachat et al. 1987, 1990; Briggs and Schachat 1989; Härtner and Pette 1990).

Oscillations in the cytoplasmic Ca^{2+} concentration were detected in skinned slow and cardiac muscles (Fabiato and Fabiato 1978; Stephenson and Wilkie 1981). No similar phenomenon was observed in skinned fast-twitch fibers. The skinning procedure, as well as the presence of azide and caffeine, excluded the involvement of membranous compartments in this oscillation. Inhomogeneous small contractions in certain sarcomeres, with subsequent stretch-induced affinity changes in the troponin C molecules in neighboring ones, may explain the phenomenon (Stephenson and Wilkie 1981; Edman and Flitney 1982).

Differences in the composition of myosin light chains and their different phosphorylation properties may contribute to the different Ca^{2+}-sensitivity and relaxation properties in the different muscle types as well (Bárány et al. 1965; Bárány 1967; Ashley and Moisescu 1972; Frederiksen 1980; Moore and Stull 1984). However, in 1976, using two rabbit muscles (soleus and crureus) with identical myosin ATPase activities, Brody detected a difference in their relaxation speed, proportional in size to the difference found in their SR Ca^{2+} transport activity (Brody 1976).

The molecular characteristics of the different contractile protein isoforms and their possible contribution to the relaxation properties was recently reviewed by Gillis (1985), Brutsaert and Sys (1989), and Pette and Staron (1990). Its detailed discussion is beyond the scope of this article.

The less-known mechanoelastic properties of the titin filaments may have a role in stabilizing the sarcomeric structure in contraction-relaxation and in stretch (Horowits et al. 1986; Fulton and Isaacs 1991). It may contribute to the fiber-type-specific differences observed in muscle relaxation (Stein et al. 1982). Since the intact muscles contain additional cell types and tissue elements, their amount, viscosity, and elasticity may contribute to the relaxation characteristics as well. The significance of these elements becomes more prominent under certain pathological conditions (Coulton et al. 1988).

Despite the complexity of the biochemical and biophysical factors determining the physiological relaxation process, the striking parallelism between the temperature dependence of the SR Ca^{2+} uptake process and the relaxation itself indicates this system as having the key role.

3 Molecular Systems of Muscle Activation and Inactivation

3.1 The Activation System of Muscle Contraction – Muscle-type-specific Differences in Excitation Contraction Coupling

The chain of events leading to muscle-type-specific differences in the relaxation process could start already at the level of the activation of the muscle. The general concept of muscle activation, the mechanism of excitation contraction coupling, was recently described in the following reviews: Huang (1988); Fleischer and Inui (1989); Catterall (1991); Rios and Pizzaro (1991).

The action potential stimulus reaches the depth of the muscle through the transverse tubule system as a depolarization wave. The voltage-sensitive element of the T-tubule membrane has been identified as a dyhidropyridine-sensitive calcium channel (DHP receptor) (Curtis and Catterall 1984; Borsotto et al. 1985; Rios and Brum 1987). Besides the voltage sensor

function, responsible for the intramembrane charge movement (Kovács et al. 1979), it can serve as an L-type calcium channel, letting the calcium ions from the T-tubule lumen (extracellular space) into the muscle cytoplasm (Fleischer and Inui 1989). The initial inflow of the extracellular calcium can initiate further calcium release from the SR. This mechanism of activation seems to be relevant in the cardiac muscle (Fabiato 1985). Since both fast and slow skeletal muscles can be activated even in calcium-free medium, the decisive role of the inflow channel function of the DHP receptors during the activation process can be excluded (Eusebi et al. 1980, 1985).

The cytoplasmic extramembrane portion of the DHP receptor forms connections with the so-called feet component of the junctional sarcoplasmic reticulum membrane (Tanabe et al. 1987; Leung et al. 1988; Jorgensen et al. 1989). The feet protein complex is identical with the ryanodine-sensitive calcium release channel (Block et al. 1988), composed of four similar 400- to 450-kDa subunits (Fleischer et al. 1985; Inui et al. 1987a, 1987b, 1988; Saito et al. 1988). Recently Jorgensen et al. identified a 94-kDa protein component, supposedly connecting the DHP and ryanodine receptor complexes in the junctional space (Jorgensen et al. 1992).

Detailed evidence for the molecular heterogeneity between the Ca^{2+}-release complexes of different muscle types is now emerging. Chua and Dulhunty found the sensitivity toward depolarization higher in rat soleus than in the fast EDL muscle (Chua and Dulhunty 1988). In K^+ contracture experiments the half maximal tension required a depolarization value of -14 mV in EDL. In the soleus, this level of activation was already achieved at -28 mV. In the same experiments the inactivation of the T-tubule voltage sensor was 2.5 times faster in the EDL than in the soleus (Chua and Dulhunty 1988).

Burke et al. found similar action potential characteristics in all the different motor units of the cat gastrocnemius muscle (Burke et al. 1973). In 1974, Hanson detected significant differences in the resting membrane potential and action potential properties of rat fast and slow muscles (Hanson 1974). Fast fibers had a higher resting potential and higher amplitude and shorter duration of the spike of the action potential than the slow fibers. The negative after-potential was higher in fast fibers as well.

Detailed studies on the amino acid sequence as well as on the molecular structure of the voltage sensor–Ca^{2+} release channel complexes will provide a better understanding of their muscle-type-specific differences (Wagenknecht et al. 1989; Takeshima et al. 1989; Zorzato et al. 1989, 1990).

Muscular dysgenesis, an inherited muscle disorder in the mouse, is caused by a defect of the DHP receptor, L-type calcium channel protein of the T-tubule membrane (Adams et al. 1990; Adams and Beam 1990; Powell 1990). This genetic disease proved to be an excellent tool for the

studies characterizing the structure and function of the DHP receptor, and the whole excitation-contraction (E-C) coupling process. The injection of expression plasmids carrying cDNA sequences coding for the DHP receptor molecule completely restored the E-C coupling and the T-tubule L-type calcium channel function in dysgenic muscle cells (Tanabe et al. 1988).

Two different isoforms of the DHP receptor protein have been identified so far (Tanabe et al. 1987; Mikami et al. 1989). The primary sequence of the two isoforms differs predominantly in the extramembrane segments, forming the loops directed toward the junctional SR membrane surface.

The cardiac isoform restored the L-type Ca^{2+} current in dysgenic skeletal muscle cells as well, but the E-C coupling process became cardiac type, i.e., dependent on the presence of Ca^{2+} in the extracellular space (Tanabe et al. 1990a). This clearly indicates that the ability to interact with the ryanodine receptor molecule in the junctional SR is missing in the cardiac type isoform. It cannot open the Ca^{2+}-release channel of the junctional SR membrane by a conformational change, concomitant with an intramembrane charge movement.

In an elegant set of experiments, chimeric DHP receptors containing different segments of the cardiac and the skeletal isoforms were coexpressed in dysgenic skeletal muscle cells (Tanabe et al. 1990b). The cytoplasmic region between transmembrane repeats II and III seems to be essential for the skeletal muscle-type EC coupling. The interaction of this region with the recently described 94-kD coupling protein needs to be proved (Jorgensen et al. 1992).

The L-type calcium current seems to be associated with the transmembrane segments, which are more similar in the two isoforms. This calcium channel function of the skeletal muscle isoform remains latent in normal muscles, where the voltage sensor "intramembrane dipol" function of the protein is more prominent (Kovács et al. 1979; Rios and Pizzaro 1991). Slow- and fast-muscle-type isoforms within the skeletal muscle-specific DHP receptor have not been detected so far, although more detailed studies on the field may change this picture.

In 1988, Hymel et al. purified and reconstituted the cardiac form of the ryanodine receptor complex (Hymel et al. 1988). In planar lipid bilayers this isoform showed higher sensitivity for calcium than the skeletal muscle form. Ryanodine stabilized the open state of the channel more effectively in the skeletal muscle than in the cardiac muscle isoform. They detected slight differences in the Ca^{2+} and K^+ permeability characteristics of the two isoforms as well.

In homogenates of rat fast, slow, and cardiac muscles Fehér et al. found no difference in the total amount of ryanodine receptor molecules (Fehér et al. 1988). The distribution of these molecules, however, differed

significantly. In the fast-muscle homogenates about 13%, in slow-muscle homogenates 20% of the vesicles had ryanodine-sensitive release channels, while in heart muscle homogenates the ratio of these vesicles was as high as 50%. This result indicates that in the skeletal muscle, the ryanodine receptor-calcium release channel is concentrated in the junctional region of the sarcoplasmic reticulum; in the cardiac muscle it is more diffusely distributed around the whole SR surface. The known differences between the skeletal and cardiac muscle-type E-C coupling mechanisms make the interpretation of these observations plausible (Eusebi et al. 1985; Fehér et al. 1988; Tanabe et al. 1990a, 1990b).

In 1991, Lee et al. compared the ryanodine receptor complexes of rabbit fast- and slow-muscle sarcoplasmic reticulum fractions (Lee et al. 1991). The amount of the ryanodine receptor-Ca^{2+}-release channel protein in heavy SR fraction of rabbit slow muscle was about one third of that present in the fast muscle heavy SR. The slow-muscle-type channel had about 20 times longer closed time than the fast form, although the length of the open time of the channel and most of the conductance parameters showed only slight differences between the muscle types studied. The immunological cross-reactivity of the two ryanodine receptor preparations indicates further similarities (Lee et al. 1991).

Sutko et al. recently described two different isoforms of the ryanodine receptor complex in avian fast skeletal muscle (Airey et al. 1990; Sutko et al. 1991). The functional characteristics of these isoforms, as well as their significance in the E-C coupling, are still unknown.

The fast accumulation of information on the ryanodine receptor complex may soon provide a better understanding of its muscle-type-specific differences as well (Zorzato et al. 1989, 1990; Takeshima et al. 1989; Wagenknecht et al. 1989).

3.2 The Calcium-eliminating Systems of the Plasma Membrane

Calcium elimination through the sarcolemmal membrane is performed by two separate systems: the Ca^{2+}-ATPase enzyme and the Na^{+}-Ca^{2+} exchange system. The Ca^{2+}-ATPase is an approximately 130-kD single subunit enzyme (Caroni and Carafoli 1980, 1981; Famulski et al. 1988). It is activated by calmodulin, by acidic phospholipids, and by limited proteolysis (Sarkadi et al. 1980; Caroni et al. 1983). The enzyme is a member of the P-type cation transport ATPase family (Pedersen and Carafoli 1987a, 1987b). No cross-reaction of the enzyme was found with polyclonal antibodies raised against the sarcoplasmic reticulum Ca^{2+}-ATPase (Sarkadi et al. 1988). It transports probably one Ca^{2+} ion from the cell, at the expense of the hydrolysis of one molecule of ATP (Carafoli 1991).

Four genes have been isolated so far, coding for different isoforms of the plasma-membrane Ca^{2+}-ATPase (Shull and Greeb 1988; Greeb and Shull 1989; Strehler et al. 1990). The isoforms PMCA1 and PMCA3 were detected in skeletal muscle. In heart muscle the PMCA1 and PMCA2 forms are expressed (Greeb and Shull 1989; Grover and Khan 1992). No information regarding the skeletal muscle fiber-type-specific distribution of these isoforms has been published. Even the understanding of the functional significance of the isoforms in different tissues is in a rather early phase (Enyedi et al. 1989; Grover and Khan 1992).

The activity and capacity of the plasma membrane (sarcolemmal) Ca^{2+}-ATPase alone does not seem to be sufficient for the eliminiation of the large amounts of calcium liberated during muscle activation (Garrahan and Rega 1990; Carafoli 1991). The other Ca^{2+}-transporter system in the plasma membrane is the Na^{+}-Ca^{2+} exchanger. It was found to be present in both skeletal and cardiac muscles (Kadoma et al. 1982; Lederer and Nelson 1983; Miura and Kimura 1989), although its functional role was proven only in cardiac muscle cells (Horackova and Vassort 1979). This transporter exchanges one Ca^{2+} ion for three Na^{+} ions (Blaustein 1984; Läuger 1987; Reeves 1990). The direction of the transport depends on the actual Na^{+} concentration on the two sides of the membrane. Therefore, the continuous functioning of the system as a Ca^{2+} eliminator requires the existence of the Na^{+} gradient across the sarcolemma, maintained by the $Na^{+}K^{+}$-ATPase (Caroni and Carafoli 1983; Blaustein 1984; Post et al. 1988).

The lack of specific inhibitor substances for the Na^{+}-Ca^{2+} exchanger complicates the studies on the system. Its role in the regulation of the active state of the muscles, particularly in cardiac muscle, and its involvement in adaptation processes remains a challenging question for future research (Reeves 1990; Pietrobon et al. 1990).

3.3 The Role of the Mitochondria in Muscle Calcium Regulation

The mitochondrial content of the different muscle types largely reflects their metabolic character. Morphometric studies in mouse cardiac muscle detected about 37.5% volume density for mitochondria (Eisenberg 1983). In the slow oxidative muscle of guinea pigs, the mitochondria occupied about 4.9% of the total volume. In the fast oxidative part of the vastus lateralis this value was 8.2%, while in the fast glycolytic region it was only 1.9% (Eisenberg and Kuda 1975, 1976). In rat anterior tibial muscles Thornell et al. measured the volume density of mitochondria between 1.3% and 9.3% (Thornell et al. 1987). Usually the fibers with shorter contraction time contained fewer mitochondria. In the soleus the mitochondria occupied between 5.5% and 8.3% of the fiber volume. The correlation between

the contraction speed and the mitochondrial content was less clear in the individual soleus fibers (Thornell et al. 1987).

Although the relatively large intramitochondrial volume and the in vitro measurable Ca^{2+} uptake gave indications for the participation of this organelle in the regulation of the cytoplasmic calcium level (DeLuca and Engstrom 1961; Vasington and Murphy 1962), deeper kinetic and biochemical characterization of the mitochondrial calcium transport revealed evidence against this theory. The relatively low Ca^{2+} affinity and the slow rate of the uptake process made the participation of the mitochondria in the intracellular Ca^{2+} homeostasis unrealistic (Crompton et al 1976; Vercesi et al. 1978; McCormack and Denton 1984; Somlyo et al. 1985; Lukács and Kapus 1987). The electron-dense particles in the mitochondrial matrix, previously considered calcium deposits, revealed only weak Ca signals with X-ray microanalysis (Bond et al. 1984; Somlyo et al. 1985).

The real role of the intramitochondrial calcium content and its fluctuation is more important in the regulation of some metabolic enzymes involved in the terminal substrate oxidation, such as pyruvate dehydrogenase, NAD-dependent isocitrate dehydrogenase, alfa-oxoglutarate dehydrogenase (Denton et al. 1972; McCormack and Denton 1979, 1984).

Under certain pathological conditions Ca^{2+} may accumulate in the mitochondrial matrix space (Carafoli 1982). This can be observed in severe, almost certainly irreversible cell damage. In the Ca^{2+} regulation of the contraction-relaxation of muscles the mitochondria do not seem to have any role.

3.4 Cytoplasmic Calcium-binding Proteins in Different Muscle Types: Parvalbumin

According to the estimation of Carafoli, only 0.1% of the total cellular calcium content is in free ionized form (Carafoli 1987). The rest is bound to various calcium-binding proteins and phospholipids. The general properties of these calcium-binding components were reviewed by Pietrobon et al. (1990). Acidic phospholipids have a large intracellular calcium-buffering capacity. Their functional significance is less well understood (Dawson and Hauser 1970).

One group of the cytoplasmic calcium-binding proteins transduces the signal of calcium binding to other structural proteins and/or to metabolic enzymes (Carafoli 1987; Heizmann and Hunziker 1990; Pietrobon et al. 1990). The main representative of this group in muscle tissues is troponin C (Ebashi 1963; Potter and Gergely 1975; Potter and Johnson 1982). The other important member of this group, calmodulin, is present in skeletal and cardiac muscles only in low amounts (Nairn and Perry 1979). Its functional

role is in the regulation of the sarcolemmal calcium pump, and in regulating the phosphorylation of phospholamban, myosin light-chain kinase, and some metabolic enzymes (Klee and Vanaman 1982). The low abundance of calmodulin in muscle tissues makes its calcium-buffering role negligible.

The other group of cytoplasmic calcium-binding proteins, the major one being parvalbumin, act as real buffers. The buffering capacity of these proteins is largely limited by their actual concentration in the cell. In the skeletal muscles of lower vertebrates and in fast-twitch muscles of small mammals the amount and binding capacity of parvalbumin is sufficient to influence calcium distribution in the fibers (Carafoli 1987; Pietrobon et al. 1990).

Parvalbumin is a 12-kDa member of the EF-hand-type calcium-binding protein family. It has an acidic isoelectric point and three so-called EF hand domains, formed by repetitive helix-loop-helix motifs, suitable for the reversible, high-affinity binding of calcium ions (Kretsinger 1980; Gerday 1982). In parvalbumin only two of these domains are functional; the third very similar domain does not participate in Ca^{2+} buffering, probably because of the deletion of two amino acids from the sequence (Declerc et al. 1988).

Parvalbumin is present in large amounts in fish, amphibian, and reptile muscles (Hamoir 1968; Pechere et al. 1971). Among mammalian muscle types, the fast-glycolytic IIB fibers contain the largest amount of this protein. IIA fibers contain much less, while the slow-oxidative, type-I fibers are almost free of this cytoplasmic calcium-binding protein (Blum et al. 1977; Celio and Heizmann 1982; Heizmann 1984; Leberer and Pette 1986). Schmitt and Pette recently published a microanalytic study on the fiber-type-specific distribution of parvalbumin in rabbit muscles (Schmitt and Pette 1991). The highly sensitive fluorometric sandwich ELISA system indicated 2–5 μg/g muscle weight in type-I fibers. In the IIA fibers this value was 17–29 μg/g muscle; in IIB fibers the results were less homogeneous, scattering between 75 and 1340 μg/g muscle. The presence of some further subtypes, e.g., type IID/X, may be responsible for the large diversity detected within the IIB fiber population (Schmitt and Pette 1991).

The high concentration of parvalbumin in fast-glycolytic muscles suggested a possible role for this protein as a soluble relaxant factor (Gillis et al. 1982; Heizmann et al. 1982; Gillis 1985). This theory was further supported by some experiments which detected faster decline in the calcium signal than was expected on the basis of SR uptake function alone (Gillis et al. 1982; Gillis 1985). Computer-simulation experiments indicated parvalbumin to be the main calcium sink in the early phase of relaxation (Gillis et al. 1982; Gillis 1985). In the later phase this calcium is transported into the lumen of the sarcoplasmic reticulum, rendering the parvalbumin molecules

suitable for calcium buffering again. Some other computer-simulation experiments, however, denied this possible role of parvalbumin, limiting its involvement in calcium buffering only to repeated contractions (Robertson et al. 1981).

The amount of parvalbumin is different in the fast-twitch muscles of different mammals. Usually, the muscles of smaller animals with faster contraction-relaxation parameters are richer in parvalbumin (Heizmann et al. 1982; Leberer and Pette 1986; Simoneau et al. 1989). The IIB fibers of larger mammals contain less parvalbumin, reflected perhaps in the slower relaxation speed (Barnard et al. 1971; Luff 1981). However, the higher transport activity of the sarcoplasmic reticulum Ca^{2+}-ATPase may compensate for the lower parvalbumin level in these animals. In cardiac and slow skeletal muscles parvalbumin is hardly detectable and therefore has no calcium-buffering role.

4 The Sarcoplasmic Reticulum System

4.1 Structural Differences in the Sarcoplasmic Reticulum System of Different Muscle Types

The first observers of the specialized muscle endoplasmic reticulum system, at light-microscopic resolution, were Veratti and Retzius (Retzius 1890; Veratti 1902). Porter and Palade detected the same system in early electron-microscopic studies and named it sarcoplasmic reticulum (Porter and Palade 1957). For the detailed structure of the sarcotubular system, revealed by more sophisticated morphological methods, see the review by Peachey and Franzini-Armstrong (1983).

The transverse tubular (T) system is continuous with the sarcolemmal membrane. It forms extensive invaginations, connecting the extracellular space deep into the muscle fibers (Ezermann and Ishikawa 1967; Peachey 1980). The T tubules run across the muscle fibers at the ends of the A-band regions, in some species near to the Z-disc area (Peachey and Franzini-Armstrong 1983).

The sarcoplasmic reticulum represents a continuous membrane-bounded compartment, occupying the space between the myofibrils (Peachey 1965; Sommer and Johnson 1979; Franzini-Armstrong 1980; Eisenberg and Eisenberg 1982). In the middle portion of the sarcomeres, the SR compartment is thin, forming the longitudinal tubules. Toward the end of each sarcomere they extend into the so-called terminal cisternae.

The T tubules form junctions with the sarcoplasmic reticulum at the terminal cisterna region. In the fast-twitch fibers usually two terminal cisternae with one T-tubule segment form the so-called triads. The diads more

typical for the slow skeletal and cardiac muscles consist of one terminal cisterna region and a T-tubule segment (Johnson and Sommer 1967; Kelly 1969; Eisenberg and Eisenberg 1982; Mitchell et al. 1983). The area of the terminal cisterna surface which forms contact with the T tubule is called the junctional SR. The rest of the terminal cisterna surface, together with all the longitudinal tubules, is called free SR. The whole free SR shows high homogeneity in its chemical composition, reflecting a similar functional role, and free lateral mobility of the membrane elements within these regions.

The junctional complex forms the structural basis of the excitation-contraction coupling. Its characteristics were briefly discussed in Sect. 3.1. The general structural localization of the sarcoplasmic reticulum is similar in the fast and slow skeletal as well as in cardiac muscles (Eisenberg 1983). Morphometric studies have indicated the volume ratio of the SR in the fast-twitch fibers to be around 7%–8%, in the slow-twitch fibers of different species between 0.5% and 4% (Eisenberg 1983). The morphometric analyses represent usually rough estimates of the real volume distribution; therefore, large variations within the same muscle type and between different species have been reported. Still, the greater abundance of the SR system in the fast muscles matches well with the faster relaxation speed of this muscle type (Luff and Atwood 1971; Schmalbruch 1979; Eisenberg et al. 1987) (Fig. 3).

The sarcoplasmic reticulum can be isolated as a microsomal fraction from muscle homogenates by differential centrifugation (Nakamura et al. 1976). The average yield is about 1 mg/g muscle tissue, which represents

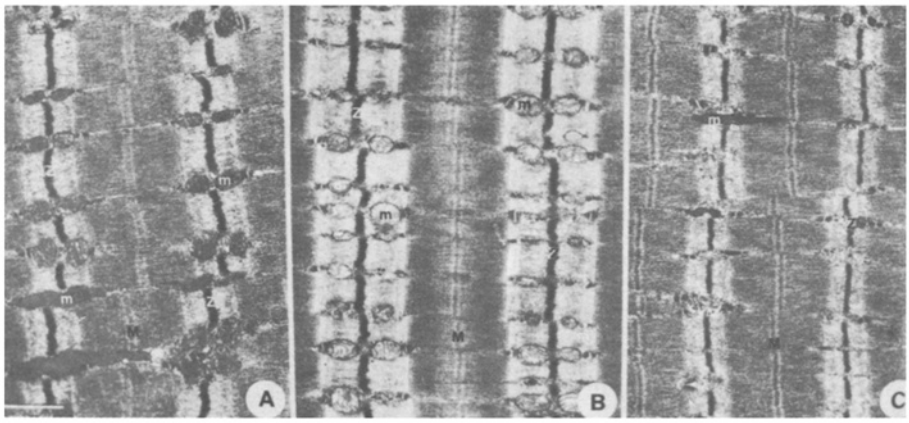

Fig. 3A–C. Longitudinal plastic sections of anterior tibial muscle fibers at low magnification: **A** fiber from mapped slow-twitch unit, enzyme histochemically a type-1 fiber; **B** and **C** fibers of mapped fast-twitch units, enzyme histochemically designated as type-2A fiber (**B**) and type-2B fiber (**C**). *M*, M band; *m*, mitochondria; *Z*, Z disc. *Bar A–C*, 1 μm. (From Thornell et al. 1987)

approximately 5%–10% of the total SR in the muscle (Gillis 1985). Preparations from slow muscles provide lower yields, about 0.5 mg/g muscle (Lee et al. 1991). Usually, the actual yield is highly influenced by the homogenization method and by the amount of the contaminating non-SR membrane elements present in the preparation (Dux and Martonosi 1984). Highly purified SR preparations and further isolated subfractions, such as heavy or light SR, terminal cisternae, triads, and junctional membrane, can be obtained after gradient fractionations combined with pressure treatment of the microsomes. The functionally intact SR vesicles can be separated after loading with calcium in the presence of oxalate inside the vesicle lumen (Bonnet et al. 1978).

Electron-microscopic studies described the isolated sarcoplasmic reticulum as forming round, sealed vesicles 80–100 nm in diameter (Ebashi and Lipmann 1962). The average diameter of the vesicles from slow-muscle preparation is about 20%–25% smaller (Lee et al. 1991); this difference indicates nearly two times greater volume for the SR vesicles prepared from fast muscles. However, the presence of non-SR elements in the preparations may be responsible for this difference (Dux and Martonosi 1984).

The surface of the isolated SR vesicles is covered with 4-nm-diameter surface particles in a density of about 13 000–16 000/μm^2. These particles represent the extramembrane portions of the calcium transport ATPase (Jilka et al. 1975). On freeze-fracture replicas they are represented as 8.5-nm intramembrane particles, localized exclusively on the outer, cytoplasmic fracture face (Jilka et al. 1975). Their lower number compared with the number of the surface particles was one of the first indications of the possible oligomerization of this enzyme (Vanderkooi et al. 1977).

The density of the intramembrane particles was two to three times lower in slow-twitch fibers (Bray and Ryans 1976; Zubrzicka-Gaarn et al. 1982; Gauthier and Hobbs 1986). In some studies, less difference was found in the local density of the intramembrane particles between fast and slow fibers; however, the total area in slow fibers covered with intramembrane particles was more limited (Beringer 1976; Ryans et al. 1975).

Using the freeze drying-rotary shadowing method, Ferguson and Franzini-Armstrong compared the density and distribution of the SR Ca^{2+}-ATPase molecules in different guinea pig muscles (Ferguson and Franzini-Armstrong 1988) (Table 1). In the soleus they found a density of 25 100/μm^2, in the fast vastus lateralis muscle 32 100/μm^2. In the free terminal cisterna region the enzyme covered 65.5% of the surface in slow muscle, but 99.2% in the fast muscle. On the longitudinal SR surface these values were 81.3% and 99.8% respectively.

The density of the enzyme in the slow terminal cisternae was 16 500/μm^2, in the fast muscle 31 900/μm^2. In the longitudinal tubule fractions

Table 1. Morphometric characteristics of the sarcoplasmic reticulum in guinea pig slow and fast muscles (From Ferguson and Franzini-Armstrong 1988)

	Soleus		White vastus lateralis
1. ATPase density of isolated SR (μm^{-2})	25 100		32 100
2. fTC, ATPase-occupied area (%)	65.5		99.2
3. lSR, ATPase-occupied area (%)	81.2		99.8
4. fTC surface to fiber volume ($\mu m^2/\mu m^3$)	0.18		0.264
5. lSR surface to fiber volume ($\mu m^2/\mu m^3$)	0.73		0.91
6. fTC, ATPase/SR surface area (μm^{-2})	16 500		31 900
7. lSR, ATPase/SR surface area (μm^{-2})	20 400		32 100
8. fTC, ATPase per fiber volume (μm^{-3})	3 000		8 400
9. lSR, ATPase per fiber volume (μm^{-3})	14 900		29 200
10. Total ATPase per fiber volume (μm^{-3})	17 900		37 600
11. Fast-to-slow ratio		2.10	
12. ATPase per SR surface (μm^{-2})	19 600		32 000
13. Fast-to-slow ratio		1.63	

fTC, free terminal cisterna; lSR, longitudinal sarcoplasmic reticulum

these values were 20 400 in slow and 32 100 in fast guinea pig muscles. The ratio between the slow and fast muscles was 2.1 when they compared the total Ca^{2+}-ATPase content with the fiber volume. Comparing the Ca^{2+}-ATPase content with the SR membrane surface area, the ratio between the slow and fast muscles was 1.63 (Ferguson and Franzini-Armstrong 1988). They concluded that the difference between the two muscle types was in the total area covered by Ca^{2+}-ATPase rather than in the density of the enzyme in the areas where it was present.

4.2 Chemical Composition of the Sarcoplasmic Reticulum

4.2.1 Lipid Components

Purified sarcoplasmic reticulum vesicles contain about 0.6 mg phospholipid/mg protein, which corresponds to 90–100 lipid molecules/ATPase molecules (Martonosi and Beeler 1983). In contrast to other cell membrane fractions, the SR is very poor in cholesterol. The lipid phase is necessary to provide a tight seal around the protein molecules, in order to prevent the leakage of calcium from the SR lumen. The fluidity of the lipid phase is needed to allow the molecular mobility and rearrangement of proteins during the transport cycle, as well as to resist the mechanical stresses generated by contraction and relaxation (Martonosi and Beeler 1983).

Zubrzicka-Gaarn et al. detected an about 20% lower phospholipid/protein ratio in slow-muscle SR preparations than in those derived

from fast muscles (Zubrzicka-Gaarn et al. 1982). However, the large scattering of the data and the higher percentage of cholesterol in the slow-muscle membrane suggests some contamination by non-SR elements in their preparations. The sarcoplasmic reticulum membrane contains the enzymes of the phospholipid biosynthesis and degradation (Waku et al. 1977), although the turnover of phospholipids in the SR membrane is low, compared with that in the endoplasmic reticulum membrane of other cell types (Martonosi and Halpin 1972). The SR membrane does not participate in the homoviscous temperature adaptation of the membrane systems in cold-blooded animals, above all fish (Cossins et al. 1978).

Nevertheless, the lipid phase of the sarcoplasmic reticulum membrane does not seem to play a key role in the regulation adaptation processes. The alterations in the lipid composition that have been described in different pathological conditions are more likely the results of contaminations by other muscle membranes or membranes from other cell types.

4.2.2 Protein Components, Muscle-type-specific Differences

The 110-to 115-kDa Ca^{2+}-transport ATPase represents about 85% of the total protein content in fast-muscle sarcoplasmic reticulum preparations (Martonosi 1969b). In slow-muscle SR its ratio is lower. Zubrzicka-Gaarn et al. detected 20% Ca^{2+} -ATPase in rabbit soleus SR preparations (Zubrzicka-Gaarn et al. 1982). Additional protein components of the SR fractions are calsequestrin, high-affinity calcium-binding proteins, and different classes of glycoproteins (Meissner 1975; Michalak et al. 1980; Leberer et al. 1990).

The 63-kDa calsequestrin is localized in the lumen of the terminal cisternae. Its high calcium-binding capacity suggested a function in extending the storage capacity of the SR, and in preventing the buildup of a potential gradient across the membrane during the uptake process (MacLennan et al. 1974; Campbell et al. 1983). Electron-microscopic observations reflected structural connections between the calsequestrin molecules in the terminal cisterna lumen and the inner surface of the junctional membrane area (Brunschwig et al. 1982; Saito et al. 1984; Franzini-Armstrong et al. 1987). These data suggested some supportive role in the transfer of the stored calcium to the release site; however, recent experiments have indicated that the bulk of the calcium bound to the calsequestrin remains unchanged during the release process (Volpe and Simon 1991).

Lee et al. found that the calsequestrin content in slow-muscle terminal cisternae is about 32% of the amount present in the fast-muscle samples, in agreement with a previous, similar observation by Zubrzicka-Gaarn et al. (Lee et al. 1991; Zubrzicka-Gaarn et al. 1982). Using a sandwich ELISA detection method, Leberer and Pette observed 50% less calsequestrin in

rabbit slow muscles than in fast ones (Leberer and Pette 1986). The concentration of this protein was not different between the fast IIA and IIB types. Their quantitation method is certainly more accurate than the densitometric analysis of stained gel patterns used by Zubrzicka-Gaarn et al. (1982) and Lee et al. (1991) (Table 2).

Different glycoproteins have been isolated from the SR membrane. They were identified as calcium-binding proteins having close genetic relationships with each other (Campbell and MacLennan 1981; Leberer et al. 1990; Movsesian et al. 1990). The 160-kDa Ca^{2+}-binding glycoprotein sarcalumenin was more abundant in the longitudinal tubule fraction (Leberer et al. 1990). In an earlier study, Zubrzicka-Gaarn et al. found significantly less of this glycoprotein in slow muscle than in fast muscle SR (Zubrzicka-Gaarn et al. 1982).

Wiehrer and Pette described a 30-kDa protein fraction more abundant in the slow muscle SR (Wiehrer and Pette 1983). They suggested that the ratio between the amount of Ca^{2+}-ATPase and the amount of this protein could be used to characterize the fiber-type origin of the preparation, a ratio of 0.7 being typical for the slow, one of 12.5 for the fast-muscle membrane. Very similar ratios (0.5 and 8 respectively) were published recently by Lee et al. (1991).

Table 2. Sarcoplasmic reticulum Ca-ATPase, calsequestrin, and parvalbumin content[a] as determined by sandwich ELISAs in extracts of rabbit skeletal muscles of histochemically defined fiber composition (From Leberer and Pette 1986)

Muscle	Fiber type			ATPase	CS	PA
	2B	2A	1			
	(%)			(μg/g wet wt.)		
Psoas (white portion), $n = 7$	100	0	0	2640 ± 330	243 ± 17	805 ± 53
Vastus lateralis superficialis, $n = 6$	100	0	0	3458 ± 476	239 ± 5	1200 ± 30
Vastus lateralis profundus, $n = 3$	82 ± 3	15 ± 4	3 ± 2	3022 ± 342	202 ± 9	924 ± 45
Tibialis anterior (whole muscle), $n = 7$	52 ± 1	43 ± 1	5 ± 1	2940 ± 398	275 ± 15	602 ± 18
Tibialis anterior (deep portion), $n = 3$	49 ± 4	45 ± 4	6 ± 1	2620 ± 350	259 ± 25	541 ± 31
Tibialis anterior (superficial portion), $n = 5$	84 ± 7	12 ± 5	4 ± 2	3340 ± 342	253 ± 20	727 ± 42
Extensor digitorum longus, $n = 12$	66 ± 1	30 ± 1	4 ± 1	2682 ± 421	243 ± 36	544 ± 26
Soleus, $n = 10$	0	5 ± 1	95 ± 1	554 ± 46	131 ± 25	4.4 ± 1.0
Semitendinosus, $n = 4$	0	6	94	524 ± 148	155 ± 9	4.8 ± 0.7

[a] Data represent mean values ± SD. CS, Calsequestrin; PA, parvalbumin; n, number of muscles

In a comparative study of identically prepared microsomal fractions from six different rat muscles the Ca^{2+}-ATPase represented 50%–60% of the total protein content in the mainly fast-type levator ani, semimembranous, and extensor digitorum longus muscles (Dux and Martonosi 1984). In the diaphragm, with a fiber population of mixed contraction properties, it represented 30% of the microsomal proteins, while in the slow soleus muscle it constituted only 16%. In the microsome fraction prepared from the myocardium only 8% of the protein content was Ca^{2+}-ATPase, based on densitometric gel scans (Dux and Martonosi 1984). The amount of the calsequestrin in all fast-type muscles was similar. In the soleus and cardiac muscle samples it remained below the detection level with Coomassie blue staining. The other protein components of the samples seemed to be more characteristic for the metabolic type of the muscles than for their contraction-relaxation speed (Fig. 4).

A 94-kDa protein band was clearly present in the levator ani and semimembranous samples. This band is identical with the glycogen

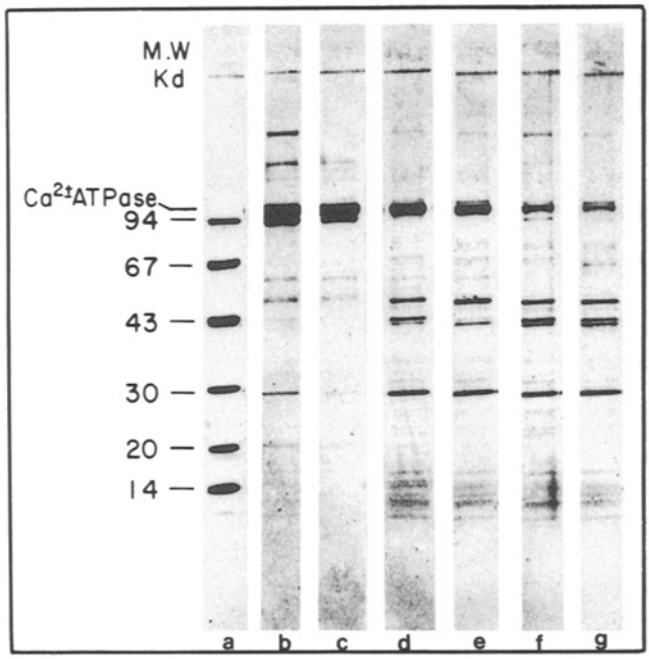

Fig. 4. SDS-polyacrylamide gel electrophoresis pattern of microsomal proteins from six different muscle types of rat. *a*, Molecular weight marker; *b*, m. semimembranosus; *c*, m. levator ani; *d*, m. extensor digitorum longus; *e*, diaphragm; *f*, m. soleus; *g*, cardiac muscle (left ventricle). Note differences between glycolytic (*b*, *c*) and oxidative (*d*–*g*) muscles in the amount of phosphorylase and several protein bands in the range of 43–60 kDa. (From Dux and Martonosi 1984)

phosphorylase, which explains its presence in the microsomes of the mainly fast glycolytic muscles. In the fast oxidative-glycolytic EDL this band was missing, as it was in the slow-oxidative soleus and cardiac muscles. Several lower molecular weight proteins (54, 45, 43, and 30 kDa) were present in nearly equal quantities in all muscles with an oxidative metabolic character (EDL, diaphragm, soleus, cardiac muscle) without respect to their contraction type.

A small amount of the 30-kDa band was present in the fast semimembranous sample, even less in the microsomes derived from the levator ani (Dux and Martonosi 1984). The different amounts of contaminating non-SR membrane elements in some samples may be responsible for the presence of these additional protein bands. Typical protein components, enzymes of the endoplasmic reticulum system, may be detected in minor quantities in the SR membrane as well (Ohnoki and Martonosi 1980). The identification and immunohistochemical localization of the 30-kDa protein would certainly improve its usefulness as a slow-type SR marker (Wiehrer and Pette 1983; Lee et al. 1991).

The sarcoplasmic reticulum fraction of cardiac and slow skeletal muscles contains an important low-molecular-weight regulatory protein phospholamban (Kirchberger et al. 1974; Tada et al. 1974). Its structure and function are described in Sect. 5.8.

5 The Sarcoplasmic Reticulum Ca^{2+}-ATPase Enzyme

5.1 The Reaction Cycle of the Sarcoplasmic Reticulum Calcium Pump

The Ca^{2+}-ATPase enzyme is the main protein component of the longitudinal sarcoplasmic reticulum and of the free terminal cisterna regions in the SR (Martonosi and Beeler 1983). This single subunit integral membrane protein belongs to the family of the P-type cation transport ATPase (Pedersen and Carafoli 1987a). Its reaction cycle involves cyclic rearrangements of the enzyme between two main conformations, called E1 and E2 (De-Meis and Vianna 1979). In the E1 (high calcium affinity) conformation, the enzyme binds two Ca^{2+} ions on the cytoplasmic side of the membrane. Upon the binding and subsequent hydrolysis of one ATP molecule the enzyme forms an acid-stable phosphorylated intermediate (Yamamoto and Tonomura 1967; Makinose 1969; Martonosi 1969a; Froelich and Taylor 1976). In the following step the enzyme undergoes a conformational rearrangement; the calcium ions become occluded inside the protein and finally reappear at the luminal side of the membrane (Waas and Hasselbach 1981; Glynn and Karlish 1990). The conformation in this phase is called

E2. Its low calcium affinity helps the release of the translocated calcium ions into the lumen of the SR (Ikemoto 1975). Finally, the enzyme returns to the E1 conformation, following a step of dephosphorylation (Inesi et al. 1970; Kanazawa et al. 1971).

Under certain conditions the transport cycle is fully reversible. In the presence of ADP, in calcium-free medium the enzyme can pump out the previously loaded calcium from the lumen, with a concomitant synthesis of ATP (Hasselbach 1978; DeMeis and Vianna 1979). This process requires rather aphysiological conditions, and it is far too slow to be significant in activating Ca^{2+} release in the muscle (Martonosi 1984).

The SR Ca^{2+}-ATPase is not selective for ATP as energy source. Other high-energy phosphate donors such as acetyl phosphate and creatine phosphate can energize the transport as well (DeMeis and Hasselbach 1971; DeMeis and DeMello 1973; Yates and Duance 1976). As judged from the affinity constants for these substances, they do not play a role under physiological conditions (Pucell and Martonosi 1971).

Recently, a close association of the enzyme creatine kinase with the sarcoplasmic reticulum membrane was observed. This association may serve as the ATP-regenerating system of the Ca^{2+} transport during sustained muscle activity. More details on this theory can be found in Walliman et al. (1992).

5.2 Functional Differences Between the Fast- and Slow-muscle Sarcoplasmic Reticulum

The activity of the sarcoplasmic reticulum Ca^{2+}-ATPase enzyme measured by Zubrzicka-Gaarn et al. in rabbit slow-muscle SR was about four times lower than that in fast muscles (Zubrzicka-Gaarn et al. 1982). However, the values were almost identical when they were calculated on the basis of the amount of the Ca^{2+}-ATPase protein present in the sample. This indicates that the difference in the specific activity is merely a result of the presence of a large, inactive, non-Ca^{2+}-ATPase protein mass in the slow-muscle preparations.

The intact sealed vesicles which constitute the muscle sarcoplasmic reticulum (microsome) preparations provide a unique opportunity for the functional characterization of the enzyme. The ATP-dependent calcium uptake process, usually with the calcium-precipitating anion oxalate inside the vesicles, was widely used for the characterization of SR fractions from different muscle types. In 1964, Sreter and Gergely observed a maximal calcium uptake capacity of 6.76 μmol/mg protein in rabbit fast-muscle microsomes. In the microsomal fraction from the slow soleus this capacity was only 0.35 μmol/mg protein. The addition of azide to the medium did not change the uptake capacity in fast-muscle microsomes, while in

the slow-muscle preparations it caused about 50% inhibition (Sreter and Gergely 1964). Since azide is a known inhibitor of the mitochondrial calcium uptake, this different behavior indicated that the microsomal fraction from slow muscle contained a large amount of contaminating mitochondrial membranes. This rather high, nearly 20-fold difference was not found later between the calcium uptake functions of slow- and fast-muscle microsomes.

In 1969, Sreter reported a sixfold difference between the calcium uptake capacity of fast and slow rabbit muscle microsomes, both in the presence and in the absence of oxalate (Sreter 1969). However, the initial rate of the uptake process was 12 times higher in the fast-muscle samples. The seasonal variation in the uptake velocity described in the same paper may be an early observation of hormonal influences on SR activity, although the lack of further studies in this area makes the exclusion of technical artifacts difficult (Sreter 1969). A sixfold difference was found between rabbit fast- and slow-muscle microsomes in their calcium uptake rate and capacity by Zubrzicka-Gaarn et al. (1982).

Comparing different muscle types of guinea pigs, Fiehn and Peter found the maximal calcium uptake capacity in sarcoplasmic reticulum fractions in predominantly IIA and IIB type muscles to be 5.2–5.5 μmol/mg protein. In the type I soleus this value was 2.8 μmol/mg protein (Fiehn and Peter 1971). The maximal velocity of the uptake process was 1.3 μmol/mg protein/min in fast muscles, 0.35 in the slow ones. The K_m value for calcium was uniformly 4 μM for all muscles tested.

The microsomal fractions prepared from different muscle types may contain different amounts of contaminating non-SR elements (Sreter and Gergely 1964; Dux and Martonosi 1984). The separation procedure may generate artificial functional differences due to the heterogeneous sensitivity toward homogenization or centrifugation steps. Therefore, the technique developed to measure calcium uptake in whole-muscle homogenates made a great contribution to the quantitative characterization of the sarcoplasmic reticulum function in different muscle types (Briggs et al. 1977; Leberer et al. 1987).

In fast-muscle homogenates of rats, Briggs et al. found five times higher calcium uptake velocity than in the slow soleus. In homogenates of the fast m.extensor digitorum of cats the calcium uptake was six times higher than in the slow soleus homogenates (Briggs et al. 1977). These data were in agreement not only with the differences in relaxation speed between these muscles, but also with the immunochemically (sandwich ELISA) determined ratios of the Ca^{2+}-ATPase content of fast and slow muscles (Leberer and Pette 1986) (Table 2).

Fehér et al. found the ratios between the calcium uptake rate of cardiac, slow, and fast skeletal muscle homogenates to be 1 : 3.8 : 14.4 respectively.

The total uptake capacity of the different homogenates was more similar, giving a ratio of 1 : 0.75 : 1.5. It is interesting that the total uptake capacity was less in slow muscles than in the cardiac muscle. The nearly 2:1 ratio between the fast and slow skeletal muscle total uptake capacity fits with other morphometric data (Fehér et al. 1988; Ferguson and Franzini-Armstrong 1988).

Recent technical developments seem to make possible the measurement of the Ca^{2+}-ATPase activity in homogenates as well (Everts et al. 1989; Simonides and Hardeweld 1990; Matsushita and Pette 1992), making the quantitative characterization of the sarcoplasmic reticulum system even more accurate.

The steady-state concentration of the phosphorylated intermediate form of the calcium pump enzyme is accepted as a good indicator for the number of active enzyme molecules in the sarcoplasmic reticulum membrane (Martonosi and Beeler 1983). Heilmann et al. found the level of the phosphorylated intermediate form to be 15–40 times higher in fast than in slow rabbit muscles (Heilmann et al. 1977). Zubrzicka-Gaarn et al. detected about four times higher Ca^{2+}-, Mg^{2+}-dependent phosphoprotein formation in rabbit fast microsomes than in the ones prepared from slow muscles (Zubrzicka-Gaarn et al. 1982). Interestingly, the values became almost identical in the two muscle types when they were calculated on the basis of the Ca^{2+}-ATPase protein content determined by gel densitometry.

All the quantitative differences in sarcoplasmic reticulum functional parameters between different muscle types can reflect differences in the total amount or in local density of similar elements, as well as a genuine diversity in the transport system and in its regulatory mechanism. Recent developments in the immunochemical, molecular genetic characterization of the sarcoplasmic reticulum calcium-ATPase isoforms, as well as the specific identification of the enzyme through crystallization, have provided more insights into this area.

5.3 Molecular Structure of the Sarcoplasmic Reticulum Ca^{2+}-ATPase as Revealed by Enzyme Crystals

The extramembrane portion of the sarcoplasmic reticulum Ca^{2+}-ATPase can be visualized on the surface of the vesicles by negative staining. The transport protein forms intramembrane particles on the outer, cytoplasmic fracture face after freeze fracturing of the SR (Jilka et al. 1975).

Addition of vanadate to the sarcoplasmic reticulum vesicles in a calcium-free medium generated highly ordered two-dimensional crystalline arrays of the Ca^{2+}-ATPase (Dux and Martonosi 1983a). The structural units of these crystals are enzyme dimers, the lattice parameters are a: 65.9 Å, b: 114.4 Å, γ: 77.9° (Taylor et al. 1984, 1986) (Fig. 5).

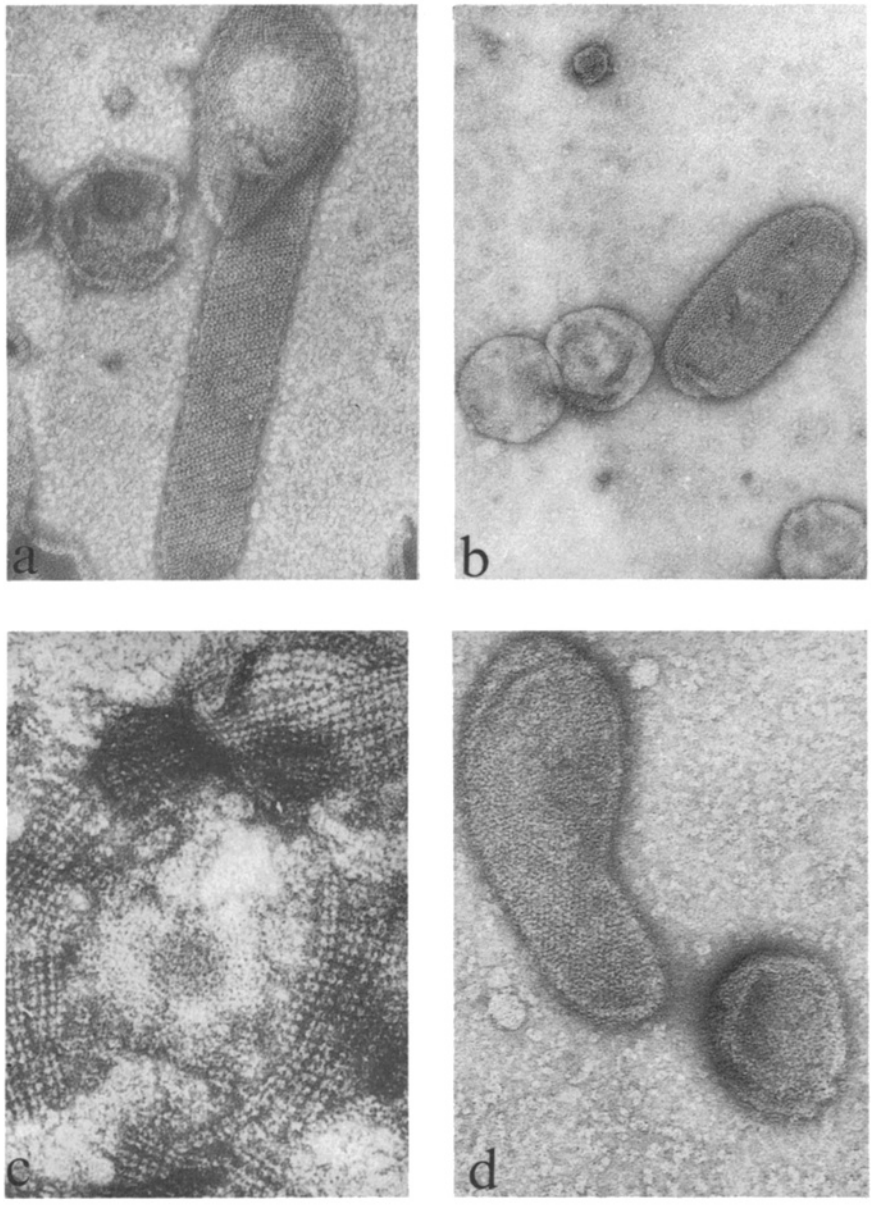

Fig. 5a–d. Two-dimensional crystals of the sarcoplasmic reticulum Ca^{2+}-ATPase. **a,b,c** Crystals in the E2 conformation of the enzyme, induced by 5 m*M* vanadate in calcium-free medium. Rabbit sarcoplasmic reticulum vesicles from fast m. longissimus dorsi **a** and slow m. soleus **b** muscles. The crystallized vesicles were disrupted into isolated Ca^{2+}-ATPase dimer chains by hyposmotic treatment of the preparation **c**. **d** Ca^{2+}-ATPase crystals in the E1 conformation state, induced by 10^{-5} *M* $PrCl_3$ at pH 7.5. Sarcoplasmic reticulum vesicle from rabbit m. longissimus dorsi (fast) muscle

Negative-stained, deep-etched, rotary-shadowed, and freeze-fractured crystallized membranes revealed the structure of the extramembrane portion of the enzyme as a nearly trigonal, pear-shaped body, representing about two thirds of the total protein mass (Peracchia et al. 1984; Ting-Beall et al. 1987; Taylor et al. 1984, 1986, 1988a; Castellani et al. 1985) (Fig. 6). The intramembrane portions of the two molecules forming the dimers seem to diverge obliquely toward the neighboring dimer chains inside the lipid bilayer (Taylor et al. 1988a).

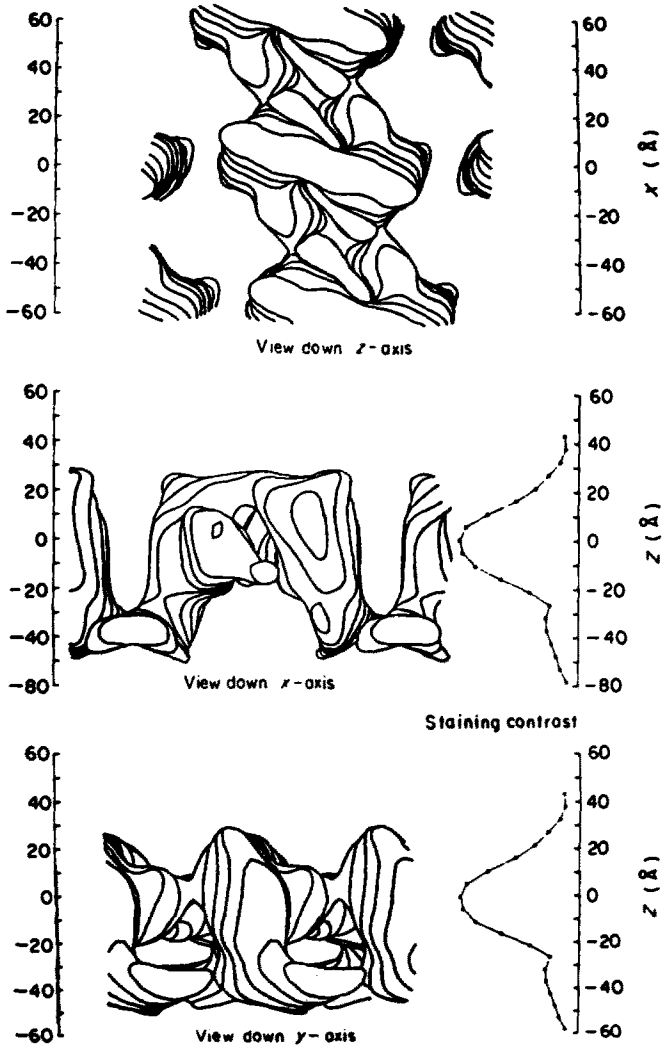

Fig. 6. Reconstructed surface contour views of the E2-type two-dimensional crystals of sarcoplasmic reticulum Ca^{2+}-ATPase. (After Taylor et al. 1986)

The sarcoplasmic reticulum Ca^{2+}-ATPase can be crystallized in two-dimensional arrays with enzyme monomers as the structural units as well (Dux et al. 1985a). Different lanthanides and calcium ions at slightly alkaline pH promote this crystal form, with unit cell dimensions of a: 66 Å, b: 52 Å, γ: 110° (Dux et al. 1985a). Freeze-fracture electron micrographs indicate the reorganization of the intramembrane portion of the enzyme in this crystal form as well; however, the moderate stability of this structure makes the fine-structural analysis more difficult (Ting-Beall et al. 1987).

The vanadate in calcium-free medium stabilizes the enzyme in the low calcium affinity E2 conformation (Pick 1982; Dux and Martonosi 1983c; Jóna and Martonosi 1986). Calcium and lanthanides promote the E1 conformation (Grisham 1983). Additional identification of the crystalline enzyme forms with the E1 and E2 conformations was achieved by limited tryptic cleavage and fluorescence spectroscopy (Dux and Martonosi 1983b; Jóna and Martonosi 1986).

The existence of monomer- and dimer-type two-dimensional Ca^{2+}-ATPase crystals is not sufficient to answer the question regarding the functional role of enzyme oligomerization in ion transport. The different crystal forms reflect only the possibility of forming both monomeric and dimeric structures from the same enzyme (Dux and Martonosi 1983a; Dux et al. 1985a). The functional role of the SR Ca^{2+}-ATPase oligomerization was recently reviewed by Andersen (1989). The question is still far from being settled. Although every reaction step of the ATP hydrolysis and calcium translocation can be performed in solubilized, monomeric preparations, at the very high local protein density in the native SR membrane the role of protein-protein interactions in the regulation and stabilization of the system remains possible (Heegaard et al. 1990; Klein et al. 1991).

Isolated sarcoplasmic reticulum vesicles from fast and slow skeletal and cardiac muscles revealed identical two-dimensional calcium-ATPase arrays in calcium-free media in the presence of vanadate (Dux and Martonosi 1984) (Fig. 5). This indicates similar structural disposition and local density for the enzyme in different muscle types. The percentage ratio of the crystal-covered vesicles showed close correlation with the calcium-ATPase protein content and its specific activity in the microsome fractions (Dux and Martonosi 1984) (Fig. 7). The local density of the calcium pump enzyme, therefore, does not seem to be much different in fast and slow skeletal and cardiac muscle sarcoplasmic reticulum membranes.

In fast-twitch muscle microsomes of the rat and the rabbit, about 70%–90% of the vesicles were covered with Ca^{2+}-ATPase crystals. In slow and cardiac muscle preparations this value was around 6%–10% (Dux and Martonosi 1984). This difference is close to that observed between fast and slow muscles in their SR Ca^{2+}-ATPase content, calcium uptake, and

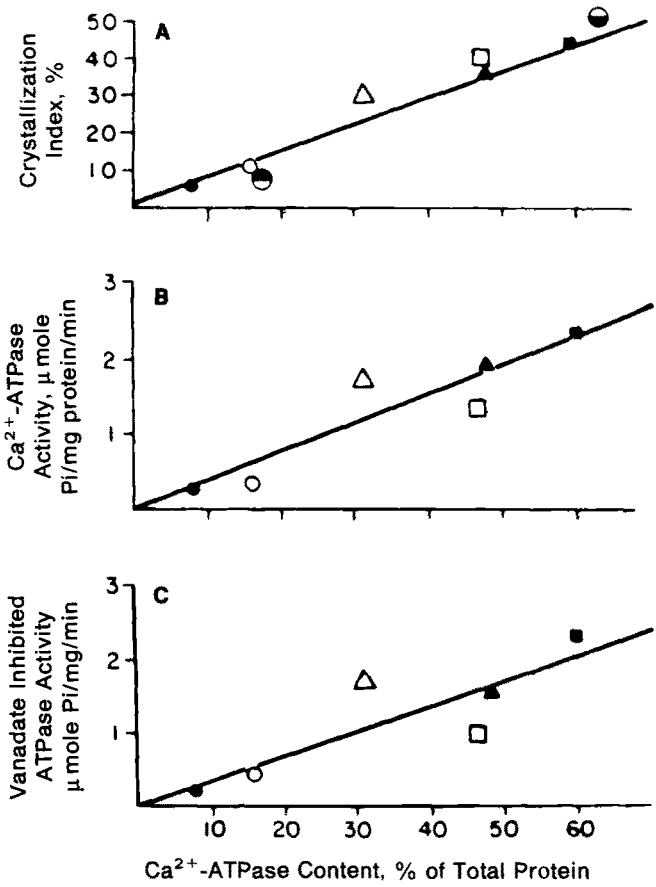

Fig. 7A–C. Correlation between Ca^{2+}-ATPase content and crystallization index (**A**), Ca^{2+}-activated ATPase activity (**B**), and 0.1 m*M* Na_3VO_4-inhibited portion of Ca^{2+}-activated ATPase activity (**C**) in different rat muscle types. Ca^{2+}-ATPase content was determined from gel densitograms. ▲, M. semimembranosus; ○, m. soleus; □, m. extensor digitorum longus; △, diaphragm; ■, m. levator ani; ●, cardiac muscle; *panel A* ◓, rabbit fast muscle; ◒, rabbit slow muscle. Lines are regression lines. Correlation coefficients were 0.96 in **A**, 0.91 in **B**, and 0.83 in **C**. (From Dux and Martonosi 1984)

calcium-dependent ATP hydrolysis activities (Briggs et al. 1977; Zubrzicka-Gaarn et al. 1982; Leberer and Pette 1986).

The almost identical ATPase activity and phosphoprotein concentration levels in fast and slow muscles, calculated on the basis of Ca^{2+}-ATPase content int the preparations by Zubrzicka-Gaarn et al. (1982), are in good agreement with the crystallization results. They indicate that the presence of larger amounts of contaminating non-SR membrane elements in slow and cardiac muscle microsomes may be responsible, at least in part, for the measurable functional differences. However, the lateral diffusion of the

calcium-ATPase enzymes may generate two-dimensional crystalline array patches even at low local densities (Dux 1985). The presence of vesicles completely covered with enzyme crystals in slow and cardiac muscle preparations reduces the likelihood of this explanation (Dux and Martonosi 1984).

In slow-muscle SR vesicles separated by calcium oxalate loading the Ca^{2+}-ATPase still represented only 30% of the total protein content, in contrast to the 70%–85% observed in fast muscles (Zubrzicka-Gaarn et al. 1982). This result is comparable to the Ca^{2+}-ATPase local density ratio of 1.6–2.1 observed by Ferguson and Franzini-Armstrong between fast and slow muscles of guinea pigs (Ferguson and Franzini-Armstrong 1988).

All the applied methods have certain shortcomings. Despite these technical problems, the six- to seven fold difference between the functional parameters of the SR Ca^{2+}-transport system in fast and slow muscles seems to be a combined result of two factors. These are the different total SR membrane and Ca^{2+}-ATPase content in the muscle and some genuine differences between the Ca^{2+}-ATPase isoforms regarding their specific activity and regulation mechanism.

5.4 Immunochemical Differences Between Fast and Slow Sarcoplasmic Reticulum Ca^{2+}-ATPases

Immunochemical and immunohistochemical studies indicated quantitative differences in the SR Ca^{2+}-ATPase content between fast and slow skeletal and cardiac muscles (Jorgensen et al. 1982, 1983; Leberer and Pette 1986). Polyclonal Ca^{2+}-ATPase antibodies detected the weakest reactivity in cardiac and in slow skeletal muscles. Both fast-fiber types contained far more enzyme. Type-IIB fibers showed a somewhat stronger reaction than IIA fibers did (Jorgensen et al. 1982, 1983; Maier et al. 1986b; Krenacs et al. 1989) (Fig. 8).

The qualitative distinction between calcium-ATPase isoforms with antibodies is more complicated. Isoform-specific immunreactivity of some antibodies was reported (Damiani et al. 1981; Volpe et al. 1982; Kaprielian and Fambrough 1987). The high level of sequence homology between the fast and slow isoforms (Brandl et al. 1986) explains the wide cross-reactivity with different polyclonal antibodies. The competitive ELISA system applied by Leberer and Pette was able to distinguish between the fast and slow isoforms to a limited degree (Leberer and Pette 1986).

Identification of the sequence of the Ca^{2+}-ATPase isoform genes makes easier the preparation of real isoform-specific antibodies (Jorgensen et al. 1988; Tunwell et al. 1991). Some isoform-specific monoclonal antibodies proved to be useful in fiber-typing, membrane characterization studies. The slow-type-specific monoclonal antibody was suggested to react with the

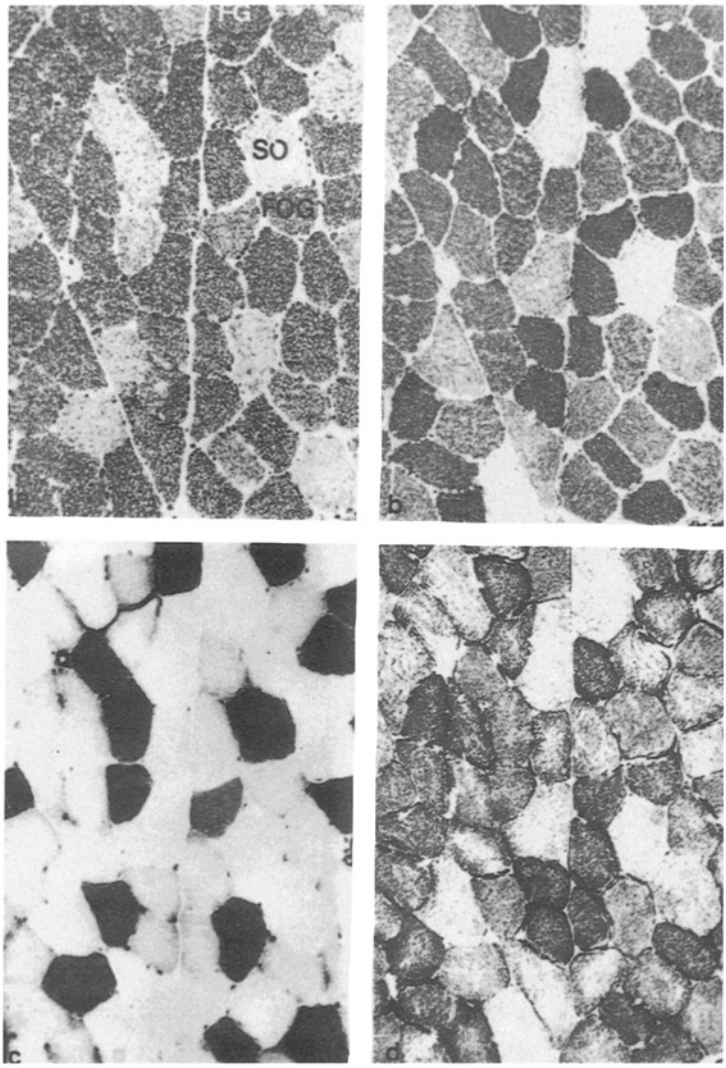

Fig. 8a–d. Consecutive frozen sections of rat gastrocnemius muscle, immunostained for SR Ca^{2+}-ATPase **a** and myoglobin **b** and enzyme histochemically stained for myosin ATPase after preincubation at pH 4.3 **c** and succinate dehydrogenase. **d** Slow oxidative (*SO*) fibers with strong myosin ATPase activity stained slightly for Ca^{2+}-ATPase, while fast glycolytic (*FG*) and fast oxidative glycolytic (*FOG*) fibers show intense SR Ca^{2+}-ATPase staining. (From Krenács et al. 1989)

phospholamban-binding site of the enzyme, since this regulatory protein is absent from the fast sarcoplasmic reticulum (Jorgensen et al. 1988; Mabuchi et al. 1990).

The recent identification of the phospholamban-binding sequence, as well as its presence in the fast Ca^{2+}-ATPase isoform, makes this speculation unlikely (James et al. 1989). Conformational changes i.e., modification of certain enzyme domains, may confuse the immunochemical characterization of the isoform composition by altering the immunoreactivity of the enzyme (Leberer et al. 1987, 1989; Mabuchi et al. 1990; Molnár et al. 1990; Dux et al. 1990; Kaprielian et al. 1991; Matsushita et al. 1991).

5.5 Structural Information on the Sarcoplasmic Reticulum Ca^{2+} Pump, Revealed by Chemical Modification and Limited Proteolysis Methods

Chemical modification of certain regions in the sarcoplasmic reticulum Ca^{2+}-ATPase provided some information on the structure of the enzyme. The competitive inhibitory effect of fluorescein isothiocyanate (FITC), bound to the Lys 515 residue, on ATP binding indicated the close relation of this domain with the nucleotide-binding site (Pick and Karlish 1980; Mitchinson et al. 1982; Champeil et al. 1988). Borohydride reduction identified the phosphorylated residue of the enzyme as Asp 351 (Degani and Boyer 1973).

Early experiments on the SH group reactivity revealed the localization of the protein mass in the membrane bilayer (Hasselbach and Elfvin 1967; Martonosi 1976; Andersen and Moller 1977; Yamada and Ikemoto 1978). The reactivity of the SH groups and the lysine residues was found to change during the transport cycle (Martonosi 1976; Yamamoto and Tonomura 1977; Andersen and Moller 1977). Combined modification experiments with limited proteolytic cleavage of the enzyme identified the SH groups essential for ATP hydrolysis, phosphorylation, calcium binding, occlusion, and translocation (Yamamoto and Tonomura 1977; Kawakita et al. 1980; Davidson and Berman 1987; Ancos and Inesi 1988).

SH group-bound spin labels provided information on the mobility and oligomerization of the enzyme (Hidalgo and Thomas 1977; Napier et al. 1987; Squier et al. 1988). New spin labels, with well-characterized binding sites and better spectral and binding characteristics, seem likely to make these observations even more specific in the future (Horváth et al. 1990, 1992). No systematic comparative study of the chemical modification of the fast and slow Ca^{2+}-ATPase has been published so far.

The sarcoplasmic reticulum Ca^{2+}-ATPase is cleaved by trypsin first at the Arg 505 residue, leading to the generation of two 53- to 55-kD fragments, called A and B (Migala et al. 1973; Inesi and Scales 1974). The second cleavage takes place on the A fragment at Arg 198, yielding two subfragments called A1 and A2 (Thorey-Lawson and Green 1975). The fragmentation of the enzyme does not interfere with the ATP-dependent

phosphorylation of the enzyme until this point, revealing the phosphate-binding site on the A fragment, and subsequently on the A1 subfragment (Stewart and MacLennan 1976). This retention of the phosphate binding makes it possible to visualize the SR Ca^{2+}-ATPase and its cleavage products in mixed membrane preparations and homogenates. The plasma membrane Ca^{2+}-ATPase was successfully distinguished from the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase in different cell types with this method (Sarkadi et al. 1988).

The FITC attached to Lys 515 on the enzyme is carried by the B fragment, indicating that the ATP-binding site (or part of it) is localized around the first cleavage site (Abramson and Shamoo 1978). The fluorescence responses of the attached FITC helped to characterize the conformation-specific structural rearrangements of the cleavage products (Dux et al 1985b; Jóna and Martonosi 1986; Ancos and Inesi 1988).

The accessibility of the second tryptic cleavage site (Arg 198) is highly sensitive to the actual conformation of the enzyme (Dux and Martonosi 1983b; Dux et al. 1985a; Andersen et al 1986; Imamura et Kawakita 1989). In the E1 conformation, the hydrolysis of this peptide bond takes place rapidly. The removal of calcium from the medium and the subsequent addition of vanadate completely protected this region from the tryptic cleavage, by rendering the enzyme into the E2 conformation (Dux and Martonosi 1983b; Dux et al. 1985a). In this conformation even the B fragment seems to be protected from further degradation (Dux et al. 1985b). Antibody labeling experiments detected the second tryptic cleavage site buried deep inside the protein mass of the extramembrane domain (Molnár et al. 1990; Tunwell et al. 1991).

Limited proteolysis with chymotrypsin gave products similar to those observed with trypsin (Ancos and Inesi 1988). Thermolysin and subtilysin yielded multiple small fragments, indicating that these enzymes attack several peptide bonds simultaneously on the Ca^{2+}-ATPase (Ancos and Inesi 1988). Recently, a well-controlled vanadate-induced photocleavage of the SR Ca^{2+}-ATPase was reported. This may add valuable new data to the structural information collected by limited proteolysis methods (Végh et al. 1990; Molnár et al. 1991a).

In certain species the tryptic cleavage of the sarcoplasmic reticulum Ca^{2+}-ATPase gave products different from those observed in the rabbit and rat enzyme (Ohnoki and Martonosi 1980; Dux et al. 1989). In carp microsomes the first cleavage generated an 80-kDa fragment, carrying the phosphate group, and several smaller fragments labeled with FITC (Sarkadi et al. 1988; Dux et al. 1989; Hieu et al. 1992). Although the carp enzyme cross-reacted with polyclonal antibodies generated against rat fast Ca^{2+}-ATPase, the lack of two-dimensional crystal formation in the presence of

EGTA and vanadate, together with the unique cleavage pattern, indicates interesting discrepancies in the structure and disposition of the enzyme, making it a suitable target for further studies of SR evolution (Dux et al. 1989; Hieu et al. 1992).

Wuytack et al. detected slight differences between the apparent molecular weights of the phosphorylated, tryptic cleavage products of the fast and slow calcium-ATPase isoforms (Wuytack et al. 1989). This molecular-weight difference cannot be explained by the different lengths of the remaining amino acid sequences (Brandl et al. 1986, 1987; Korczak et al. 1988). It is caused by some unknown difference in the migration characteristics, most probably in charge distribution. Even without a clear explanation, it represents a sensitive and useful method for the characterization of calcium-ATPase isoform composition at the protein level. The potential of the chemical modification methods in combination with limited proteolysis of the enzyme is probably far from being completely exploited in the characterization of sarcoplasmic reticulum Ca^{2+}-ATPase isoforms.

5.6 The Primary Amino Acid Sequence of the Sarcoplasmic Reticulum Ca^{2+}-ATPase, a Possible Three-dimensional Folding Model

The full-length amino acid sequence of the sarcoplasmic reticulum Ca^{2+}-ATPase was first derived from its complementary DNA sequence (MacLennan et al. 1985). The hydrophobicity plot of the 1001 amino acid sequence suggested ten transmembrane helices. Both the N and the C terminal of the protein, together with two thirds of the total mass, are located on the cytoplasmic side of the membrane (Taylor et al. 1986; Matthews et al. 1989). Antibody and fluorescence labeling detected only minimal amounts of the enzyme protein protruding from the luminal membrane surface (Clarke et al. 1990a; Molnár et al. 1991b).

The large cytoplasmic extramembrane portion of the enzyme is formed by three major domains. The nucleotide binding, the phosphorylation, and the energy transduction step respectively were attributed to these domains (MacLennan et al. 1985; Brandl et al. 1986) (Fig. 9). The primary sequence of the enzyme did not contain any segment similar to the common calcium-binding EF-hand structure (Persechini et al. 1989).

The five transmembrane helices toward the N terminal of the enzyme continue into a narrow stalk segment, connecting the extramembrane domains. This stalk region is very rich in negatively charged glutamate residues, suggesting a possible localization for the calcium-binding site (MacLennan et al. 1985; Brandl et al. 1986). The stalk segment is clearly visible in the two-dimensional crystals of the enzyme with both negative staining and frozen hydrated electron microscopy (Castellani et al. 1985;

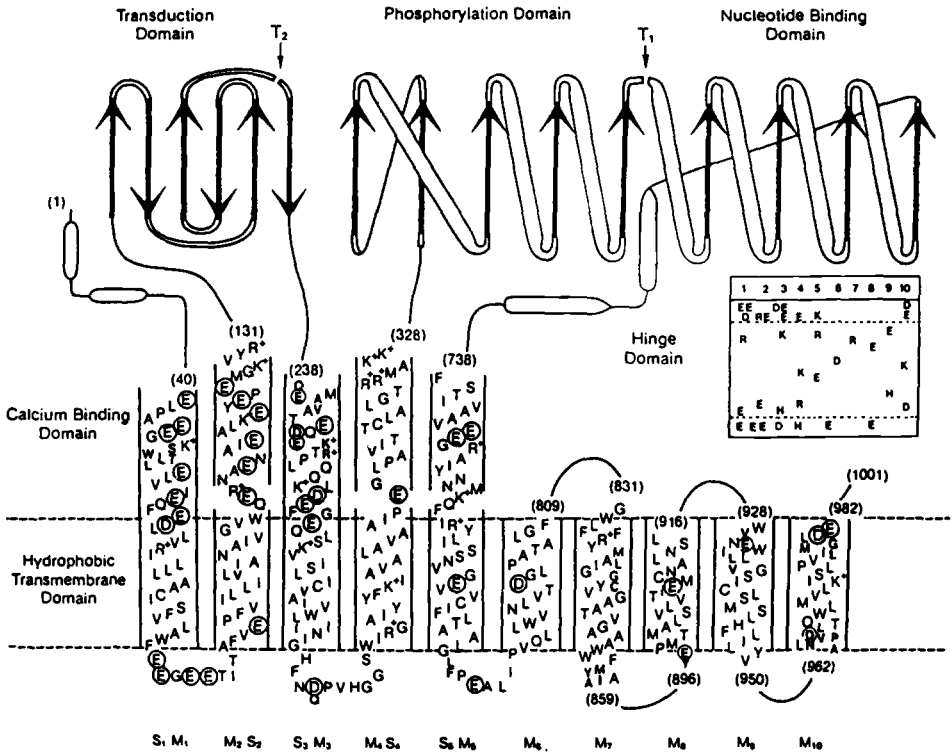

Fig. 9. Proposed tertiary folding model of sarcoplasmic reticulum Ca^{2+}-ATPase, based on the hydophobicity plot of the primary sequence of the enzyme. (From Brandl et al. 1986)

Taylor et al. 1984, 1986, 1988a). The three extramembrane domains are oriented into a trigonal arrangement, reflected by the contour of the molecule both in E1- and in E2-type two-dimensional crystals (Dux et al. 1985a; Taylor et al. 1986) (Fig. 6). This trigonal arrangement provides the steric possibility for the phosphorylation of the Asp 351 residue by the gamma phosphate group of the ATP. The ATP-binding site is located further along the primary sequence, around the first tryptic cleavage site (Arg 505) and the FITC binding site (Lys 515) (Taylor and Green 1989).

Since the discovery of the complete primary sequence, systematic site-directed mutagenesis experiments have helped to prove or modify the proposed three-dimensional folding model of the SR Ca^{2+}-ATPase. MacLennan's group managed to insert and express Ca^{2+}-ATPase gene constructs in COS-1 cells. The infected cells acquired 20–50 times higher calcium transport rates. The presence of the sarcoplasmic reticulum Ca^{2+}-ATPase enzyme was detected with western blotting and immunohistochemical techniques (Maruyama and MacLennan 1988).

Interestingly, almost all kinds of mutant enzymes were expressed, and many of them showed little or no change in calcium binding, calcium transport, phosphorylation, and dephosphorylation properties (Andersen et al. 1989; Vilsen et al. 1989). Most of the negatively charged residues in the stalk region were replaced by uncharged amino acids without any major effect on the calcium binding and transport function (Clarke et al. 1989a). These results disprove one of the reasonable predictions of the proposed model.

The charged residues in the transmembrane helices 4, 5, 6, and 8 were necessary for normal calcium transport function. These four helices are the most likely candidates for the formation of the calcium-binding site and the hydrophylic channel across the membrane (Clarke et al. 1989b; Green 1989).

For the nucleotide-binding function the highly conservative sequence around the phosphate-binding Asp 351 and sequences around residues 519, 604, 629, and 703 seemed to be essential (Maruyama et al. 1989; Clarke et al. 1990b). These results supported the proposed model of Taylor and Green for the ATP-binding site, as a cavity on the cytoplasmic surface of the enzyme, formed by the folding of four peptide segments, localized further apart from each other along the primary sequence (Taylor and Green 1989). Amino acid residues Thr 181, Gly 182, and Glu 183, as well as the whole hinge domain, seem to be essential for the conformational transitions between the E1-P and E2-P forms of the enzyme (Clarke et al. 1990c; Vilsen et al. 1991a).

The localization of the calcium-binding site within the membrane bilayer supports the possible gating mechanism of the ion transport channel through a slight tilting motion of the transmembrane helices 4, 5, 6, and 8 (MacLennan 1990; Inesi et al. 1990; Vilsen et al. 1991b). This transport mechanism model seems to be consistent with the lack of major rearrangements of the secondary structure during the calcium translocation process (Csermely et al. 1986; Arrondo et al. 1987; Barth et al. 1991).

5.7 Isoforms of the Sarcoplasmic Reticulum Ca^{2+}-ATPase

Further studies of the sarcoplasmic reticulum Ca^{2+}-ATPase gene revealed three main isoforms (Brandl et al. 1986, 1987; Lytton et al. 1989; Burk et al. 1989). Burk et al. called the gene expressed in fast skeletal muscles SERCA1 (Burk et al. 1989). It has two different products generated by alternative splicing. The SERCA1a form is present in adult, the SERCA1b form in neonatal fast muscles (Brandl et al. 1987; Burk et al. 1989). The neonatal form contains seven additional amino acids at the C-terminal end. The functional role of this difference is unknown (Brandl et al. 1987; Burk et al. 1989; Lytton et al. 1991).

The SERCA2 gene has a shorter, 997-amino acid splice product called SERCA2a. This form is present in the slow skeletal and cardiac muscles. The 2b variant, having 41 additional amino acids at the C-terminal end, is present in smooth muscle, brain, and many other cell types in low quantities (Burk et al. 1989; Lytton et al. 1989).

The third member of the SERCA gene family (SERCA3) was detected in spleen, stomach, intestine, lung, uterus, and pancreas cells (Burk et al. 1989; Grover and Khan 1992). No alternative splice product of this gene has been found so far.

Brandl et al. compared the sequence of the two main muscle isoforms, the fast adult-type SERCA1a and the slow/cardiac type SERCA2a in rabbit (Brandl et al. 1986, 1987). The adult fast isoform contained four additional amino acid residues. Three of these amino acids were located at the C terminus of the protein; one extra amino acid was added to the sequence in the vicinity of the first tryptic cleavage site (Arg 505) (Brandl et al. 1986, 1987). Sequences and lengths of the individual isoforms seem to show some variability between different species (Campbell et al. 1991).

The fast and slow/cardiac isoforms show about 85% homology in general. In the rabbit they differed in 164 amino acid residues; 66 of them represented alterations with minor physicochemical significance, so-called conservative replacements. Most differences were found at the N-terminal end of the molecule. At the phosphorylation domain and at the nucleotide-binding domain about 34% of the amino acids differed between these two isoforms. The most conserved regions were the transmembrane helices, the stalk region, and the so-called hinge domain connecting the phosphorylation and ion-transporting regions (Brandl et al. 1986).

The pospholamban-binding sequence was found to be present in both the slow/cardiac and the fast isoforms (James et al. 1989), interestingly enough, because of the lack of phospholamban in the fast-muscle sarcoplasmic reticulum (Kirchberger and Tada 1976; Heilmann et al. 1977; Jorgensen and Jones 1986; Leberer et al. 1989).

The sequence of the third intracellular organellar calcium pump protein (SERCA3) has about 75% homology with both the fast and slow/cardiac isoforms (Burk et al. 1989; Lytton et al. 1989, 1991). It seems to have a segment suitable for cAMP-dependent protein kinase phosphorylation (Burk et al. 1989). This observation is in agreement with the early data indicating that calcium uptake into the endoplasmic reticulum of nonmuscle cells is activated by cAMP-dependent protein kinase and calmodulin (Bygrave and Tranter 1978; Moore and Kraus-Friedmann 1983; Gerok et al. 1990).

Despite the remarkable developments in the field of sarcoplasmic reticulum Ca^{2+}-ATPase molecular biology and sequence analysis, the complete understanding of the structure and function of the different isoforms

will require the high-resolution X-ray crystallography analysis of three-dimensional Ca^{2+}-ATPase crtystals (MacLennan 1990).

Generation of three-dimensional crystals of the fast-muscle sarcoplasmic reticulum Ca^{2+}-ATPase isoform was successful in solubilized membrane preparations (Dux et al. 1987; Pikula et al. 1988; Taylor et al. 1988b). Improved methods of crystal formation promise to provide high-resolution structural data on the enzyme. With frozen hydrated electron microscopy a resolution of 6A has been achieved so far (Taylor et al. 1988b; Stokes and Green 1990a,b). Comparative studies on the three-dimensional crystals of the different Ca^{2+}-ATPase isoforms still have a long way to go.

5.8 Different Regulation Mechanisms in Sarcoplasmic Reticulum Membranes – Phospholamban

The Ca^{2+}-dependent ATPase activity, the initial rate of calcium uptake, and the steady-state level of phosphorylated intermediate formation was found to be five to ten times lower in cardiac and slow muscles than in the fast-muscle SR system (Shigekawa et al. 1976; Sumida et al. 1978; Wang et al. 1979; Fehér et al. 1988). The similar crystal structures observed in the different isoforms (Dux and Martonosi 1984) and the high level of homology in the amino acid sequences (Brandl et al. 1986, 1987) suggest general similarities in the molecular mechanism of the ATP-dependent calcium transport in fast and slow skeletal, as well as in cardiac muscles. Additional differences in regulatory mechanisms, in interactions with other proteins, lipid elements of the sarcoplasmic reticulum membrane, or with other substances in the muscle, may explain the differences in functional performances, especially under in vivo conditions.

The decay phase of the calcium signal in cardiac muscle was shortened by beta-adrenergic stimulation. The relaxation of the heart became faster, as part of the positive inotropic-chronotropic response (Tada et al. 1974; Kirchberger and Tada 1976; Wollenberger and Will 1978). A similar response was detected in slow skeletal muscles, although it attracted less interest (Bowman and Zairis 1958; Bowman and Raper 1967; Marsden and Meadows 1970).

The sarcoplasmic reticulum membrane of these muscle types contains a 22- to 25-kDa pentameric protein, phospholamban (Katz and Remtulla 1978; LePeuch et al. 1979; Jorgensen and Jones 1986). The amount of phospholamban/Ca^{2+}-ATPase in slow-muscle sarcoplasmic reticulum was only 20% that detected in cardiac muscle (Jorgensen and Jones 1986). The explanation of this difference is the presence of some fast fibers in the slow-muscle preparations, rather than a different stoichiometric ratio between the phospholamban and the Ca^{2+}-ATPase in slow and cardiac muscles.

The phospholamban can be phosphorylated with cAMP-dependent, calmodulin-dependent, and, at least in vitro, phospholipid-dependent protein kinases (Katz and Remtulla 1978; LePeuch et al. 1979; Movsesian et al. 1984; Jakab and Kranias 1988). The phosphorylation of the phospholamban increases the calcium affinity of the calcium-ATPase enzyme and leads to a twofold activation of the ATP hydrolysis and calcium transport functions (LePeuch and Demaille 1989). The dephosphorylated phospholamban binds to the calcium-ATPase, probably in its E2 conformation (James et al. 1989). Following the phosphorylation by either kinase system it dissociates from the enzyme, and a concomitant charge rearrangement on the membrane surface may be responsible for the increased calcium affinity of the ATPase (Chiesi and Schwaller 1989; Colyer and Wang 1991).

The effects of the different kinase systems on the phosphorylation level of the phospholamban are not additive. Kimura et al. observed a similar activation of the calcium pump by the binding of a monclonal antibody to the phospholamban in the cardiac sarcoplasmic reticulum membrane (Kimura et al. 1991). The effect of the monoclonal antibody binding was not additive to the effects of the phosphorylation induced by different kinase systems. This observation indicates that the removal of the phospholamban from a certain region of the calcium-pump protein is more important in the activation than the phosphorylation-induced charge redistribution.

There are interesting similarities between the sequence and structure of the phospholamban and the calmodulin-binding domain of the plasma membrane (PM) calcium-ATPases (Chiesi et al. 1991). The calmodulin-binding domain exerts an inhibitory effect on the PM Ca^{2+}-ATPase. The binding of calmodulin removes this domain from the active region of the enzyme, with a concomitant activation of the transport function (Carafoli 1987, 1991). Similar activation of the PM Ca^{2+}-ATPase can be achieved by the proteolytic cleavage of the calmodulin-binding domain (Sarkadi et al. 1980). This mechanism indicates an interesting evolutionary analogy in the control mechanism of the plasma membrane and the cardiac and slow-type sarcoplasmic reticulum calcium transport systems. The fast-skeletal muscle sarcoplasmic reticulum system, facing a more strenuous functional demand, may have lost this down-regulation mechanism during its history of specialization.

James et al. identified the sequence of the SR Ca^{2+}-ATPase responsible for the phospholamban binding (James et al. 1989). Since the same sequence was present not only in the slow/cardiac isoform, but in the fast muscle Ca^{2+}-ATPase as well, the absence of phospholamban from fast SR seems to be responsible for the insensitivity of this muscle type to beta-adrenergic stimulation. Indeed, in reconstituted proteoliposomes the fast isoform reacted to the phosphorylation of the added phospholamban in the

same way as the slow/cardiac isoform of the enzyme does (Szymanska et al. 1991). Therefore, the regulation of the phospholamban expression seems to be a decisive element in the diversity of cardiac, slow, and fast skeletal muscle sarcoplasmic reticulum functions.

Additional differences in the regulatory mechanism of the Ca^{2+}-ATPase isoforms may surface in the future. They will provide interesting questions for research into muscle relaxation and sarcoplasmic reticulum in the coming years.

6 Participation of the Sarcoplasmic Reticulum System in Alterations of the Muscle Fibers

6.1 Sarcoplasmic Reticulum Function in Muscle Differentiation and Development

It is not completely understood why the endoplasmic reticulum membrane of muscle cells differentiates into the highly specialized sarcoplasmic reticulum. The most likely explanation is that following the myoblast fusion the surface-to-volume ratio of the myotubes, later myofibers, becomes unfavorable for handling the large amount of calcium to be released and eliminated through the plasma membrane (Peachey and Porter 1959; Kretsinger 1990).

During embryonic development the extent and level of specialization of the sarcoplasmic reticulum system reflects the actual muscular activity of the embryo. In early embryonic muscles the amount of the Ca^{2+}-ATPase nearly equals the amount detectable in nonmuscle cells (Gerok et al. 1990). The calcium transport activity in muscles of 10-day-old chicken embryos had increased about ten fold by the time of hatching. A further five fold increase was detected during the first 6 weeks of postnatal development (Boland et al. 1974; Tillack et al. 1974). Similar tendencies were found in the ATPase activity and phosphorylated enzyme level. The total calcium-ATPase protein content and the density of the freeze-fracture intramembrane particles showed a somewhat smaller increase (Tillack et al. 1974).

The techniques measuring the different functions and components of the sarcoplasmic reticulum system have varying levels of accuracy and reliability, especially in embryonic muscles. Still, the disproportionate increase in some parameters of the sarcoplasmic reticulum during embryonic development may indicate some qualitative transitions, besides the increase in the total amount of these proteins (Gauthier and Hobbs 1986).

The sarcotubular system continues its development after birth (Luff and Atwood 1971). In the fast EDL muscle of the mouse the T-tubule volume density at birth was 0.08%. It increased about fivefold (to 0.4%) in adult

animals. In the slow soleus muscle a similar fivefold increase elevated the volume ratio of the T tubules from 0.04% to 0.2% (Luff and Atwood 1971). The volume density of the sarcoplasmic reticulum in EDL increased from 1.1% to 5.5%, in the soleus from 1.7% to 2.9% during this period of life.

Two-dimensional Ca^{2+}-ATPase crystal formation was detected first in the muscles of 18-day-old chicken embryos (Dux 1985). The enzyme crystals covered only small portions of certain vesicles at this age. This indicated the possibility that the synthesis and membrane insertion of the enzyme take place at distinct circumscribed areas of the initially protein-free phospholipid membrane. Lateral diffusion and consecutive aggregation of the enzyme molecules may cause the increase of the local concentration in certain areas as well, making the crystal formation possible.

The sarcoplasmic reticulum vesicles from embryonic sheep cardiac muscle contained 60% less calcium ATPase protein than those in the hearts of adult animals (Mahony and Jones 1986). The transport activity reached 70% of the level observed in adult cardiac SR. This observation indicates a higher transport turnover rate for the embryonic form of the enzyme, and a possible isoenzyme shift during development. There are indications that the embryonic cardiac muscle depends more on calcium elimination through the sarcolemmal membrane than on the sequestration function of the sarcoplasmic reticulum (Seguchi et al. 1986).

Immunohistochemical staining using monoclonal antibodies indicated that the primary chicken myotubes synthesize the slow/cardiac Ca^{2+}-ATPase isoform (Kaprielian and Fambrough 1987). Later, in the secondary myotubes, both the fast isoform and the slow/cardiac isoform were detectable. The synthesis rate of the fast isoform began to increase dramatically on the 19th day of embryonic development. Kaprielian and Fambrough concluded that the timing and regulation of the sarcoplasmic reticulum Ca^{2+}-ATPase isoform shifts during muscle maturation and are different from the timing and regulation of the myosin heavy chains and other muscle-specific proteins (Kaprielian and Fambrough 1987; Gunning and Hardeman 1991).

Molecular genetic analysis identified a Ca^{2+}-ATPase isoform as the embryonic splice product of the SERCA1 gene, the so-called SERCA1b (Brandl et al. 1987; Lytton et al. 1989; Burk et al. 1989). Species-specific differences and similarities should be clarified before one directly carries over these molecular genetic observations to the embryonic development of the chicken (Campbell et al. 1991).

Sutko et al. reported isoform differences between the expressed forms of the ryanodine receptor/foot protein complexes during the embryonic development of the chicken (Sutko et al. 1991). Further characterization of these isoforms and their functional differences may reveal important regulatory

factors in the organization and assembly of the muscle fibers. In muscle cell line cultures BC3H1 and C2C12, Airey et al. observed ryanodine receptor expression even before fusion took place (Airey et al. 1991).

The factors involved in the regulation of the sarcoplasmic reticulum differentiation and development are not completely known. The intracellular calcium level was found to regulate the synthesis of the calcium-ATPase in muscle cell cultures (Ha et al. 1979; Roufa et al. 1981; Martonosi et al. 1982). This may represent a signal for SR differentiation in vivo as well. The elevated intracellular calcium level may be related to the increased muscle activity, beginning around the 17th or 18th day in chicken embryos (Reiser and Stokes 1982).

Other factors such as hormones, growth factors, and neural activity may participate in the complex process, transforming the smooth endoplasmic reticulum into the highly specialized, abundant, calcium-sequestrating sarcoplasmic reticulum system (Florini et al. 1991; Gunning and Hardeman 1991; Airey et al. 1991). These factors and their interrelationships constitute an important and exciting field for further research.

6.2 Relaxation and Sarcoplasmic Reticulum Function in Muscle Adaptation: Exercise and Fatigue

Fatigue, the workload which exceeds the functional and metabolic capacity of the muscle, is characterized by an increase in relaxation time in cardiac and skeletal muscles. The length and extent of the workload, or the stimulation frequency which is needed to elicit the fatigue, is different depending on the type, species origin, previous exercise history, and other parameters of the muscle under study (Hochachka 1990).

The different muscle types behave differently when subjected to repetitive stimulation (Brown and Euler 1938; delPozo 1942; Hanson 1974). In fast-twitch rat muscle, repeated stimulations rapidly increased the amplitude of the twitch tension; a similar reaction was not observed in the slow soleus muscle (Hanson 1974). In 1968, Close and Hoh observed a maximum 1.9-fold increase in the twitch tension following repetitive stimulation of rat fast muscles. Both low- and high-frequency trains of stimuli (20 Hz and 300 Hz respectively) were able to increase the contraction tension (Close and Hoh 1968). This reaction is called the staircase phenomenon, following repetitive stimulation at low frequency, and post-tetanic potentiation, after a high-frequency stimulation protocol.

Short stimulation trains at either frequency were not able to change the contraction time. The half relaxation time of the muscles was even found to be shortened. Longer trains of stimuli, however, caused a significant lengthening in both the contraction and half relaxation time (Close

and Hoh 1968). The explanation of the staircase phenomenon and post-tetanic potentiation is still not completely clear. There is a close correlation between the myosin light-chain phosphorylation and the presence of contraction potentiation in different rat muscles (Moore and Stull 1984). This, or other modifications of the contractile proteins, may be responsible for the altered contraction parameters. Other possible explanations may be the increased release of activating calcium and/or the decreased functional performance of the calcium-eliminating, -buffering systems following repeated stimulation (Close and Hoh 1968; Klug et al. 1988; Williams 1990).

Altered neural stimulation patterns are reflected in the relaxation properties of the muscles. Chronic low-frequency stimulation resulted in the slowing of the relaxation speed in fast-twitch muscles (Pette et al. 1972, 1973; Salmons and Sreter 1976). However, Eerbeck et al. found lengthened relaxation at both 5–10 Hz and 20–40 Hz chronic stimulation of cat muscles (Eerbeck et al. 1984). In their experiments the half relaxation time increased by 212%, well beyond the 140% increase in the total twitch contraction time. The stimulated fast-twitch muscles became even slower than the normal slow cat muscles. The different stimulation protocols and the different species under study make the direct comparison of these experiments complicated (Simoneau and Pette 1988).

The biochemical background of the altered relaxation in chronically stimulated muscles is first of all probably an early fatigue reaction of the muscle. In long-term experiments, however, it represents one of the general adaptive fiber-type alterations which take place after several weeks of stimulation or extensive training. The metabolic and ionic changes that occur during the early phase of the adaptation process may be responsible for the genetic changes, leading to the altered phenotypic characteristics of the stimulated muscles.

In 24 weeks of endurance training Ingjer detected the redistribution of fiber types in human muscles (Ingjer 1979). Histochemical methods did not detect any shifts between fast and slow types. More prominent was the shift from the fatigue-sensitive IIB type to the fatigue-resistant IIA type and, above all, the presence of the transitory form-IIC type. The contraction-relaxation properties of this IIC type are not different from those of the other fast types (IIA,IIB), although IIC fibers are more fatigue resistant than IIB fibers (Schiaffino et al. 1990).

Fifteen weeks of high-intensity training in rats increased the proportion of the type-I and -IIA fibers, with a concomitant decrease in the number of IIB fibers (Green et al. 1984). The changes involved the myosin isoform composition, SR parameters, and the parvalbumin content of the muscles.

Increased workload of the myocardium results, as an early response, in a slowing of the relaxation. The load dependence of the relaxation disappears

as an early signal of the hypoxic state of the cardiac muscle (Lecarpentier et al. 1982; Brutsaert and Sys 1989; Schwartz et al. 1990). In several studies, a decrease in the immunochemically detectable amount of the sarcoplasmic reticulum Ca^{2+}-ATPase, or in its mRNA message, was detected under these circumstances (Levitsky et al. 1989; Komuro et al. 1989; Mercadier et al. 1990). Expression of a different (fast) isoform of the SR Ca^{2+}-ATPase was never observed in cardiac muscle (Schwartz et al. 1990). Therefore, the change in the cardiac SR Ca^{2+}-ATPase content due to decreased synthesis and/or enhanced degradation seems to be an early and important step in the myocardial reaction to increased workload, ischemia, and hypoxia.

Inactivation of skeletal muscles increases the speed of their relaxation (Kim et al. 1982). Tenotomy caused shorter relaxation time in the slow soleus muscle, while tenotomized fast-twitch muscles did not change their contraction-relaxation speed (Vrbova 1963). These responses are somewhat a mirror image of the slowing relaxation in chronically stimulated or fatigued muscles.

In fatigued muscles the slowing of the relaxation is obvious. Possible sources of this response are reviewed by Green (1990). In short-term fatigue, the exhaustion of the energy donor substances in the muscle was supposed to stand behind the altered relaxation (Edwards et al. 1975). Parameters such as increased lactate, inorganic phosphate, decreased ATP and creatine phosphate levels, acidosis, and increased temperature were suspected to interfere with the actomyosin cross-bridge dynamics and sarcoplasmic reticulum functions (Nakamaru and Schwartz 1972; Edwards et al. 1975; Sahlin et al. 1981; Scherer and Deamer 1986; Byrd et al. 1989a,b).

The role and importance of these individual parameters is still not completely clear. Sahlin et al. did not observe a slowed relaxation in iodoacetamide-poisoned muscles which did not accumulate lactate during exercise (Sahlin et al. 1981). However, Edwards et al. detected a change in the relaxation time of IAA-poisoned muscles as well (Edwards et al. 1975).

Accumulation of calcium and/or other ions, and a subsequent failure of the action potential and the excitation contraction coupling, was suspected as a primary cause of muscle fatigue (Bianchi and Narayan 1982a,b). The elevated calcium content in the sarcoplasmic reticulum terminal cisternae found by X-ray microanalysis in exhausted muscles may support this concept (Gonzalez-Serratos et al. 1978).

The failure of the calcium-elimination system in metabolically fatigued muscles was suggested by many studies. A transitory drop in the SR calcium uptake and ATPase activity was found in horses after high-intensity exercise (Byrd et al. 1989a,b). The sarcoplasmic reticulum function recovered to the normal level within 60 min after cessation of the excessive workload. It nearly followed the recovery of the muscle pH and temperature to the

normal level (Byrd et al. 1989a,b). Very similar trends were observed in the relaxation properties and SR Ca^{2+} uptake of human muscles during exercise (Gollnick et al. 1991). The decreased SR function was observed above all in type-I and -IIA fibers. Fewer alterations were found in the IIB fibers (Fitts et al. 1982; Byrd et al. 1989a,b).

The fatigue reactions are not identical after high- and low-frequency stimulation of the muscles (Metzger and Fitts 1987). Although the early changes in the relaxation and in the action potential parameters are similar, the intracellular pH becomes more acidic, the SR function more depressed in low-frequency fatigue. The recovery from this form of muscle fatigue is a slower process (Metzger and Fitts 1987; Green 1990).

Evidence has been published for both the calcium release and the calcium uptake functions of the sarcoplasmic reticulum as being the main cause of the altered relaxation in fatigued muscles (Dawson et al. 1980; McIntosh and Kupsch 1987; Metzger and Fitts 1987; Westerblad et al. 1991; Westerblad and Lennergren 1991). Detailed characterization of this phenomenon is still and exciting question at the borderline of muscle physiology and biochemistry.

Chronic low-frequency stimulation of rabbit fast-twitch muscles leads to the slowing of the relaxation within 4–5 days (Pette et al. 1973, 1976; Brown et al. 1976). The ATPase activity and calcium uptake function drops to about 50% of the normal level during this time (Heilmann and Pette 1979; Leberer et al. 1987; Simoneau et al. 1989), while the immunochemically detectable amount of Ca^{2+}-ATPase remains unaltered. The reduced level of SR function remains unchanged for about the following 30–40 days of stimulation (Leberer et al. 1987). After 40–50 days of stimulation the amount of the SR Ca^{2+}-ATPase begins to decline, with a concomitant shift in its isoform composition. The synthesis of the slow/cardiac isoform in the stimulated fast muscles is accompanied by the synthesis of phospholamban, normally present only in cardiac and slow skeletal muscle SR (Leberer et al. 1989; Briggs et al. 1990).

Chronic low-frequency stimulation caused an early dramatic decrease in the parvalbumin content of the muscle (Klug et al. 1983a,b; Leberer and Pette 1990). During the first 5 days of stimulation the muscle looses about 50% of its original parvalbumin content. After 21 days, its level falls below 7% of the control value (Klug et al. 1988). Despite the clear parallelism between the relaxation properties and parvalbumin content of a muscle, the changes of the two parameters do not show complete linearity: 90% reduction of the parvalbumin content was found in muscles with 60% elongation of their relaxation time (Klug et al. 1988). The disappearance of the staircase phenomenon after chronic low-frequency stimulation of fast muscles with a parallel drop in the parvalbumin content supports the

importance of this protein in calcium buffering during the increased muscle activation in repeated stimulation (Gillis et al. 1982; Gillis 1985; Klug et al. 1988).

Sreter et al. detected a fivefold increase in the resting calcium ion concentration in chronically stimulated fast-twitch muscles (Sreter et al. 1987). This elevated calcium level may be the result of the decreased parvalbumin content and the depressed SR function in the muscle. On the other hand, it may just represent an unspecific response, a transitory imbalance in the calcium homeostasis of the muscle, since a similar increase was observed at the cessation of the stimulation as well (Sreter et al. 1987).

The same authors detected the slow isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase after only a few days of low-frequency stimulation in fast muscles (Mabuchi et al. 1989, 1990). The apparent difference between their results and those from other laboratories (Leberer et al. 1989; Briggs et al. 1990) may be due to slightly different stimulation patterns, to the appearance of a unique slow isoform in the stimulated muscle, as suggested by Mabuchi et al. (1990), or to the limited sensitivity of the antibodies used for the immunochemical studies, detecting slight conformational rearrangements in the calcium-ATPase molecules as the synthesis of a new isoform. The mRNA message of the slow isoform was seen to increase only after much longer stimulation periods (Leberer et al. 1989).

The early partial inactivation of the calcium-ATPase molecules in low-frequency stimulation does not seem to be the result of the degenerative reactions observed in about 10% of the fibers (Gambke et al. 1985; Maier et al. 1986a). Reduced ATP and FITC binding, together with altered tryptic cleavage at the T1 site, indicates a modification of the ATP-binding site in at least a certain population of the enzyme (35%–50%), leading to the inactivation of these molecules (Leberer et al. 1987; Dux and Pette 1990; Dux et al. 1990; Matsushita et al. 1991; Matsushita and Pette 1992). The mechanism of this modification is not known yet; it may be a response to metabolic changes, chemical factors in certain fibers, or fiber regions.

The reduced calcium uptake of the sarcoplasmic reticulum is likely an early factor in the induction of other steps in the complete, function-dependent adaptive transformation of fast skeletal muscles under chronic low-frequency stimulation (Pette 1990a, 1991).

6.3 Responses of the Sarcoplasmic Reticulum System to Altered Thyroid States

Recent observations indicate that the hormones of the thyroid gland have a profound effect on the contractile parameters of the muscles. Hypofunction of the thyroid gland results in prolonged relaxation, while hyperfunction

or administration of thyroxin shortens the relaxation time. It turns the contraction parameters of slow muscles into those of the fast type (Fitts et al. 1980; Beekman et al. 1989; Dulhunty 1990; Everts 1990).

The sarcoplasmic reticulum with its calcium-ATPase enzyme is one of the targets of the thyroid hormone effect. Both fast and slow skeletal, as well as cardiac muscles are involved in the reaction (Suko 1973; Fitts et al. 1980; Rohrer and Dillmann 1989; Beekman et al. 1989; Simonides et al. 1990). The effect is more obvious in the slow and cardiac muscles, probably because the SR membrane in the fast muscle is already almost saturated with the calcium pump protein, leaving little space for further increases in the local density of the enzyme.

In the slow and cardiac muscle SR membranes the density of the intramembrane particles increased proportionately to the shortening of the relaxation time (Dulhunty 1990). Analysis of the Ca^{2+}-ATPase mRNA messages indicated that in the cardiac muscle only the slow/cardiac isoform of the enzyme was expressed (Rohrer and Dillmann 1989). In the slow fibers a moderate increase in the slow Ca^{2+}-ATPase synthesis rate was accompanied by an about 150-fold increase in the synthesis rate of the fast isoform (Simonides et al. 1990).

With immunohistochemical detection methods Nunes et al. described a more pronounced increase in the slow isoform synthesis in the slow muscles of hyperthyroid animals (Nunes et al. 1985). However, the limited isoform selectivity of many antibodies, together with possible changes in antibody affinities, may make detection of the real increase in the fast isoform synthesis by nucleic acid analysis difficult (Simonides et al. 1990).

Despite the exciting new developments in this area, there is still no complete understanding of the factors involved in SR adaptation in altered thyroidal states. In the cardiac SR the increased phosphorylation of phospholamban under thyroxin treatment has been described as a possible factor in the increased calcium pump activity and in the consecutive improvement of the relaxation function (Suko 1973; Limas 1978). An interesting opposite regulatory effect of thyroid hormones was detected on the expression of calcium-ATPase and phospholamban in cardiac muscle. In euthyroid hearts the ratio of calcium-ATPase to phospholamban was 0.81 (Beekman et al. 1989). In hypothyroid animals this ratio decreased to 0.32, while in hyperthyroid heart it became as high as 1.69 (Beekman et al. 1989). This shift in the Ca^{2+}-ATPase/phospholamban ratio may contribute to the increased pump activity, by eliminating the inhibitory effect of the dephosphorylated phospholamban on at least a certain portion of the Ca^{2+}-ATPase molecules. Since the cardiac muscle seems to synthesize only the slow/cardiac isoform of the enzyme, even in hyperthyroid animals (Rohrer and Dillmann 1989), this situation generates an interesting change in the stoichiometric ratio of

the calcium pump and its regulator, phospholamban. Further studies in this area seem to be necessary.

Thyroid hormones influence the synthesis of many other elements of muscle tissues. Major quantitative and qualitative changes take place in the expression of the Na^+, K^+-ATPase, and myosin isoforms under the influence of the thyroid hormones as well (Chizzonite and Zak 1984; Izumo et al. 1986; Ismail-Beigi et al. 1986; Russell et al. 1988; Kirschbaum et al. 1990). Chronic low-frequency stimulation and thyroid hormones seem to influence antagonistically the expression of fast isomyosins (Kirschbaum et al. 1990). This pioneering observation suggests that the thyroid hormones are probably one of the key elements in the delicate chain of interactions of muscle activity, neural influences, hormones, and growth factors determining the ultimate phenotype of a given muscle, and making the functional adaptations, the plasticity of the muscles possible (Müller et al. 1991). This regulatory action of the thyroid hormones, together with other hormones and growth factors, is an attractive field for future sarcoplasmic reticulum research.

6.4 Involvement of the Sarcoplasmic Reticulum System in Muscle Diseases

Several forms of muscle diseases are associated with slowed relaxation, or with a defective restoration of the resting state after contraction. Certain functions of the sarcoplasmic reticulum were found to be altered in muscle diseases. In most cases these alternations are the outcome of changes in the fiber-type composition of the affected muscle (Webster et al. 1988), the accumulation of nonmuscle cells within the samples tested, and the contamination of the SR preparations with other membrane elements. Therefore, these changes should be considered secondary events during the development of muscle diseases. A comprehensive review of the role of the sarcoplasmic reticulum in muscle diseases was recently published by Martonosi (1989).

The protracted relaxation sometimes observed in X-chromosome-linked progressive dystrophies of human beings, mice, and dogs can be explained in part by the higher vulnerability of the fast-twitch fibers, leaving the slow fibers, with their slower relaxation, behind (Dubowitz and Brooke 1973; Nonaka et al. 1981; Dangain and Vrbova 1988; Webster et al. 1988; Fink et al. 1990). The dystrophic process, with repeated fiber degeneration, regeneration, and subsequent fibrosis, may leave the mechanoelastic components of the affected muscles damaged as well (Coulton et al. 1988).

Despite the several reported alternations in the chemical composition and functional performance of the sarcoplasmic reticulum in progressive dystrophies, the primary cause of the disease is associated with a defect

in the sarcolemmal membrane and its cytoskeletal supportive system, the dystrophin and its associated glycoprotein complex (Hoffmann et al. 1987a, 1988; Arahata et al. 1988; Campbell and Kahl 1989; Ervasti et al. 1990; Yoshida and Ozawa 1990; Ibraghimov-Beskrovnaya et al. 1992). Indeed, the local density of the SR Ca^{2+}-ATPase and its disposition and molecular structure were found to be unchanged by two-dimensional enzyme crystallization in human progressive dystrophy (DMD) patients (Dux and Martonosi 1983d).

The intensive mechanical stress affecting the muscle membranes during contraction and relaxation requires a strong anchoring system to connect the membranes to the cytoskeleton (Waugh et al. 1974; Nunzi and Franzini-Armstrong 1980; Lazarides 1980). These elements play an important role in protecting the barrier function of the membranes during mechanical stress, stretch, and compression. The membrane-cytoskeletal protein dystrophin was first mistakenly associated with the triad region of the sarcotubular system (Hoffmann et al. 1987b; Watkins et al. 1988). In the light of recent results this does not seem to be the case, but other filamentous elements are certainly involved in this function (Fulton and Isaacs 1991). Their detailed characterization seems to be an interesting question for future studies.

In Dy-type mouse muscular dystrophy, Leberer et al. found 30%–50% lower specific calcium transport activity, together with an unchanged amount of immunoreactive Ca^{2+}-ATPase protein (Leberer et al. 1988). In this study the measurements were made on whole muscle homogenates; therefore, the differences reflect genuine inactivation of certain portions of the calcium pump molecules. No structural defect of the SR Ca^{2+}-ATPase was found in this dystrophy (Dux and Martonosi 1983d).

In chicken hereditary muscular dystrophy Kaprielian et al. found synthesis of the slow calcium-ATPase isoform in fast-twitch muscles to persist beyond its presence in normal animals (Kaprielian et al. 1991). There was no difference in the disappearance of the fast isoform from the slow muscles of normal and dystrophic chicks. These results indicate interesting differences in the regulation of Ca^{2+}-ATPase isoform expression and its possible involvement in chicken hereditary muscular dystrophy. Since both the fast and slow isoforms can form similar two-dimensional arrays, the normal structural features of the enzyme in SR vesicles derived from dystrophic chicken muscle is in agreement with the results of Kaprielian et al. (Dux and Martonosi 1983d, 1984).

Brody's disease, or silent myotonia, is the only known relaxation disorder associated primarily with a defect of the sarcoplasmic reticulum Ca^{2+}-ATPase (Brody 1969). The fast isoform of the enzyme is absent. This causes extremely prolonged relaxation, especially after resting periods. Interestingly, the absence of the fast isoform does not lead to fiber degeneration,

or dystrophic reactions, most probably because of the compensatory increase in the sequestering function of the unaffected slow Ca^{2+}-ATPase isoform, and perhaps other calcium sequestering systems (Karpati et al. 1986; Danon et al. 1988). The lack of dystrophic signs in Brody's disease indirectly excludes the significance of SR Ca^{2+}-ATPase defects in other muscle disorders with severe muscle devastation (Martonosi 1989).

The junctional sarcoplasmic reticulum membrane and its excitation-contraction coupling function is involved in mouse muscular dysgenesis. The genetic defect of the T-tubule voltage sensor, dihydropyridine receptor, molecules causes a general disruption of muscle differentiation and development (Powell 1990; Adams and Beam 1990).

In malignant hyperthermia, the calcium-release function of the sarcoplasmic reticulum system is involved. The gene encoding the ryanodine receptor calcium-release channel complex is defective, causing calcium overloading of the affected muscles (Nelson 1988; Knudson et al. 1990; MacLennan et al. 1990). This leads to extreme activation of the contractile apparatus and subsequent exhaustion of the metabolic reserves of the muscle (Nelson 1988; McCarthy et al. 1990; Davies 1990). These events usually cause delayed relaxation in the affected muscles, without the primary involvement of the calcium-elimination systems (Everts et al. 1992).

The accumulation of calcium in muscle fibers with a consecutive activation of calcium-dependent proteases is a common feature in the late phase of several muscle diseases (Dux et al. 1978; Bertorini et al. 1982; Turner et al. 1988, 1991; MacLennan et al. 1991). In part, this can be considered a secondary, aspecific sign of the general disruption of the regulation processes in the muscle (Martonosi 1989). The decrease in the amount of the soluble calcium buffer, parvalbumin, in some muscle diseases may contribute to this process as well (Haiech et al. 1979; Stuhlfauth et al. 1984; Klug et al. 1985; Jockusch 1990).

7 Questions Regarding Muscle Relaxation at the Border Between Physiology and Biochemistry: Future Perspectives

The large amount of information gathered during the past few years on muscle relaxation and the structure and function of the sarcoplasmic reticulum makes a comprehensive description of the field more and more difficult. The key role of the sarcoplasmic reticulum and its Ca^{2+}-ATPase enzyme in the regulation of the relaxation properties of a muscle can be generally accepted. Regulation in the short term can be by changes in ionic concentrations, pH, and metabolites in the intracellular space of the muscle. This short-term regulation usually involves the active centers, substrate-binding

sites of the enzyme, in the slow/cardiac isoform – the phosphorylation of the regulatory protein phospholamban. The long-term regulation by neural impulses, muscle activity, and certain hormones can influence the expression of the different isoforms.

The application of highly sophisticated biochemical, molecular biological methods using isolated, reconstituted membrane and enzyme preparations usually eliminates the real, dynamically changing environment in which the SR performs its functions in living muscles. The assay media for ATPase activity and Ca^{2+} transport measurements are suitable for the determination of the maximal velocity and maximal transport speed of the system under such optimal conditions, which are not necessarily present in the muscle during the measurements of the relaxation properties with physiological methods.

Adaptation of biochemical ideas and modifications of the methods may bring us closer to the real complexity of the problem in the future. Promising new developments are the more sophisticated techniques for the study of SR functions, both ATP hydrolysis and calcium transport, in whole-muscle homogenates (Everts et al. 1989; Simonides and Hardeweld 1990; Matsushita and Pette 1992).

The application of highly specific inhibitors of SR Ca^{2+}-ATPase, such as cyclopiazonic acid and thapsigargin, may make these methods even more reliable (Seidler et al. 1989; Kijima et al. 1991; Lytton et al. 1991; Sagara and Inesi 1991; Sagara et al. 1992). According to some investigations, thapsigargin may be suitable even for distinguishing between the Ca^{2+}-ATPase isoforms (Kijima et al. 1991); however, other studies detected identical sensitivity for all SERCA isoforms when they were in the low-Ca-affinity E2 conformation (Lytton et al. 1991; Sagara and Inesi 1991; Sagara et al. 1992). The conformational specificity of thapsigargin inhibition may provide an additional tool for structural studies of the enzyme.

Despite the accepted key role of the Ca^{2+} uptake system in muscle relaxation, the complexity of other factors should be considered when one is interpreting physiological phenomena in molecular, biochemical terms. The same differences in the physiological contraction-relaxation parameters of different muscle types can arise in many different ways from the combination of several biochemical parameters. Sometimes even a set of biochemical changes that compensate for each other may remain undetected at the physiological level. The highly sensitive, sophisticated calcium-detected methods with double-fluorescent labeling seem to be promising for an understanding of the complexity of the calcium-handling systems in contraction and relaxation (Simon et al. 1991; Klein et al. 1991).

The differences in the relaxation properties of different muscle types can be explained in part by quantitative differences in identical molecu-

lar systems. Some differences are related to qualitative differences in the systems, i.e., different isoforms or unique regulatory mechanisms. The slow skeletal muscles (fibers) represent an exciting combination of these factors. Some systems such as Ca^{2+}-ATPase, troponin-C isoforms, or expression of phospholamban share the characteristics of the cardiac muscle. Others are identical with the ones observed in the fast skeletal muscles (DHP receptor, calsequestrin, perhaps the ryanodine-receptor isoforms) (Wilkinson 1980; Barndl et al. 1986; Damiani et al. 1986; Fliegel et al. 1987, 1989; Pan et al. 1990). A better understanding of the heterogeneous regulation of these parameters in slow muscles will help the emerging new muscle-replacement treatments of cardiac, skeletal, and smooth muscle diseases (Acker and Stephenson 1987; Chacheques et al. 1988; Pette 1990b; Partridge 1991).

The recent explosive increase in information on the molecular structure and function of the calcium-release and -uptake systems of the sarcoplasmic reticulum may bring us closer to an explanation of some phenomena in muscle physiology, such as low- and high-frequency fatigue, post-tetanic potentiation, the staircase phenomenon, load dependence of relaxation, and their differences in slow and fast skeletal and cardiac muscles, as well as in muscles of animals with different body size, body temperature, and living environment.

The success of the intensive research using analytical techniques during the past few years has provided more complex, synthetic approaches to the studies of relaxation at the borders between muscle biochemistry, molecular biology, and physiology. As Professor Ebashi formulated it in his recent article, "Perhaps we are at the beginning of a renewed and prosperous age in muscle science" (Ebashi 1991).

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Rev. Physiol. Biochem. Pharmacol., Vol. 122

Barnacle Muscle: Ca^{2+}, Activation and Mechanics

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1 Introduction

Large crustacean muscle fibres have been used extensively for physiological study of excitation contraction (E-C) coupling because of their large size and robust nature Fatt and Katz 1953; Caldwell and Walster 1963; Hoyle and Smyth 1963; Falk and Fatt 1964; Hagiwara and Naka 1964; Hagiwara and Nakajima 1966; Hagiwara et al. 1964a,b, 1968; Edwards et al. 1964; Ashley and Ridgway 1970). The large size of the fibres allows them to be used both for voltage clamp techniques using an axial electrode system, and for studies on ion and solute transport. Consequently, many aspects of their membrane physiology, ion and solute regulatory mechanisms together with ion diffusion are relatively well understood (Brinley 1968; Danielson et al. 1971; Nelson and Blaustein 1980; Russell and Blaustein 1974; Russell and Brodwick 1979; Ashley et al. 1972, 1974; Ashley and Lea 1976, 1978; Carruthers 1983; Baker and Carruthers 1983; Dipolo 1972; Ashley et al. 1978; Caputo 1973; Hinke and McLaughlin 1967; Hinke 1970; Lakshminarayanaiah and Rojas 1973; Keynes et al. 1973; Kushmerick and Podolsky 1969; Stephenson et al. 1981). However, their mechanical

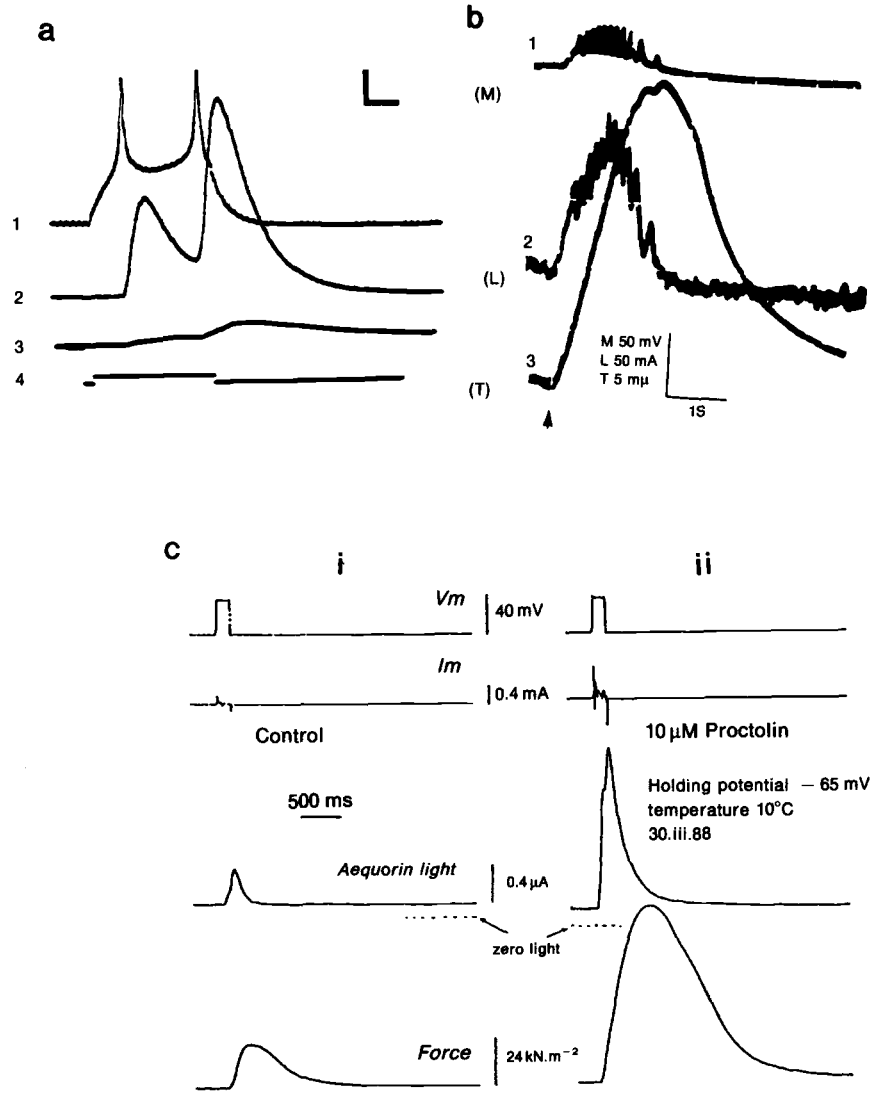


Fig. 1. **a** Effect of potassium citrate, injected internally, on the fibre responses. *Trace 1*, membrane response; *trace 2*, calcium-mediated (acquorin) light emission; *trace 3*, isometric tension; *trace 4*, calibration pulse and stimulus mark. Nominal intensity, 1.6; nominal duration, 300 ms. Calibrations: vertical – trace 1, 10 mV; trace 2, 3.8×10^{-9} l; trace 3, 0.35 g; trace 4, 1 V calibration pulse; horizontal – 100 ms. Citrate concentration estimated assuming uniform dilution inside the fibre as 5–6 mM, external saline contained 20 mM calcium and 100 mM magnesium. Resting light emission, 0.68×10^{-9} l; temp., 11°–12°C. (From Ashley and Ridgway 1970). **b** The effect of 1 mM L-glutamate in standard artificial sea water (ASW) applied at *arrow* upon the membrane potential (M), aequorin-light response (L), and isometric tension response (T) of a single barnacle muscle fibre. Temp. 10°–12°C. (From Ashley and Campbell 1978). **c** Proctolin (Revest 1987) potentiates both force and aequorin light signals in response to voltage clamp steps. (i) control and (ii) in presence of 10 μM proctolin. Temp. *ca.* 12°C. (From Griffiths 1990)

characteristics (Hoyle and Abbott 1967) and certain aspects of activation-contraction coupling have been less well studied. Clearly, force responses to various types of stimuli (Fig. 1) do not follow precisely the time course of free calcium concentration change recorded with either the photoprotein aequorin (Ridgway and Ashley 1967; Ashley and Ridgway 1968, 1970), the metallochromic indicator arsenazo III (Dybyak and Scarpa 1982; Ashley 1983) or with the fluorescent Ca^{2+} indicator, fura 2 (Timmerman and Ashley 1986). As relatively little is known about crustacean muscle mechanics, it was of value to compare the mechanical properties of barnacle muscle with those of other better studied preparations, particularly frog. In a later part of the review we describe some experiments which explore the mechanical properties of this crustacean preparation, and relate these to the likely characteristics of its cross-bridge cycle and with the kinetics of contraction and relaxation. This review does not deal in any detail with the numerous studies on Ca^{2+} ion movements (Caldwell 1964; Ashley et al. 1972; Ashley et al. 1974a; Ashley 1967; Lederer and Nelson 1983, 1984) on pH regulation (Boron 1977; Boron et al. 1978; Knakal et al. 1985) or on the ^{31}P NMR (nuclear magnetic resonance) studies (Dubyak and Scarpa 1983).

2 Evidence for a Transient Free $[Ca^{2+}]$ Change

Although the potentially useful indicator properties of the photoprotein aequorin were reported by Shimomura and coworkers (Shimomura et al. 1962, 1963) it was not until 1967 that this molecule was first used within a biological system, a slowly contracting, striated crustacean muscle fibre, to detect a transient free Ca^{2+} response as a result of electrical activation (Ridgway and Ashley 1967). The initial observations made with this method indicated that the free calcium and force did not rise and fall concomitantly; there was an appreciable delay between the maximum of the aequorin light emission, and hence free calcium in the cell and maximum force. This rather unexpected finding led to the immediate suggestion that the free calcium concentration change within the muscle cell may be controlling the rate of force development, so that the Ca^{2+}-dependent reactions which produced force were rate controlling steps and were out of equilibrium with the free calcium concentration, at least following a brief electrical stimulation (Ashley and Ridgway 1968, 1970).

The delay between the free calcium concentration change and force in a short duration transient is well demonstrated in the experimental record shown in Fig. 2, as the result of a single voltage-clamp pulse applied to an isolated barnacle muscle fibre.

Three main points arise from this record: (1) the comparison of the time course of the free calcium response with that of the force response which

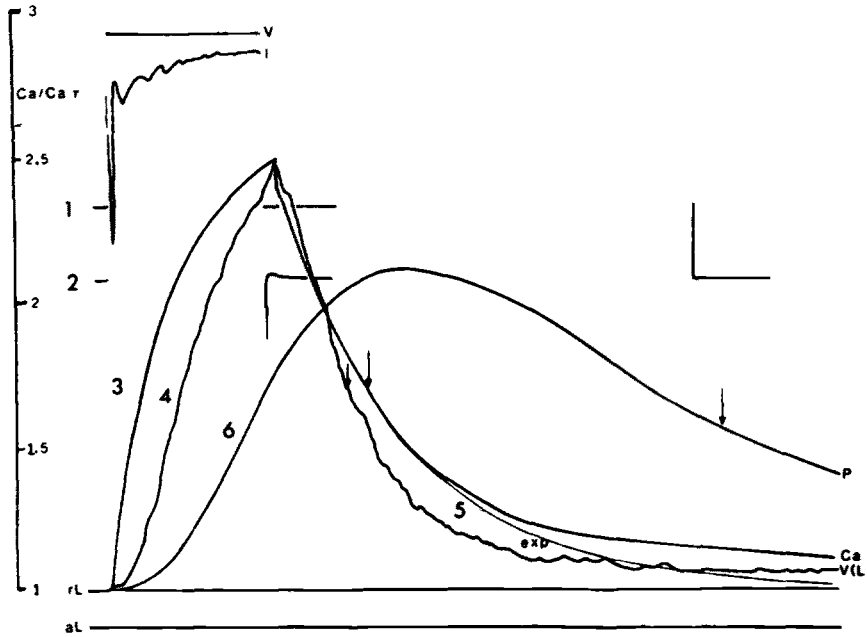


Fig. 2. Results of a single voltage clamp pulse applied to an aequorin-injected crustacean muscle fibre showing deconvolution of light record. (ΔV = +45 mV; holding potential = minus 47 mV; temp., 18°C. Gains: vertical (1) 20 mV; (2) 100 μA; (4) 50 nA; (6) 0.021 kg cm^{-2}; horizontal, 100 ms. *Trace 1*, membrane voltage (V); *trace 2*, membrane current; *trace 3*, calculated free Ca^{2+}, from a deconvolution and normalisation of the light response [V(L)] according to

$$Ca^n \rightarrow ACa \rightarrow L + \frac{dL}{dt} \cdot 1/R \rightarrow V + RC\left(\frac{dv}{dt}\right)\left[V + RC\left(\frac{dv}{dt}\right)\right]$$

where RC = 10 ms, A is a constant of the order of unity, 1/k = 11 ms, and n = 2.8; *trace 4*, aequorin-light response [V(L)]; *trace 5*, indicates decay of calcium, if exponential; *trace 6*, isometric tension, r(L) is resting light a(L) is zero light. Arrows indicate the half-time of the decay of light [V(L)] and calculated free Ca^{2+} and that on the force trace indicates the position of half-relaxation of force. Temp. 16°–18°C. (From Ashley 1983 and Ashley and Lignon, unpubl)

has already been mentioned, shows that the free [Ca^{2+}] rises faster than force; (2) during the relaxation phase of the force response, free [Ca^{2+}] falls more rapidly than force; and (3) the magnitude of the transient free [Ca^{2+}] change (from 0.07–0.1 μM to 2-10 μM) is far smaller than the total number of myofibrillar calcium-binding sites in the cell (140–200 μM barnacle troponin C (BTnC); two calcium-specific sites (Collins et al. 1991); relatively high affinity, rapid calcium buffering system). If all of these sites need to be occupied during maximum activation then the *total* amount of calcium released must be much greater than the magnitude of the simple *free* Ca^{2+} transient recorded with aequorin.

As the reactions which involve calcium and which result in force production appear to reach a steady state slowly, at least for the time course of the pulses used in Figs. 2 and 3I, it should be possible to prolong the free Ca^{2+} elevation in order to allow both the free calcium and force to come close to a steady state balance. This can be achieved experimentally by prolonging the duration of the voltage-clamp pulse, and the result is illustrated in Fig. 3II. In this case, force begins to reach a steady level in about 3–5 s at this free [Ca^{2+}] and temperature.

The second point concerns the falling phase of the calcium transient and, in particular, the relation between the free calcium concentration and force during the relaxation phase of the mechanical event. At first sight, it

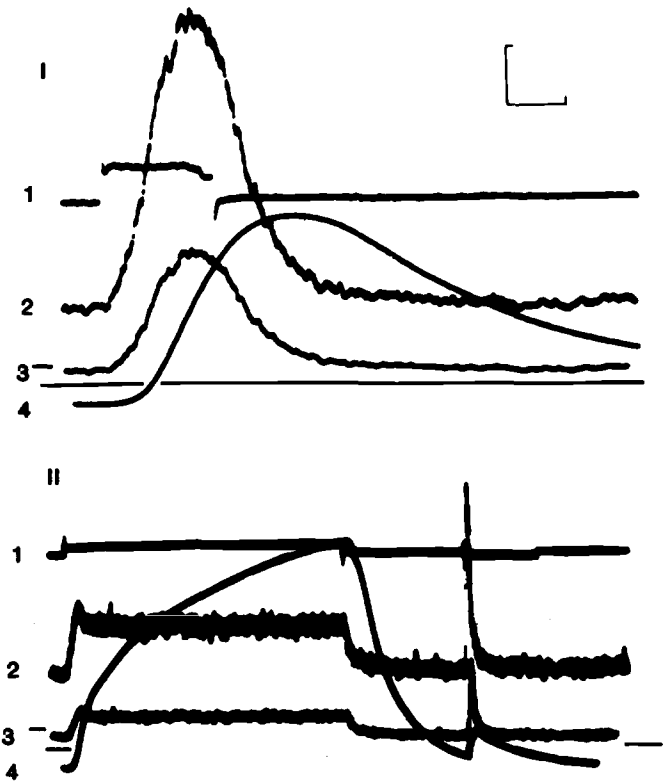


Fig. 3. Result of a short (I) and a long (II) voltage clamp pulse applied to an aequorin injected crustacean muscle fibre where light was recorded at high and low gain. In both: *trace 1*, membrane current; *trace 2*, aequorin-light at high gain; *trace 3*, aequorin light at low gain; *trace 4*, isometric force. *Solid horizontal lines* illustrate the respective baseline for the two light records. Gains: vertical, (1) 50 μA, (2) 20 nA, (3) 50 nA, (4) 0.08 kg cm^{-2}; horizontal, (I) 100 ms, (II) 1 s, (I): 200-ms pulse, ΔV = +20 mV, (II) 5-s pulse; ΔV = +12 mV; holding potential, −53 mV; time constant of light trace, 10 ms. The transient pulse toward the end of II is artifactual. Temp., 10°–12°C. (From Ashley 1983 and Ashley and Lignon, unpubl)

would appear that the free Ca^{2+}, as indexed by the aequorin light emission, has returned to its resting value at, or just before, the onset of relaxation. However, as the relation between aequorin light and free calcium concentration is nonlinear (Shimomura et al. 1962, 1963; Ashley 1970; Moisescu et al., 1975; Allen et al. 1977), small but highly important changes in free calcium concentration may be neglected; and this is particularly relevant at free Ca^{2+} values close to the resting free calcium concentration (Fig. 2). Therefore, in order to determine the free calcium concentration at any particular instant during the course of the transient response, it is important that the light signal be deconvoluted (Ashley and Moisescu 1972a,b; Figs. 2 and 13). The deconvolution protocol shows that there is an appreciable and elevated free calcium concentration during the whole phase of force relaxation (Ashley and Lignon 1981; Ashley 1983). Ashley and Ridgway

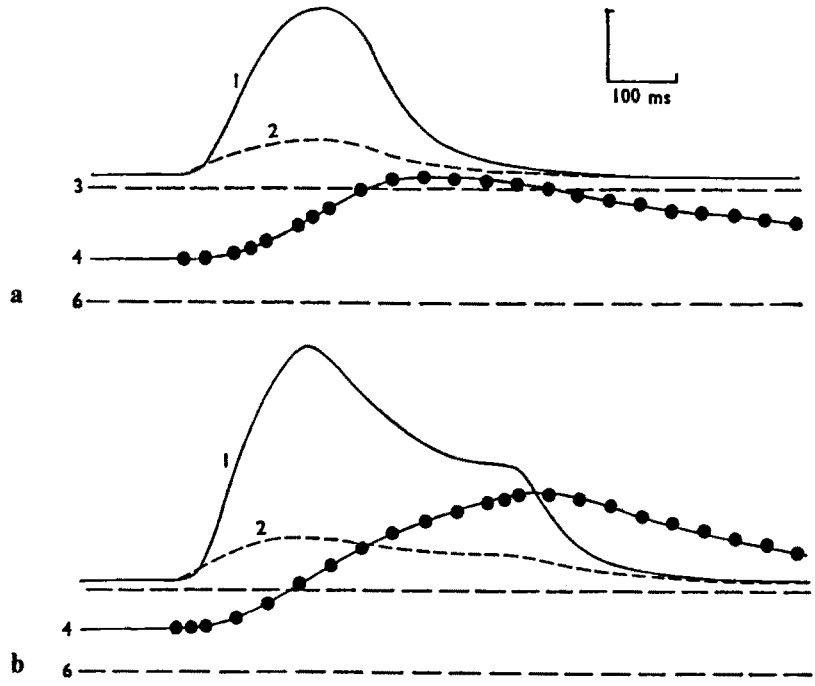


Fig. 4. Effect of short- and long-duration stimuli upon the time relations between aequorin light emission (*trace 1*), calculated free calcium from aequorin kinetics ($k_3 = 80\ s^{-1}$ at 10°C; Equ (1) *trace 2*); baseline for light and free calcium (*trace 3*), isometric tension (*trace 4*); •, tension calculated from Ashley and Moisescu (1972a); baseline for tension and calcium bound (*trace 5*). $P_{max'}$ maximum tension per response for (**a**) is 5.6%, and for (**b**) is 8.3% P_o (maximum obtainable tension). Temp., 10°–12°C. The same normalisation of ± 4% was used for (**a**) and (**b**). Tracings were from the same isolated *Balanus nubilus* muscle fibre. Calibration bar: vertical, *trace 1*, 3.8 nlm; *trace 2*, $Ca_r \times 6.67$; *trace 4*, 2.5 g (2.6% of P_o). (From Ashley and Moisescu 1972a)

(1970) proposed that calcium was still attached to the contractile system during the later phase of the relaxation process, and returned to the sarcoplasmic reticulum (SR) relatively rapidly at low calcium concentrations. This suggestion later formed the basis of models of Ca^{2+} fluxes (Figs. 4 & 7; Ashley and Moisescu 1972a,b; Ashley and Moisescu 1973b; Ashley et al. 1974c) and subsequently developed by others for frog (Robertson et al. 1981; Gillis et al. 1982; Baylor et al. 1983; Melzer et al. 1986; Stein et al. 1988). The fact that deconvolution of high gain aequorin light records, obtained during the later phases of relaxation, indicates an appreciable elevation of the free Ca^{2+} at this time, gives some support to this notion of prolonged Ca^{2+} buffering by the myofilament calcium sensor, TnC. It would, of course, be possible that this elevated calcium is due to loss from soluble calcium-binding proteins in the sarcoplasm, such as parvalbumin, which load with Ca^{2+} during activity and then return the Ca^{2+} to the SR during the later stages of relaxation. This is not likely in barnacle fibres, as the concentration of parvalbumin and related soluble calcium-binding molecules is very low (less than 6 μM; Griffiths et al. 1988, 1990; Fig. 5), compared, for example with frog where millimolar concentrations of parvalbumin exist in the sarcoplasm.

This calcium flux model formed the basis of the calculations in which the transient free calcium response observed during activation was used to

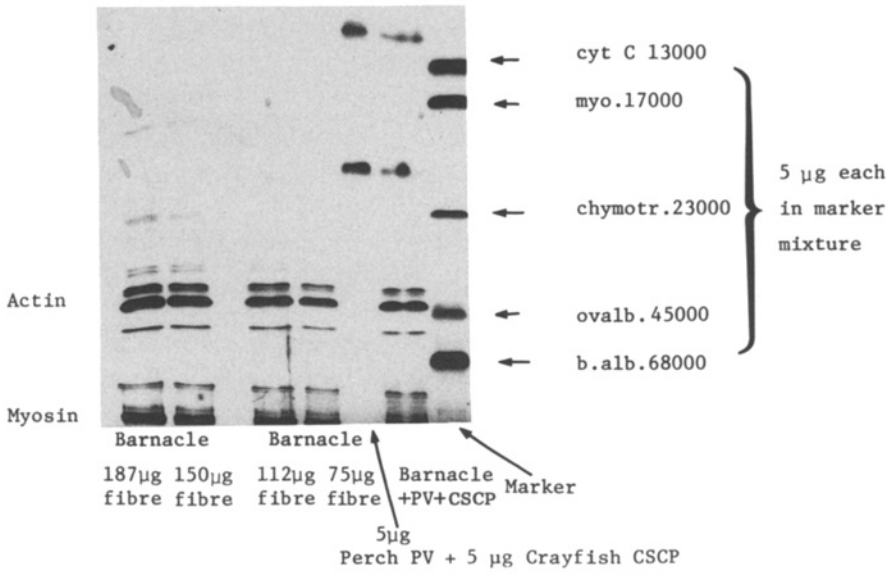


Fig. 5. Sodium dodecyl sulphate (13.5%) gel electrophoresis of barnacle muscle extract demonstrating absence of significant barnacle cytoplasmic calcium binding proteins, either of parvalbumin (*ca.* 12 kDa) or of sarcoplasmic calcium binding protein (*ca.* 22 kDa). (From Ashley and Griffiths, unpubl)

predict the time course of relative force recorded simultaneously, and also to reconstruct the free Ca^{2+} event (Ashley et al. 1974c) (see Fig. 7).

2.1 Derivation of the Free Ca^{2+} Response

To predict force quantitatively, a precise knowledge of both the time course and magnitude of the transient free calcium concentration change is required. The method for deconvolution of the light record, from which the time course of the free $[Ca^{2+}]$ change is derived, is based upon the kinetic scheme given below. This was determined from cuvette experiments in which the photoprotein was mixed rapidly (*ca.* 1 ms) with calcium and where the rise time of the resulting light emission response was observed. For simplicity, k_1 and k_{-1} are taken to be the same per site and the number of calcium ions taken nominally as 2, although the stoichiometry of the light emission: $[Ca^{2+}]$ curves suggests at least 3 (Ashley and Campbell 1979). The rise time of the light emission, which here was independent of both the calcium and magnesium concentration at a pCa < 7 ($pCa = -\log_{10}[Ca^{2+}]$), predicts an apparent rate constant of 100 s^{-1} at 20°C (80 s^{-1} at 10°C) and is virtually the value of k_3 in Eq. 1, since the first step (k_1) is considered to be rapid.

$$2Ca^{2+} + Aeq \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 2Ca - Aeq \xrightarrow{k_2} X \xrightarrow{k_3} Y^* \xrightarrow{fast} Y + h\nu \qquad (1)$$

The steady-state decay of light on the other hand is slow in comparison and is governed by the product of k_2 (the maximum rate of utilisation at low pCa; 1.3 s^{-1} at 20°C) together with the relative concentration of the component ($2Ca^{2+}$-aequorin). Based upon this relatively rapid rise time of the light emission (80 s^{-1}), the free calcium transient in vivo is translated in front of the aequorin light emission trace by some 12–13 ms at 10°–12°C, the temperature of the single muscle fibre experiments.

The exact time course of Ca^{2+} attachment to and loss from the contractile filaments when geometrically arrayed in the myofibril still remains to be determined, however, experiments with caged Ca^{2+} and caged Ca^{2+} chelators give insights into this process (Sect. 8.3), and particularly the use of danzylaziridine labelled TnC (TnC_{DANZ}) within intact single muscle fibres.

The third point from Fig. 2 concerns the calibration of the aequorin-light response (free calcium transient) in terms of the absolute $[Ca^{2+}]$. Estimates of the $[Ca^{2+}]_i$ in fibres at rest (Portzehl et al. 1964) based upon the measured value for the free ionised magnesium concentration in barnacle, (Ashley and Ellory 1972; Brinley et al. 1977; Van Wagoner et al. 1986; Ashley and Campbell 1979) place the resting calcium at close to 250 nM

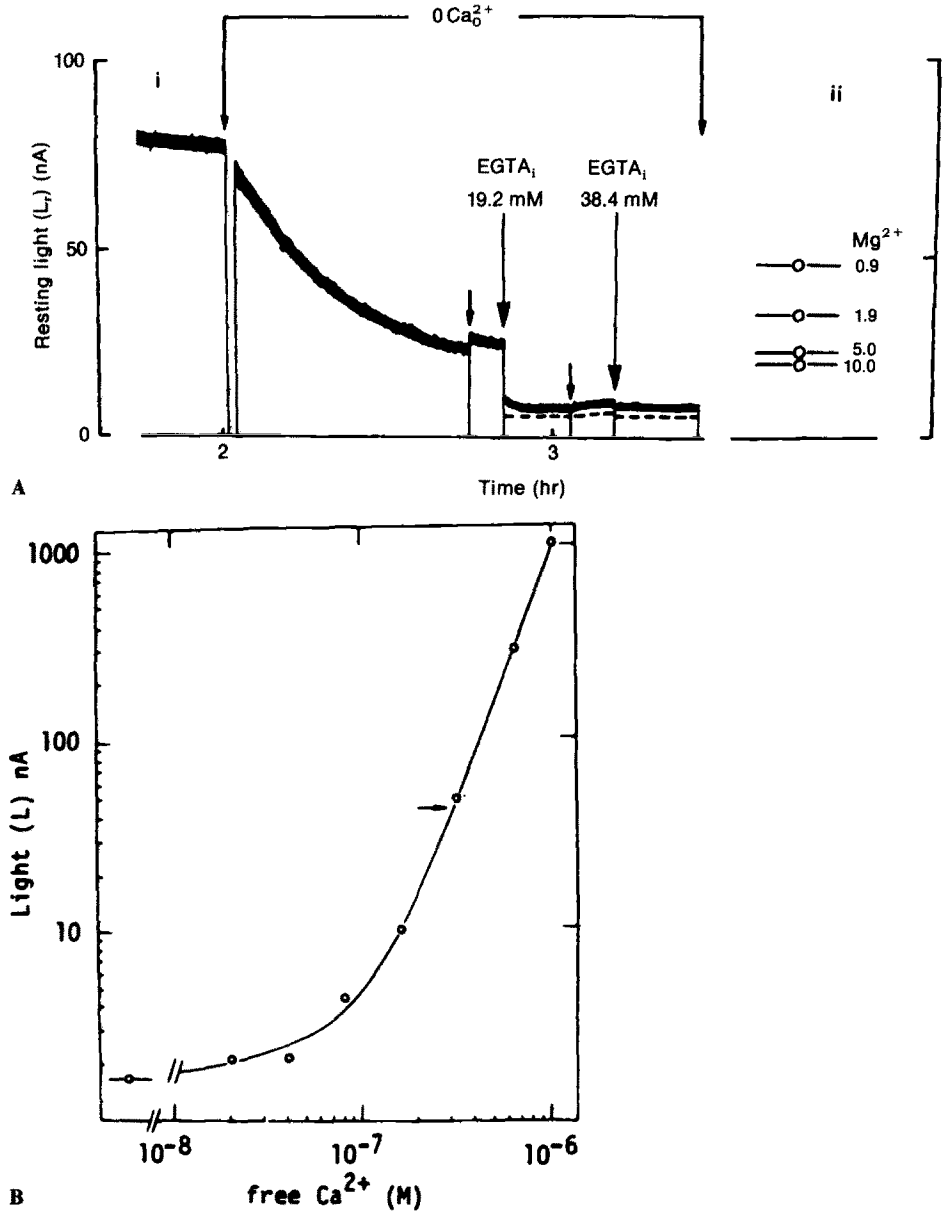


Fig. 6. A (i) The effects of Ca^{2+}-free saline and injecting a solution of dipotassium ethylene glycol bis (β-aminoethylether) N,N,N′,N′ tetraacetic acid (K_2EGTA; 1.0 M, 25 mM TES (N-tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid), pH 7.1) on the resting light emission (L_r) from a single barnacle muscle fibre. The intracellular EGTA concentrations after equilibration (19.2 and 38.4 mM kg^{-1} respectively) are estimated to have reduced the intracellular free $[Mg^{2+}]$ to 2.8 and 1.9 mM kg^{-1} respectively and increased $[K^+]_i$ to 188 and 226 mM kg^{-1} respectively. Control injections of 3 M KCl were ineffective on L_r. (ii) The values of the light emission in an assay solution containing: 100 mM total EGTA ($EGTA_{total}$), 260 mM $[K^+]$, 20 mM $[Na^+]$, 50 mM TES, pH 7.1 and estimated

(Fig. 6B). The observation that the relation between free calcium and aequorin light emission is closer to a 2.5 power function (Fig. 6B; Moisescu et al. 1975; Moisescu and Ashley 1977; Campbell et al. 1979; Allen et al. 1977) than the square law relation previously assumed (Ashley 1970) implies that the transient free $[Ca^{2+}]$ changes are somewhat smaller than those initially calculated (Ashley and Moisescu 1972a). In either case, the free $[Ca^{2+}]$ change during maximum activation is still considerably less than the likely change in total calcium bound to TnC on the myofibrils, assuming both Ca^{2+} specific (*ca.* 200 μM at calcium-specific sites) need to be occupied/TnC molecule and that all TnCs need to be fully occupied at maximum tension, P_o with a K_a *ca.* 10^6 M^{-1}. The discrepancy between the change in *free* calcium and the *total* calcium change, measured by metallochrome dye experiments in frog fibres (Miledi et al. 1982; Melzer et al. 1986), suggests that in a short duration electrically evoked response the interior of the muscle cell is strongly and rapidly calcium-buffered, mainly by the myofibrils. However, to date, no measure has been made of bound Ca^{2+} on the thin attached filament during a force response, but the $STnC_{DANZ}$ experiments suggests that Ca^{2+} attaches before, rather than at the same moment, as the cross-bridge attaches (Sections 3 & 4). A detailed analysis of these free Ca^{2+} transient and force records has also suggested mechanisms by which Ca^{2+} is released from and is taken up by the SR (Ashley et al. 1974c; Ashley and Caldwell 1974; Ashley 1978, 1983; Fig. 7). In all these calculations, the fact that the aequorin light emission exhibits a non-linear relation (Shimomura et al. 1963; Ashley 1970; Baker 1972; Allen et al. 1977) with free $[Ca^{2+}]$ implies that within cells if spatial inhomogeneities in the $[Ca^{2+}]$ exist, these could impair the accuracy of the Ca^{2+} measurements made with aequorin. However, simulations based on the free Ca^{2+} and force records determined experimentally suggest that, for slowly contracting muscle fibres, these effects are small (Fig. 8; Ashley et al. 1974c), but this may not be so for faster contracting fibres (Allen et al. 1977;

Fig. 6 (*cont.*). free $[Ca^{2+}] < 1$ nM, for free $[Mg^{2+}]$ of 0.9, 1.9, 5.0 and 10.0 mM. Light emission was measured 2 h after injection into the assay solution of the same amount of aequorin as used in the muscle fibre. *Small arrows* in (i) indicate time of insertion of injector capillary into the fibre. Fibre diameter, 1080 μm. Mean membrane potential, −47 mV. Ambient (7 nA) has been subtracted from the record. N.B. For the first 2 h after aequorin injection, fibre was in normal Ca^{2+} saline (11.8 mM); temp ca. 20°C. *Dashed line* represents L_r corrected to a free $[Mg^{2+}]$ 4.2 mM. **B** Relation between aequorin light emission and free Ca^{2+} in an assay solution of composition (Volume, 50 μl) similar to that of a muscle fibre: 180 mM $[K^+]$, 20 mM $[Na^+]$, 4.2 mM free $[Mg^{2+}]$, 50 mM $EGTA_{total}$; each (•) represents the mean of two determinations. The *arrow* points to the mean value (n = 11) of resting light from a muscle fibre that was fibre injected with the same concentration of aequorin (temp., 20°C). (From Ashley and Campbell 1979; Campbell et al. 1979)

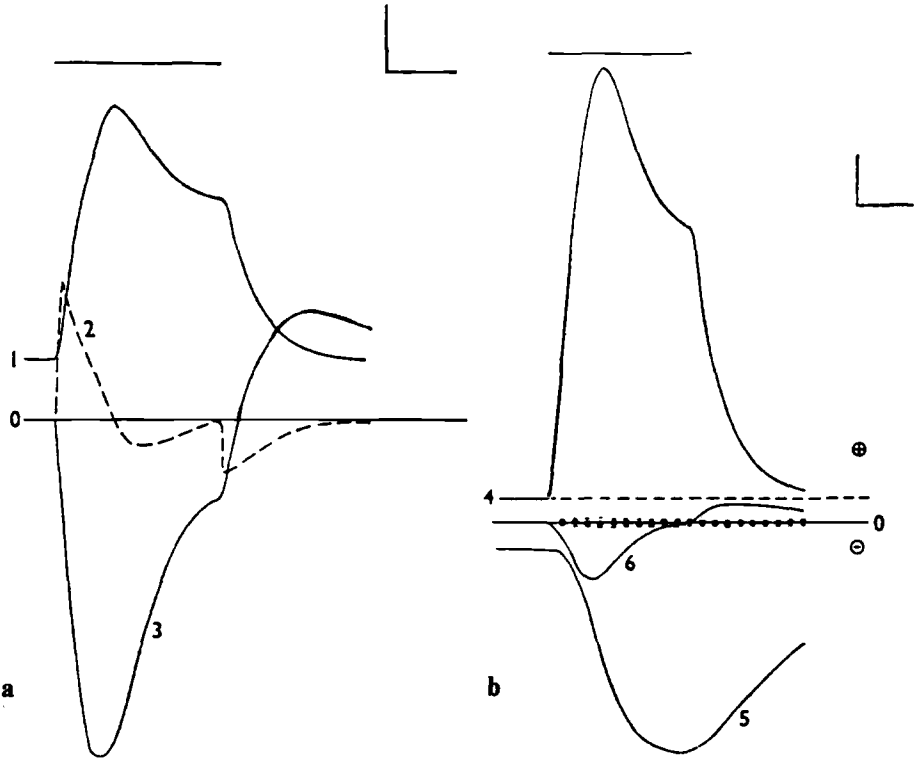


Fig. 7a,b. Time-relations during a single long-duration response of an isolated *Balanus nubilus* muscle fibre of: *trace 1*, the free calcium derived from the aequorin light emission (Fig. 4b); *trace 2*, (1+A) $(dCa/dt)_{free}$ where A is a constant of the order of unity and accommodates the rapid low-affinity calcium-binding sites ($1 \times 10^3 - 1 \times 10^4$ M^{-1}); *trace 3*, $(dCa/dt)_{myofibrils\ (M)}$; *trace 4*, $(dCa/dt)_{sarcoplasmic\ reticulum\ release\ (SRr)} + (dCa/dt)_{leakage\ (L)}$; *trace 5*, $(dCa/dt)_{sarcoplasmic\ reticulum\ uptake\ (SRu)}$; *trace 6*, $(dCa/dt)_{SR\ sites\ (SRS)}$. •, values of the expression F, where $F = -(1 + A)(dCa/dt)_{free} + (dCa/dt)_M + (dCa/dt)_{SRr} + (dCa/dt)_{SRu} + (dCa/dt)_{SRS} + (dCa/dt)_L$. The *error bars* represent 1% of the expressions calculated by numerical integration (*traces 3,4,5* and *6*). +, release of calcium into the sarcoplasm; minus, uptake. Parameters used: (1+A) $Ca_r = 10^{-7}$ M; $2c_{1,2} = 10^{-4}$ M; $aCa_r = 2.0368 \times 10^{-5}$ M s^{-1}; $b = -1.874 \times 10^{-5}$ M. s^{-1}; $D = 8.544 s^{-1}$ since $(dCa/dt)_{SRr} = B\exp(-Dt)$ when stimulation ceases, and B is the value of the regenerative release at this point in time; $(Ca_r)^2\alpha/\beta = 0.03$; $\beta = 2.15$ s^{-1}; $E = 1.68 \times 10^{-4}$ M s^{-1}, $C_s = 5 \times 10^{-6}$ M. Calibration Ca_r, vertical: (*trace 1*, Ca_r; *trace 2*, 2×10^{-6} M s^{-1}; *trace 3-trace 6* and for points (•) 1×10^{-5} M s^{-1}; horizontal; 200 ms. Temp., 10°–12°C. *Solid horizontal lines* represent the duration of the stimulus. (From Ashley and Moisescu 1973a). **a** and **b** depend upon the intensity of the depolarising pulse; E is the maximum rate of absorption by the sarcoplasmic reticulum and is achieved when (2CaS) reaches its maximum value, c_s; D is a constant; α and β are the forward and reverse rate constants for the formation of (2CaS) and $2c_{1,2}$ is total concentration of the myofibrillar calcium-binding sites. (From Ashley et al. 1974c; Ashley and Caldwell 1974)

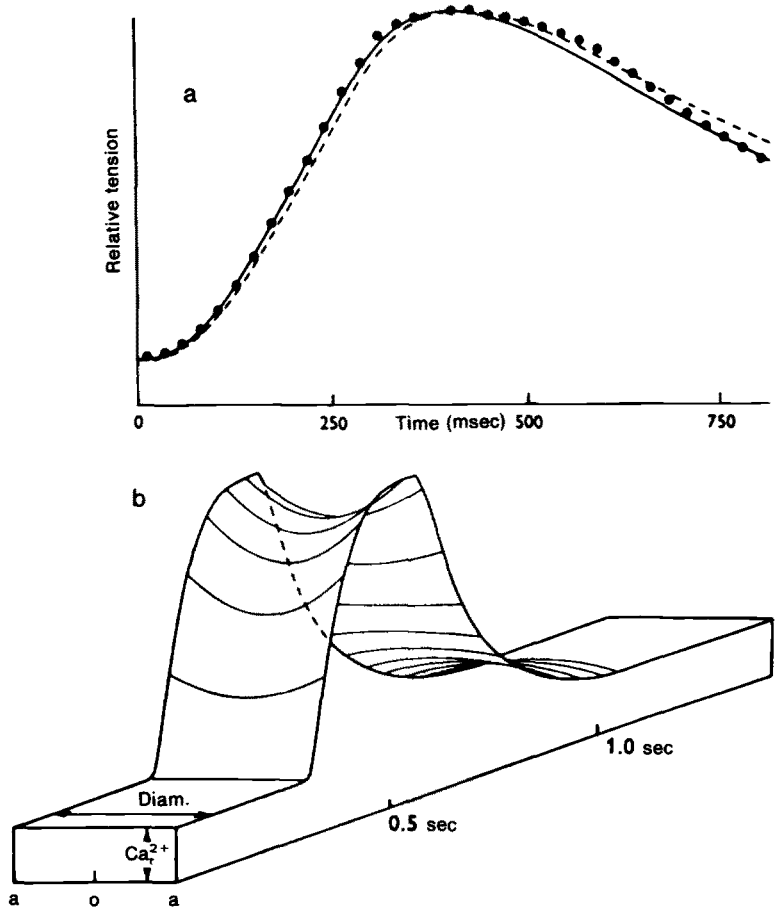


Fig. 8a,b. **a** Time course of experimentally recorded force (•). *Solid line*, the time course of relative isometric force calculated from the 'independent scheme' incorporating the process of calcium diffusion for aequorin *uniformly* distributed in the myofibril; *dashed line*, calculated relative isometric force, aequorin considered only at the surface of the myofibril. The parameters for the force calculation are $D[\partial^2 Ca(r,t)/\partial r^2) + 1/r(\partial Ca(r,t)/\partial r)] = (\partial Ca(r,t)/\partial t) + kCa(r,t)M(r,t) - kCaM(r,t)$ and where force P(t) is given by $P(t) \sim \int_0^a CaM^2(r,t)rdr$ where r is the distance from the axis of the myofibril, M is the concentration of free calcium binding sites and CaM the concentration of occupied calcium binding sites. The boundary condition is the following for Ca(r,t): *case (1)* aequorin uniformly distributed within the cell, light intensity $I(t) = \int_0^a Ca^2(a,t)rdr$; *case (2)* aequorin located at surface of myofibril (only), light intensity $I(t) \sim Ca^2(a,t)$, where a, the radius of the myofibril, is 2 μm and D, the apparent diffusion coefficient for calcium, is 6×10^{-6} cm^2 s^{-1}. **b** Three-dimensional reconstruction of the free calcium transient across the diameter of the myofibril calculated for the condition of aequorin uniformly distributed (see **a**). The complete transient is shown only at the surface of the myofibril. The *lines* joining the transients are at points of equal time and illustrate the effect of diffusion upon the time course and magnitude of the free calcium: o, axis of myofibril; a, surface of myofibril; temp., 10°–12°C. Single barnacle muscle fibre in **a** and **b**. For convenience Ca^{2+} expressed as Ca throughout legend. (From Ashley et al. 1974c)

Cannell and Allen 1984; Cannell 1986), or for the rapid (1–2 ms) changes in free Ca^{2+} observed in large nerve fibres (Baker 1972) associated with an electrical impulse.

Other groups have also suggested resting Ca^{2+} levels of 0.1–0.3 μM in relaxed muscle and 3.0–10 μM in contracting fibres from both vertebrate skeletal and cardiac muscle (Blinks et al. 1978; Ashley and Campbell 1979; Ashley et al. 1978; Allen and Blinks 1978; Tsien and Rink 1980; Marban et al. 1980; Tsien et al. 1985; Blinks et al. 1982) and in general agreement with the initial crustacean fibre experiments.

3 Ca^{2+}-Binding Studies in Isolated Fibres Using Fluorescently Labelled Skeletal Troponin C

The control and exact timing of the transient events which stem from the free calcium concentration change discussed above and which lead to the force cycle are still a matter of conjecture. It is not known in detail for example whether the free calcium change is followed by the rapid binding of calcium to the calcium sensor molecules located on the thin filament, or whether an appreciable delay in this process occurs (Ashley and Moisescu 1972a): although experiments now suggest that binding of Ca^{2+} to TnC is fast (Johnson et al. 1979) at least in the cuvette. Similarly, little is known of the timing of the events that are associated with reactions occurring after Ca^{2+} binding, such as a conformational change on the thin filament, involving tropomyosin movement, or for example, general thin filament cooperativity (Ashley et al. 1991a). Additionally, little is known as to the time relations between the free [Ca^{2+}] change and changes associated with cross-bridge movement and fibre stiffness (strongly attached, but low-force bridges) prior to the force-generating step. It is also not clear as to which product release step (ADP/Pi) from the actomyosin adenosine triphosphatase (ATPase) cycle limits the cycling of cross-bridges in intact fibres.

Some of the changes associated with activation have now been measured by using fluorescently labelled probes injected into intact barnacle muscle fibres, so that the problems associated with calcium control of the force cycle could be examined in more detail.

3.1 Danzylazaradine-Labelled Skeletal Troponin C

The danzylazaridine-labelled subunit of whole troponin from rabbit muscle, TnC (TnC_{DANZ}; Johnson et al. 1979) which shows a 2.1-fold increase in fluorescence at 510 nm when Ca^{2+} occupies the calcium specific sites (T sites)

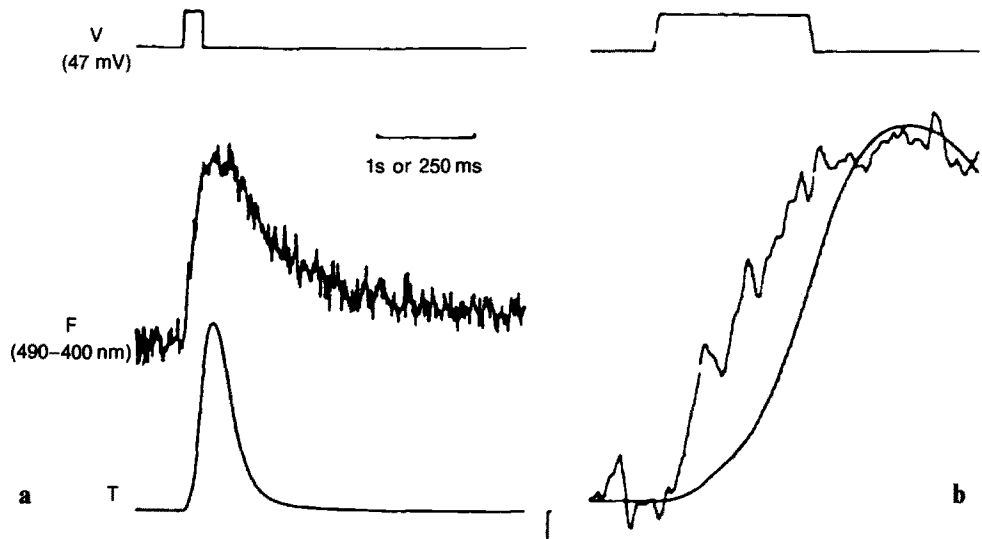
has been used. The affinity of this rabbit skeletal TnC_{DANZ} ($STnC_{DANZ}$; K_{ca} 3.3×10^5 M^{-1}) increases at the calcium specific sites by more than 10 times when incorporated into intact troponin, but decreases by a factor of more than 10 times when whole troponin is incorporated onto the actin filament (Zot et al. 1983). Hence the affinity for calcium and the time course of the consequent fluorescence change of non-filament bound $STnC_{DANZ}$ should be similar to that of whole troponin attached to the contractile filament, but yet would be unaffected by any affinity changes brought about in the native filament by cross-bridge (S1) attachment.

In barnacle, both $BTnC_1$ and $BTnC_2$ (barnacle TnC isoforms) substituted and restored force, while STnC would not substitute for force production when present in either a relaxing or activating solution at physiological ionic strength in TnC depleted myofibrils (I = 0.15 M) (Ashley et al. 1991b). Thus it is unlikely that there is appreciable exchange of $STnC_{DANZ}$ with barnacle TnC in intact fibres and hence the fluorescence signal in intact injected fibres comes solely from $STnC_{DANZ}$ *free* in the sarcoplasm of the cell. Fluorescence-polarisation experiments also suggest that little $STnC_{DANZ}$ or $BTnC_{2DANZ}$ binding occurs to the thin filament of intact barnacle fibres (Ashley et al. 1988b; Fig. 26D) at least over the time course of 24 h.

The time course of the $STnC_{DANZ}$ fluorescence signal compared to that of force development is clearly illustrated in Fig. 9a,b. The fluorescence record rises faster than force and the $t_{1/2}$ of the fluorescence rise precedes that of force by 55 ms (n = 15) at this temperature. The initial fluorescence increase is rapid and is virtually complete when force is still only at 50% of its final value, while the peak of force and the slower phase of fluorescence increase reach a maximum value at similar times.

The fact that the muscle fibre signal (490–410 nm; see legend to Fig. 9) differs in a $STnC_{DANZ}$-injected fibre from that in an uninjected fibre suggests that the signal from the injected cell arises from a change in $STnC_{DANZ}$ fluorescence as a result of Ca^{2+} being bound at the 'calcium-specific' sites of STnC.

In an attempt to explain the likely kinetics of the $STnC_{DANZ}$ signal in response to the free [Ca^{2+}] change, the occupancy of the low affinity calcium binding sites following a step change in free calcium concentration was computed, on the assumption of simple competition between Ca^{2+} and Mg^{2+} at these binding sites and the observed cuvette kinetics. Taking the free Mg^{2+} to be more than 5 mM (Ashley and Ellory 1972), the forward rate constant for magnesium binding to be (k_{on}^{Mg}) 10^4 M^{-1} s^{-1}, and the off rate (k_{off}^{Mg}) to be 50 s^{-1} (Gillis et al. 1982; Ashley et al. 1991a), the calcium specific sites were, surprisingly, 50% occupied with magnesium (Fig. 10). Although the magnesium displacement reaction is likely to be rapid, it may



$[STnC_{DANZ}]_i$ = 50 μM; Holding potential − 50 mV;
Temp. 22°C; Fibre 3 20.3.84

5% or 2.5% resting fluorescence at 490 μm
50 or 25 kN. m^{-2}
275 mV membrane potential

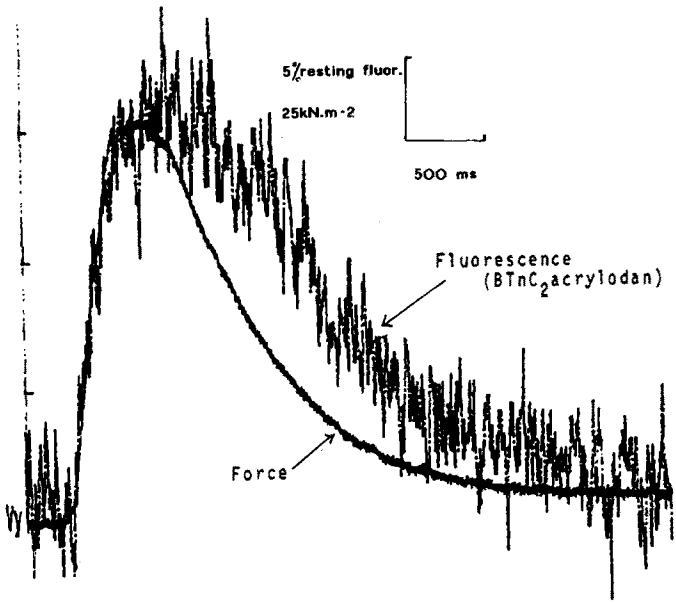


Fig. 9a-c

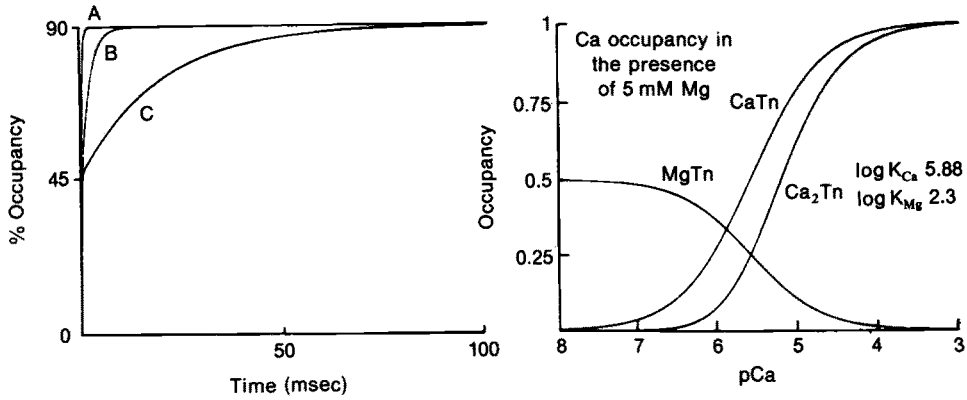


Fig. 10. Computer predictions of site occupancy as a function of $[Ca^{2+}]$ and $[Mg^{2+}]$. *Left*, time course of Ca^{2+} binding to STnC in the presence and absence of 5 mM Mg^{2+}. Free $[Ca^{2+}]$ change as step function of suitable amplitude giving 90% of maximal activation in the presence (*curves B and C*) or absence (*curve A*) of Mg^{2+}. $k_{on}^{Ca} = 10^8\ M^{-1}\ s^{-1}$, $k_{off}^{Ca} 1.3 \times 10^2\ s^{-1}$, $k_{on}^{Mg} = 10^5\ M^{-1}.\ s^{-1}$ (*curve B*) or $10^4\ M^{-1}\ s^{-1}$ (*curve C*), $k_{off}^{Mg} = 500\ s^{-1}$ (*curve B*), or $50\ s^{-1}$ (*curve C*). *Right*, equilibrium occupancy as a function of free calcium concentration $[Mg^{2+}]$ 5 mM, pK_{Ca} 5.88, pK_{Mg} 2.3. Gear method used for numerical integration; temp., 20°C. (From Griffiths et al. 1984)

be slow enough to produce some reduction in the apparent Ca^{2+} 'on'-rate to the magnesium-occupied fraction of the sites compared with the 'on'-rate to the metal-free fraction. These simulations suggest that the Ca^{2+} 'on'-rates may be reduced to the same range as the rate constant values predicted from the rise time of the $STnC_{DANZ}$ fluorescence in intact muscle fibres (Fig. 9). However, there is some additional evidence to suggest that the interaction of calcium and magnesium at the low affinity sites is more complex than a simple competition (Potter et al. 1981), in which case the value of the 'off'-rate constant for Ca^{2+} would be increased.

Although the overall time course of the fluorescence change from free $STnC_{DANZ}$ upon stimulation is not radically different from that of the time course of force development in these slow striated arthropod muscle fibres as mentioned earlier, it is appreciably slower than the kinetics derived from cuvette experiments with the native protein. This is most clearly illustrated

Fig. 9a-c. Time course of tension and TnC fluorescence responses to a voltage clamp pulse applied to a single barnacle muscle fibre. **a** Membrane potential (V), $STnC_{DANZ}$ fluorescence signal after scaled subtraction (F) and tension (T). **b** Same response, but displayed on a four times faster time trace. $[STnC_{DANZ}]_i = 50\ \mu M$. Holding potential, −50 mV; temp., 22°C. (From Griffiths et al. 1984, 1988). **c** Time course of tension and $BTnC_2$ Acryolodan fluorescence to a single voltage clamp pulse applied to a single barnacle muscle fibre. $[BTnC_2$ Acryolodan$]_i$ *ca.* 40 μM, temp., 12°C. (Griffiths, Potter and Ashley, unpublished observations)

in Fig. 9b, where the peak of the fluorescence signal and that of isometrically recorded force occur at about the same moment. There are, however, important differences in the time course of the two parameters. The initial rapid rising phase of the fluorescence difference signal usually precedes force development and is complete at a time when force is only 50% developed. This would suggest that the calcium bound to isolated $STnC_{DANZ}$ and putatively to the troponin on the intact thin filament, occurs before the onset of force, although this interpretation depends somewhat upon the precise shape of the Ca^{2+} binding/fluorescence – pCa curve. However by using double label experiments, with $STnC_{DANZ}$ as well as the photoprotein aequorin in the same cell, it is possible to show that the $STnC_{DANZ}$ fluorescence response in the cell is appreciably slower than that of the free Ca^{2+} signal aequorin (Fig. 11). This finding is *not* expected from the simple cuvette kinetics of these two Ca^{2+} indicators, where the $STnC_{DANZ}$ signal rises faster (> 2 ms) than the aequorin response (7–10 ms) following a step change in free $[Ca^{2+}]$ at 10°C. However a fast free Ca^{2+} (aequorin) and a

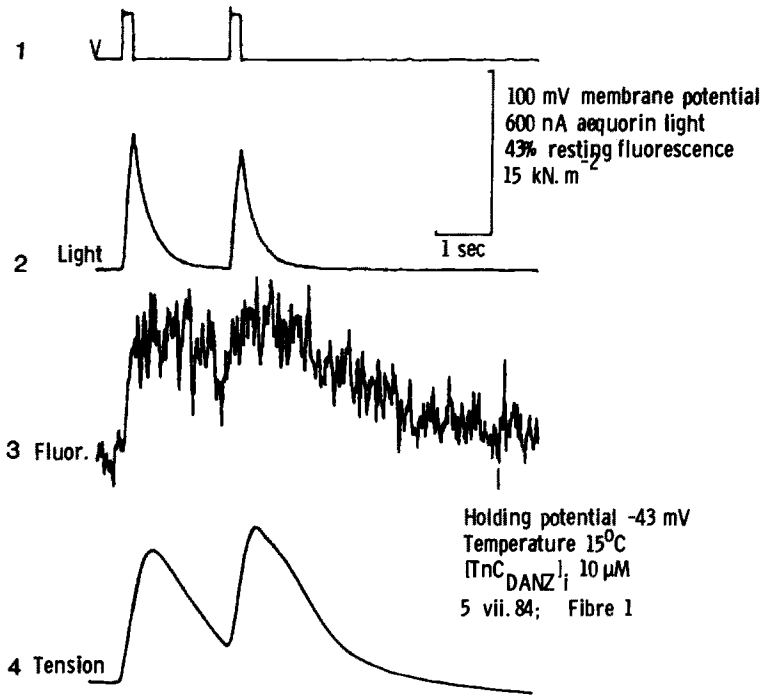


Fig. 11. Tension record from two identical voltage clamp (*trace 1*) pulses applied to single barnacle muscle fibre; aequorin light (*trace 2*) and $STnC_{DANZ}$ fluorescence (*trace 3*). Deconvoluted fluorescence and aequorin luminescence records were consecutive. Holding potential, −43 mV, Δ V + 34 mV; 200 ms pulses; temp. 15°C. $[STnC_{DANZ}]_i$ ca. 10 μM, aequorin *ca.* 1 μM. Resting luminescence, 130 nA. (From Ashley et al. 1984)

slower $STnC_{DANZ}$ response in the cell is not in discord with the observation that the free Ca^{2+} (aequorin) response precedes activation events on the myofilaments, such as the first actin layer (A2) line X-ray intensity change, a measure of attached cross-bridges (Fig. 14B) (Griffiths et al. 1988). Thus the $STnC_{DANZ}$ fluorescence and the A2 layer-line signal (strongly attached, low-force state) are both appreciably slower in time course than the time course of the free Ca^{2+} signal, while both events appreciably precede force development: more experiments are needed to clarify the nature of the slow $STnC_{DANZ}$ signal in the cell by using $BTnC_{2\ Acryoldan}$ (Fig. 9c) whose fluorescence response is very similar to the time course of force.

Perhaps one of the most interesting features of these experiments is the observation that the net change in $STnC_{DANZ}$ fluorescence decays much more slowly than force. This is seen in Fig. 9a, where the half time for the decay of fluorescence is on average twice that of force at 22°C. This result may imply that the Ca^{2+} attached to the Ca^{2+}-specific sites of free $STnC_{DANZ}$ dissociates more slowly than the decline of force. This idea is not in discord with mechanical observations where it is recognised that the exponential fall of force occurring after the isometric phase of force relaxation, at least in single frog fibres, is a phase of inhomogenous sarcomeric lengthening (Edman and Flitney 1978; Huxley and Simmons 1973; Cecchi et al. 1991) and it does not necessarily represent the true decline of the extent of activation for the 'average' sarcomere. In experiments using the sarcomere clamp, the *isometric* fall of force was considerably extended by prolonging the phase of sarcomeric homogeneity (Huxley and Simmons 1973); that is a slow decay of the high-force state. This slow force decay may well involve a component or reaction which is dependent upon the relatively slow release of calcium from the contractile system as suggested by the $STnC_{DANZ}$ decay. Whether this is ultimately the cause, or whether it is the result of the transfer of cross-bridges simply into a strongly attached, low-force state (see Cecchi et al. 1991) remains to be elucidated.

Nevertheless the potential importance of free Ca^{2+} in these events is emphasised by the finding that there is an elevated free $[Ca^{2+}]$ during the exponential phase of relaxation in frog (Cannell 1986) and also in barnacle muscle cells (see Ashley and Lignon 1981; Ashley 1983) which suggests that at this free Ca^{2+}, the activity of the sarcoplasmic reticulum is low at this stage of relaxation, since $k_{Ca_{off}}$ STnC is fast ($> 100\ s^{-1}$).

3.1.1 Double Pulse Experiments

In double pulse experiments, the free Ca^{2+} signal in response to the second pulse is smaller, yet the force signal is potentiated. Under these conditions the $STnC_{DANZ}$ signal summates (Fig. 11). The subsequent $STnC_{DANZ}$ fluorescence decay cannot, in barnacle, be attributed to a slow release of

Ca^{2+} from sarcoplasmic binding proteins (such as parvalbumins) as these are present at only very low concentrations in these muscle cells (Fig. 5) (Griffiths et al. 1990). This result implies that calcium is still attached to the free calcium sensor molecule ($STnC_{DANZ}$) at a time when externally recorded force is close to zero and is supported by the finding of a slow decay of the free Ca^{2+} (see above section). As the binding of cross-bridges to the thin (actin) filament may enhance Ca^{2+} binding to the calcium sensor TnC in barnacle (Ridgway and Gordon 1984), calcium should therefore be released more quickly from this non-filament bound protein, $STnC_{DANZ}$, than in fibres where the TnC is bound to the thin filament and cross-bridge binding enhances the TnC affinity for Ca^{2+}. Thus the apparent Ca^{2+} off-rate for the injected $STnC_{DANZ}$ (Fig. 9) should be even faster than that of the endogenous TnC, bound to the thin filaments (assuming the Ca^{2+} on-rates

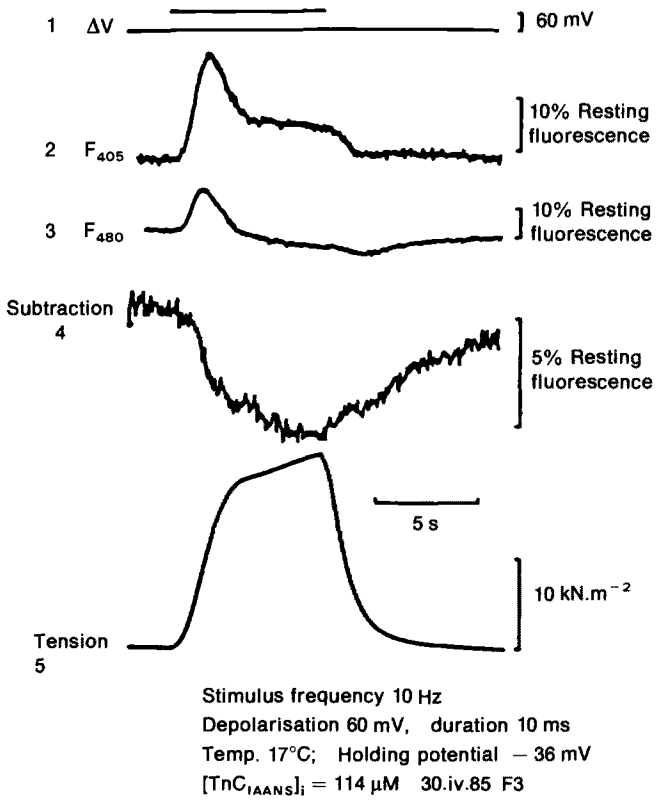


Fig. 12. $STnC_{IAANS}$ fluorescence responses from an injected single barnacle muscle fibre as a result of a long duration voltage clamp pulse. *Trace (1)* voltage; *trace (2)* 405 nm Ca^{2+}-independent and *trace (3)* 480 nm Ca^{2+}-dependent fluorescence signal; *trace (4)* subtraction of *trace 3* from *trace 2*; *trace (5)* isometric force record. Temp., 15°C. (From Griffiths et al. 1988)

to TnC are unaffected). These experiments again suggest a low SR Ca^{2+} pump activity as the cause of the slow Ca^{2+} decay during relaxation.

3.2 (4′-iodoacetamidoanilino) naphthalene–sulphonic acid (IAANS)-labelled Skeletal Troponin C

The fluorescent probe (IAANS), $STnC_{IAANS}$, which differs from $STnC_{DANZ}$ in that it is labelled at the Ca^{2+}-Mg^{2+}, site III (Zot et al. 1983) rather than the Ca^{2+} -specific sites, was axially injected into single barnacle muscle fibres and the transient fluorescence change observed at 480 nm. In short pulse duration (< 250 ms) experiments, little change in the $STnC_{IAANS}$ fluorescence difference signal was observed, despite an appreciable force transient, and under conditions where a positive transient $STnC_{DANZ}$ signal was always seen (Ashley et al. 1986b; Griffiths et al. 1988). At longer stimulus durations (> 5 s), a marked decrease in the $STnC_{IAANS}$ fluorescence was observed and this was followed, upon cessation of stimulation, by a return of the fluorescence to its initial resting value (Fig. 12). These results are consistent with a slow Ca^{2+} loading and subsequent slow unloading of the Ca^{2+}-Mg^{2+} sites of TnC. Since the Ca^{2+}-Mg^{2+} sites of STnC are virtually equivalent in affinities to those of the parvalbumins, the time course of loading and unloading of free $STnC_{IAANS}$ in these experiments should give an indication of the likely time course of the in vivo loading and unloading by Ca^{2+} of soluble Ca^{2+} binding proteins in vertebrate skeletal muscle during a force transient.

4 Time-Resolved X-ray Diffraction Studies in Intact Single Isolated Muscle Fibres

Isolated, shell-attached single crustacean muscle fibres provide a valuable preparation for time resolved X-ray diffraction studies (Griffiths et al. 1988). In these experiments it is important to produce maximum isometric force so as to maximise the X-ray signal. Single muscle fibres from *B. nubilus* were prepared attached to their shell and were pressure injected transsarcolemmally with the calcium-sensitive photoprotein aequorin (Ashley et al. 1974e).

Typical aequorin light and force records from *intact* fibres are illustrated in Fig. 13. It is clear from these records that even at high forces and high free [Ca^{2+}] (600 kN/m^2) there is still a delay of some 150 msec between deconvoluted light (free Ca^{2+}; Griffiths et al. 1990) and force. Additionally, there is still a distinct 'tail' to the aequorin light trace, when externally recorded force is low, suggesting a finite elevation of the free Ca^{2+} at this stage of the relaxation process, as seen with cannulated fibres.

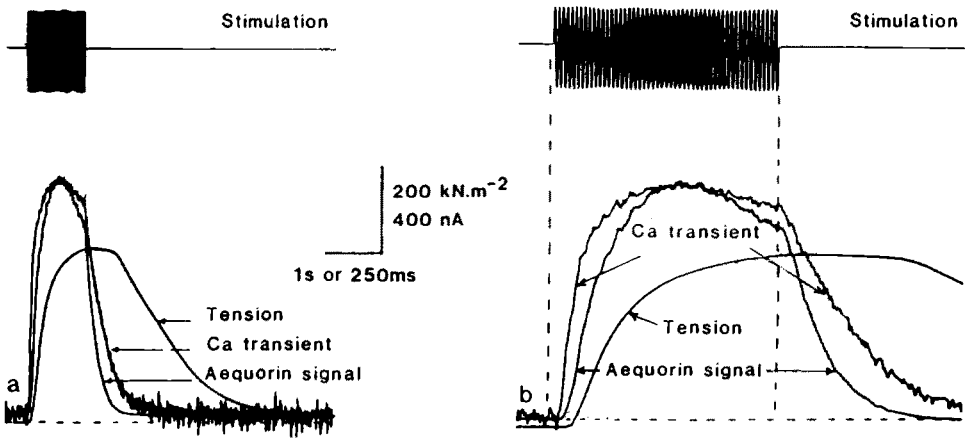


Fig. 13a,b. Aequorin light responses, free Ca^{2+} and tension during electrical stimulation of a shell-attached single barnacle muscle fibre. **a** The calcium transient may be distinguished from the aequorin light response by noting that it leads the aequorin signal during the rising phase of tension and lags during relaxation, as clearly demonstrated in the *right panel*, which shows the same record on a four times faster time base. **b** Free calcium transient was obtained from the aequorin light signal by addition of the signal itself and its first derivative (see Fig. 2 legend for details and Ashley and Moisescu 1972a). The 2.5th root of the waveform thus obtained was then calculated to yield the calcium transient. Temp., 12°C; sarcomere length 9.5 μm; stimulation 50 Hz sinusoidal. (From Griffiths et al. 1988)

Since these fibres lack significant concentrations of soluble Ca^{2+} binding proteins, such as parvalbumins, it is likely that this phenomenon represents Ca^{2+} returning to the SR from the myofilaments (TnC), as suggested by the $STnC_{DANZ}$ experiments discussed earlier. Although the extent of elevation of the free Ca^{2+} is small, it is highly significant in these intact fibres, where the resting light emission is extremely low compared to cannulated fibres, so that any elevation above resting values during the period of relaxation is readily detected.

The relation between mean sarcomere length determined by laser diffraction, aequorin light emission (free Ca^{2+}) and force development indicates that there is a distinct maximum for force development in these crustacean fibres between 8 and 10 μm [see Zachar and Zacharova (1966) concerning crayfish fibres] despite myofilament length variability in this preparation (Griffiths et al. 1988). The aequorin light emission, on the other hand, was much less dependent on sarcomere length (Fig. 14A). At 8–10 μm, isometric tensions of up to 600 kN m^{-2} can be developed and a marked change in intensity of the A2 line is used as an index of cross-bridge attachment (Fig. 14B). The half-time of this intensity change precedes that of force development by 80 ms, similar to the $t_{1/2}$ for the

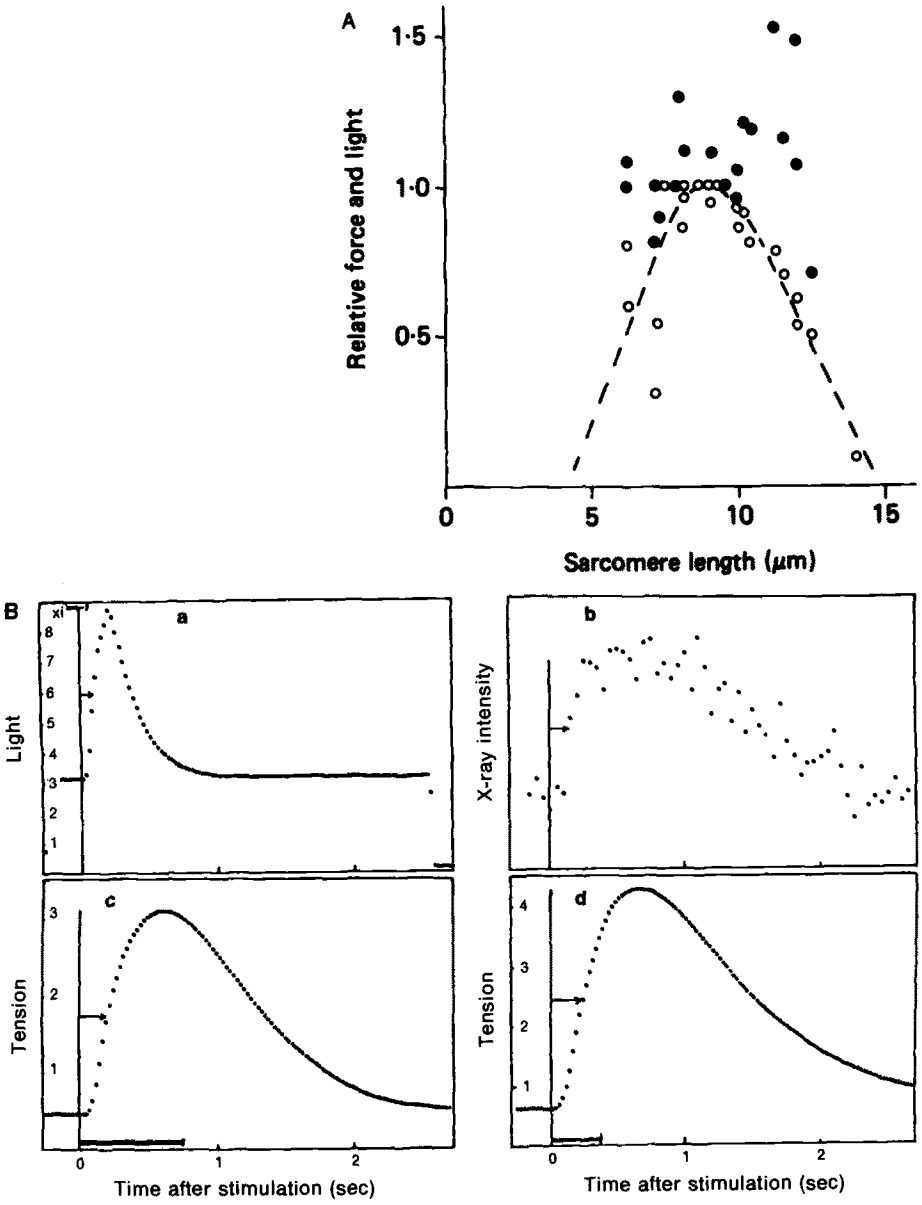


Fig. 14. **A** The relation between isometric force (○), peak aequorin light response (●) and mean sarcomere length from shell-attached single *Balanus nubilus* muscle fibres. Sinusoidal field stimulation (50 Hz) of peak-to-peak amplitude of 4–6 V, spacing 5 mm, full-length fibre; temp., 12°C. (From Griffiths et al. 1988). **B** Time course of X-ray intensity, aequorin light emission and force. *Trace* (**a**) aequorin light; A2 (first actin) layer line (axial spacing 38 nm) indicating likely cross-bridge attachment, *trace* (**b**) and isometric force (*traces* **c** *and* **d**) from a shell-attached (intact) single barnacle muscle fibre. Time resolution for force and aequorin light 25 ms and X-ray data 50 ms. 10 μm sarcomere length; temp., 12°–14°C; sinusoidal 50 Hz field-electrode stimulation. (From Griffiths et al. 1988)

$STnC_{DANZ}$ fluorescence lead over force, while that of the deconvoluted aequorin (free Ca^{2+} transient) leads force by 150–170 ms at 11°–13°C.

5 Excitation-Contraction Coupling in Barnacle Muscle Fibres

5.1 Dependence on External Ca^{2+}

E-C coupling in barnacle muscle fibres and other crustacean muscle fibres differs from that of vertebrate skeletal muscle fibres in being strongly dependent on the extracellular Ca^{2+} concentration ($[Ca^{2+}]_0$) and more closely resembles events in cardiac muscle. Both the rate and the amplitude of the electrically stimulated force response increase over the $[Ca^{2+}]_0$ range 0–100 mM (Hagiwara et al. 1968) using Mg^{2+} to replace the Ca^{2+}. Replacement of a standard saline, containing 10 mM Ca^{2+}, with Ca^{2+}-free saline causes a complete loss of inward Ca^{2+} current and force transients within 3 min at 16°C, while recovery of the contraction can be obtained within 3 min upon replacement of the Ca^{2+} saline (Hidalgo et al. 1979) or by the presence of Sr^{2+} (Franciolini 1983). The intracellular Ca^{2+} transient, recorded with aequorin, is abolished by Ca^{2+}-free saline within a few minutes (Franciolini 1982), as well as by hypertonic salines (Ashley and Ridgway 1970). Likewise, the development of tension generated by salines containing raised $[K^+]$ also requires the presence of extracellular Ca^{2+} (Edwards and Lorkovic 1967), while the injected caffeine response is initially independent of the presence of external Ca^{2+}, but requires the presence of external Ca^{2+} for its repeatability (Ashley et al. 1977; see also Bittar et al. 1974) and is suppressed by a 1–2 mM intracellular concentrations of calcium chelating agents (Ashley et al. 1965; Ashley 1967).

5.2 Relation Between Membrane Potential and Contraction

The rapidity with which E-C coupling in barnacle muscle is sensitive to the removal of extracellular Ca^{2+} suggested that either the inward Ca^{2+} current (I_{Ca}), which results from depolarisation of the sarcolemma or perhaps a superficial Ca^{2+} store, was important for the generation of the force response. The major part of the inward current in muscle fibres of barnacles and many other arthropods, is carried by Ca^{2+}, since the overshoot of the action potential shows a Nernstian relation with $[Ca^{2+}]_0$ (Fatt and Katz 1953; Fatt and Ginsburg 1958; Hagiwara and Naka 1964; Hagiwara et al. 1974) and the inward current itself can be abolished by removal of extracellular Ca^{2+} (Keynes et al. 1973). The extra influx of $^{45}Ca^{2+}$ which occurs during a depolarising pulse agrees closely with the flux calculated from the integrated inward current (Atwater et al. 1974).

Rojas and co-workers have attempted to show a direct causal relationship between I_{Ca} and the development of tension in voltage clamped, perfused barnacle fibres (Atwater et al. 1974; Hidalgo et al. 1979). At least two mechanisms could be operating here, either a simple, direct activation of the myofibrils by Ca^{2+} which enters across the sarcolemma, or an indirect activation, by the Ca^{2+} which is released from the SR, as a result of the Ca^{2+} entry (Ca^{2+}-induced Ca^{2+} release, CICR).

Over a range of depolarising steps of +20 to +120 mV, an approximately linear relation was observed between the integral of the inward current and the initial rising phase of force development in internally perfused single fibres (Fig. 15A). However, this relationship does not hold if the depolarising steps are increased further, and now the peak tension-membrane depolarisation relation reaches a plateau level which is maintained up to +230 mV (Caputo and Dipolo 1975, 1978; Fig. 15B). The current-voltage curve, on the other hand, has a bell shape (Fig. 15C), with the current amplitude decreasing at membrane potentials (E_m) above +60 mV ($[Ca^{2+}]_0$ 60 mM) to zero at an extrapolated reversal potential of +166 mV ($[Ca^{2+}]_0$ 60 mM; $[Ca^{2+}]_i$ 10^{-7} M; Keynes et al. 1973; Hidalgo et al. 1979). Thus, the tension-voltage curve has a classic sigmoidal appearance (Edwards et al. 1964) found in vertebrate skeletal muscle (e.g. Brum et al. 1986). The accompanying intracellular Ca^{2+} transient, measured with aequorin also increases sigmoidally up to E_m + 150 mV for contractions using 50 ms stimulating pulses (Ashley 1983): it does not show the bell-shaped relation observed with the current-voltage relation.

Under certain conditions, however, the tension-voltage relation of barnacle muscle ceases to be sigmoidal. For example, when the outward current is abolished either by replacement of internal K^+ by perfusion with tetraethylammonium ions (TEA^+) and caesium, or by addition of the K^+ channel blocker, 4-aminopyridine ($4\text{-}AP^+$). Under these conditions, the tension-voltage relation becomes bell-shaped, resembling the relation between the integrated inward current and E_m (Fig. 15C; Hidalgo et al. 1979). It was concluded that activation of both the inward Ca^{2+} and outward K^+ current systems is required for the tension-voltage relation

Fig. 15 (*see p. 174, 175*). **A** Relations between tension and the integral of the inward Ca^{2+} current in single perfused barnacle muscle fibre. For each set of records, *upper trace* represents the tension and the *lower traces* represent the membrane currents. Current records in Ca^{2+}-free saline above the base line in a Na^+- and Mg^{2+}-free, Ca^{2+}-containing saline below are clearly inward (*downwards*) during the pulses. The area between these two currents was measured with a planimeter. The *points drawn on top* of the tension records are proportional to these areas. Fibre internally perfused with potassium acetate and externally bathed in 60 mM $CaCl_2$ sea water. Fibre was perfused during 5 min only at a rate of 10.3 μl min; temp., 19°C. (From Atwater et al. 1974).

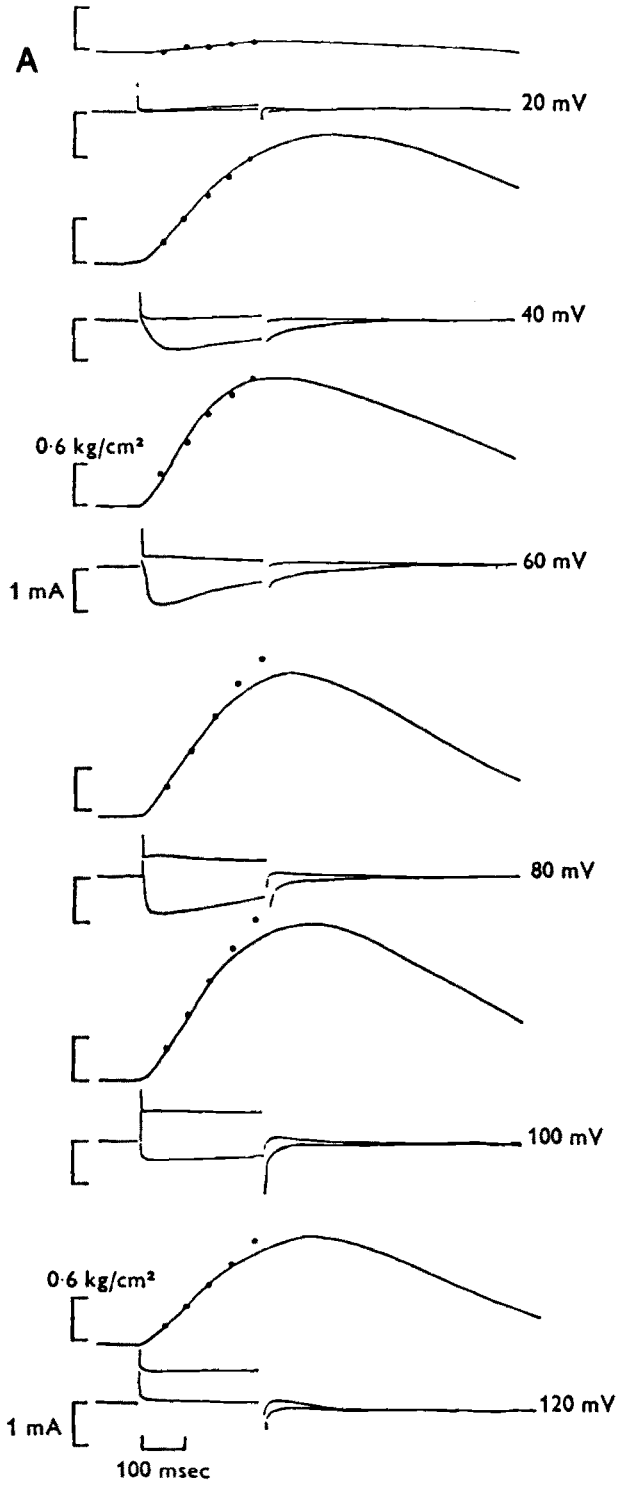

A
20 mV
40 mV
0·6 kg/cm²
1 mA
60 mV
80 mV
100 mV
0·6 kg/cm²
1 mA
120 mV
100 msec

Fig. 15A

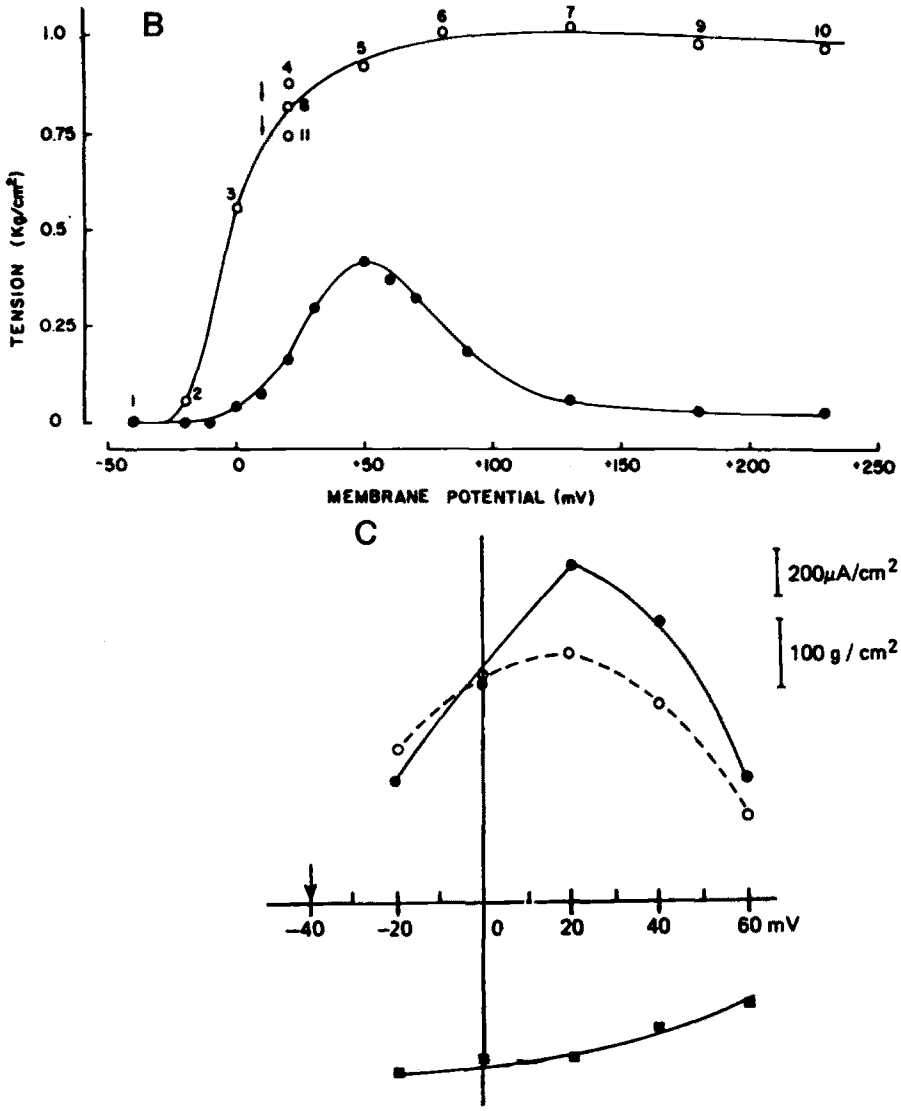


Fig. 15 (*cont.*). **B** Relations between tension and the change in membrane potential from the holding potential during a depolarising pulse. The upper curve (*white circles*) was obtained with the fibre bathed in 1 mM Ca^{2+} standard artificial sea water (ASW). The numbers indicate the order with which the pulses were applied. The lower curve (*black circles*) was obtained with the fibre bathed in 11 mM Ca^{2+} ASW plus 5 mM procaine. The pulse duration was of 40 ms; holding potential −70mV. (From Caputo and Dipolo 1975). **C** Tension, membrane current and integral of the current as a function of membrane voltage (V_m). *Ordinate*: ■–■, peak inward current; o–o, peak tension; •–•, negative value of the integral of the current, in arbitrary units. For the current calibration shown on the *top right side*, the area represents the external membrane. Internal solution: Cs^+, tetraethylammonium (TEA) and sucrose. External solution: ASW containing 10 mM Ca^{2+}; Holding potential minus 40 mV; Duration of the pulses 40 ms; temp., 16°C. (From Hidalgo et al. 1979)

to be sigmoidal and to produce a plateau level of tension, and Hidalgo and co-workers suggested a possible explanation in terms of the complex morphology of the barnacle fibre. Previously Keynes and colleagues (1973) observed that voltage clamp pulses caused oscillations in the current records. These were attributed to the Ca^{2+} and K^+ current systems being located at different sites on the sarcolemma and being separated by a series resistance, comprising the resistances of the cleft and the tubular lumen. In effect this means that the axial electrode fails to clamp all parts of the cleft and tubule membranes at the same potential as the sarcolemma. Thus it is possible that, during a voltage clamp pulse, local current loops arise within the cleft system which are not under voltage clamp control. Consistent with this idea is the loss of oscillations which accompanies removal of either the K^+ or Ca^{2+} current or by inflation of the fibre (Keynes et al. 1973). It can be envisaged that local Ca^{2+} currents may occur during a voltage clamp pulse which are capable of generating tension, even though the voltage pulse is at, e.g. +230 mV, a value at which the I_{Ca} would be zero if all the invaginations of the sarcolemma were under perfect radial clamp control. In other words, the absence of a bell-shaped tension curve does not necessarily imply the lack of participation of a trans-sarcolemmal I_{Ca}, but solely a poor radial clamp control of the voltage across the cell membrane.

A bell-shaped tension-voltage curve has been found, however, in barnacle muscle fibres exposed to 5 mM procaine (Caputo and Dipolo 1978), with the tension peaking at +50 mV and declining to zero at +150 mV (Fig. 15B) similar to the behaviour of I_{Ca}. In this case, the effect of procaine was attributed to an inhibition of the Ca^{2+} release from the SR, since in skinned fibres, procaine inhibits the SR Ca^{2+} release mechanism which is induced by either Ca^{2+} itself (CICR) or by caffeine. The remaining tension was thus attributed to direct activation of the myofibrils by the depolarisation-induced entry of Ca^{2+} across the sarcolemma. At +50 mV this tension was about 40 kNm^{-2}, compared with peak tension in non-perfused cannulated fibres of 200–250 kNm^{-2} and in intact fibres of 600 kNm^{-2}. This picture is complicated unfortunately by the fact that procaine also is able to block the outward K^+ current; thus procaine may be having the same effect as TEA^+ and $4AP^+$ as described above. A third example of a bell-shaped tension-voltage relation from crustacean muscle comes from work with crab muscle fibres by Mounier and Goblet (1987). Here in standard Ca^{2+} saline externally, there are two components of force produced in response to a voltage-clamp depolarisation: a phasic one which is dependent on I_{Ca} and a tonic one which is dependent on external Ca^{2+} and Na^+. In 50 mM TEA^+, Na^+-free saline only the phasic one was seen and this had a bell-shaped tension-voltage relation. The dependence of the tonic component on external Na^+ suggested that it arose from the I_{Ca} of a Na^+-Ca^{2+} exchange

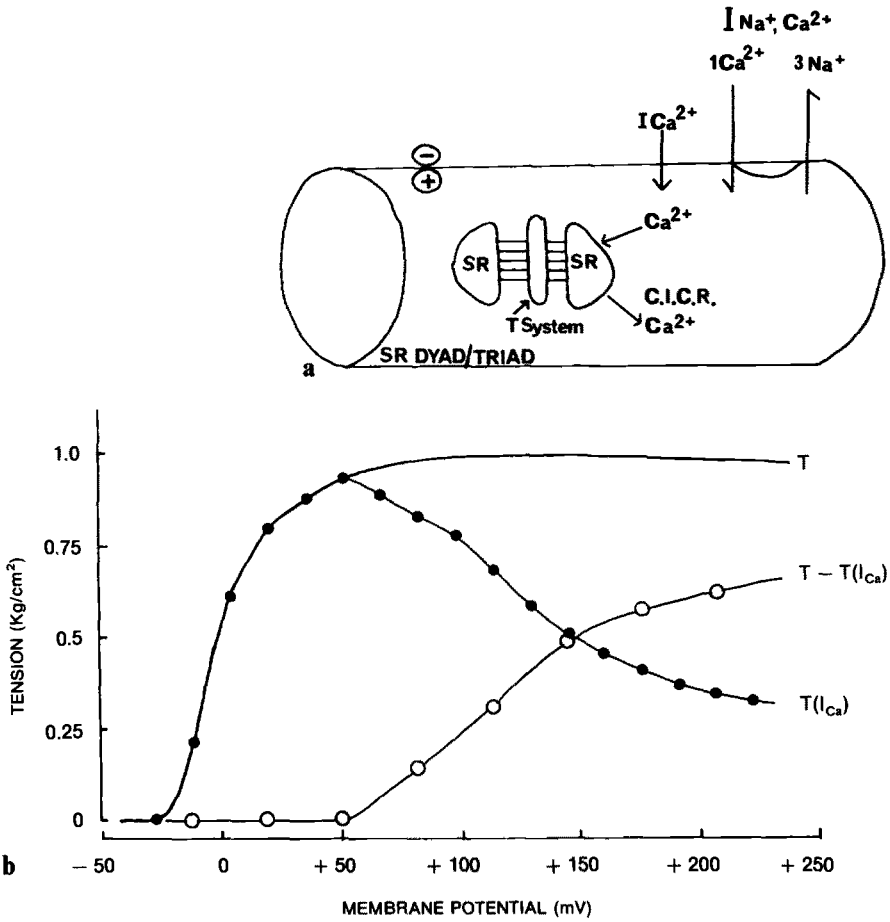


Fig. 16. **a** Schematic diagram of mechanism of action of voltage-gated calcium current I_{Ca} and inward calcium current on the Na^+-Ca^{2+} exchange system $I_{Ca(Na-Ca)}$ and influence on calcium-induced calcium release (CICR) from the sarcoplasmic reticulum (SR) of barnacle muscle. **b** The estimated contribution of inward Ca^{2+} current (I_{Ca}) (voltage-gated I_{Ca} + $I_{Ca(Na-Ca)}$) to tension responses in the experiments of Caputo and Dipolo (1975), shown in Fig. 15B, on the assumption that for E_m values more negative than +50 mV, the tension is totally dependent on $I_{Ca(Na-Ca)}$ and CICR. The tension-voltage relation in the presence of procaine to inhibit CICR (Fig. 15**b**(•)) was taken as a measure of the tension produced by direct Ca^{2+} activation of myofibrils by voltage-gated I_{Ca}. $T(I_{Ca})$, the tension component due to this and CICR, has been estimated (-•-), using the same empirical relation which was assumed for E_m values more negative than +50 mV. The difference between this curve and the original tension curve in the absence of procaine is shown also (-o-)

mechanism. Although they did not measure the voltage dependency of the tonic component, a recent study of the Na^+-Ca^{2+} exchange in cardiac cells has shown that over the E_m range of +40 to +160 mV, the exchanger inward Ca^{2+} component ($I_{Ca(CaNa)}$) increases exponentially with E_m as does

the accompanying increase in $[Ca^{2+}]_i$ (Beuckelmann and Wier 1989). Consequently, any tension resulting directly from $I_{Ca(CaNa)}$ or indirectly through CICR from the SR would be expected to increase in amplitude as E_m was made more positive. Thus, if barnacle muscle has two components of tension, one dependent on a voltage-gated I_{Ca} and the other on an exchanger $I_{Ca(CaNa)}$ (Fig. 16a) and these act independently of each other and can sum at any potential, the resulting tension-voltage relation for the summed components may appear as a single sigmoidal curve (Fig. 16b). Although there are no clear indications of two components or phases of force development in barnacle muscle, there is plenty of evidence from tracer ion studies and $[Ca^{2+}]_i$ measurements that suggest that Na^+-Ca^{2+} exchange operates across the sarcolemma of this muscle fibre (see Sect. 6.2).

The idea of an exchanger $I_{Ca(CaNa)}$-dependent component is not inconsistent with the results of Hidalgo et al. (1979) since their fibres were internally perfused with a Na^+ -free solution, presumably abolishing any inward Ca^{2+} movement via sodium-calcium exchange. A similar explanation may account for the report that in perfused fibres, the intracellular Ca^{2+} transients, recorded with the fluorescent Ca^{2+} indicator AZO-1, are abolished at large depolarisations close to the Ca^{2+} reversal potential (Vergara and Verdugo 1988). In order to reconcile the above effects of K^+ current inhibitors and procaine on the tension-voltage curve with the $I_{Ca(CaNa)}$ concept, it would be necessary to propose a pharmacological action of these agents on $I_{Ca(CaNa)}$. There is some evidence from other cells that local anaesthetics like procaine do inhibit Na^+-Ca^{2+} exchange at relatively high concentrations (Allen and Baker, quoted in Kaczorowski et al. 1989).

Thus, it is clear that the part played by Na^+-Ca^{2+} exchange in the contraction of barnacle muscle, still remains to be determined. This can be done by either the use of a specific inhibitor of either the I_{Ca}-dependent and $I_{Ca(CaNa)}$ components of force, or by examining the properties of force at very positive membrane potentials (Fig. 16b).

5.3 Is Ca^{2+} Release from the SR Involved in Excitation-Contraction (E-C) Coupling?

Several studies of barnacle striated muscle ultrastructure have been published (Hoyle et al. 1965, 1973; Selverston 1967; Gayton and Elliott 1980;

Fig. 17. A Ca^{2+} dependence of the ATPase activity in barnacle sarcoplasmic reticulum (SR). ATPase activities were measured with variable $[Ca^{2+}]$ and ethylene glycol bis (β-amino ethylether) N,N,N′,N′ tetra-acetic acid (EGTA) concentration. Free $[Ca^{2+}]$ was calculated with the use of a computer programme, taking into account the endogenous $[Ca^{2+}]$ of the SR and of the chemicals used and was measured by atomic absorption

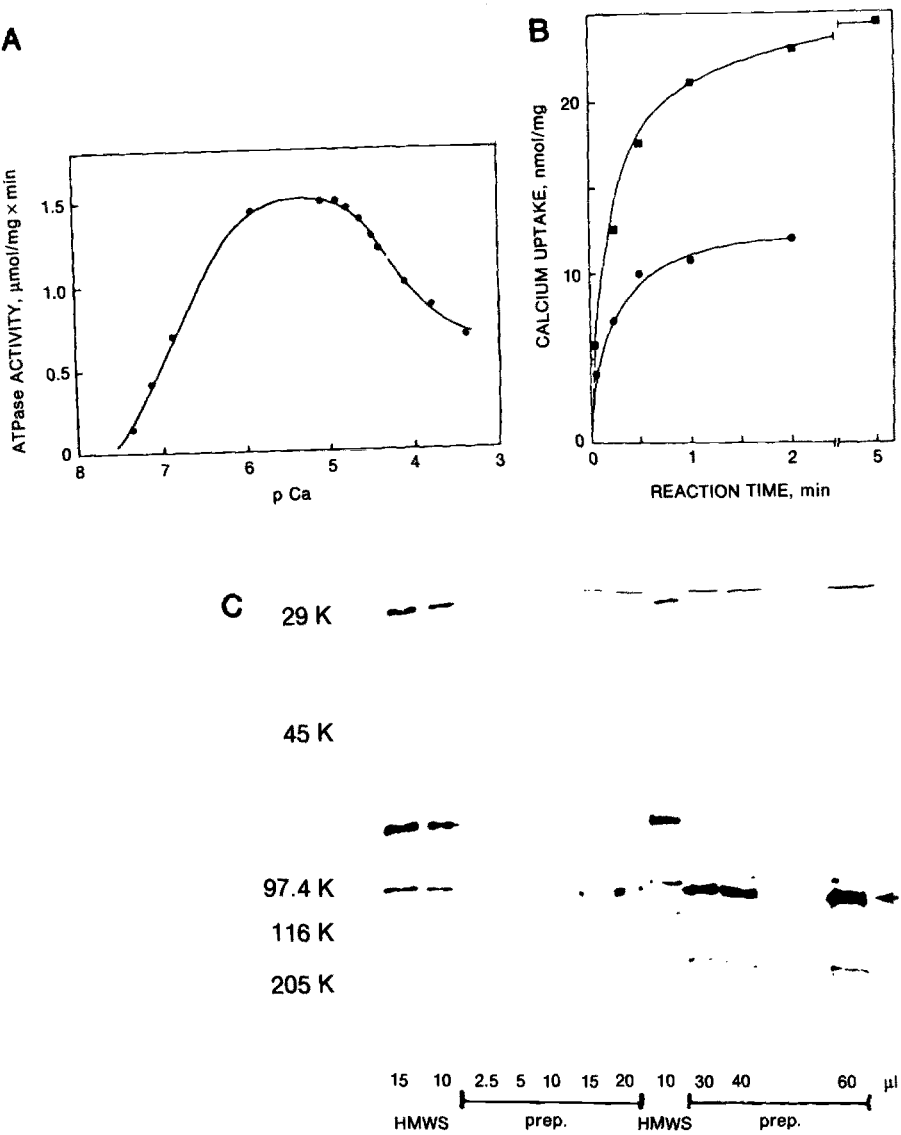


Fig. 17 (*cont.*). spectroscopy. The assay temp., was 27°C. (From Garcia et al. 1975). **B** Ca^{2+} uptake in the absence of oxalate in barnacle SR. Total [Ca^{2+}] 30 μM. Ca^{2+} uptake was measured either at 17°C (■) or 27°C (•). The solid lines represent the curves fitted to the experimental points by an equation of the general form $f(t) = AT/(k + t)$ where A is the maximum uptake value in nmol per mg and k is given in seconds. The corresponding A values used at 17°C and 27°C were 25 and 13 nmol mg^{-1}. The k value in both cases was 11 s (From Garcia et al. 1975). **C** Sodium dodecyl sulphate polyacrylamide gel electrophoresis (PAGE) of the barnacle SR fraction. The molecular weights and positions of the high molecular weight standards (HMWS) are indicated on the *left* (1 mg ml^{-1}). The *arrow* on the *right* indicates the main protein band of the SR fraction (prep.) at ca. 100 kDa. (8% Laemmli gel - PAGE blue stain - 10% MeOH/10% HOAc destain). (From Timmerman, unpubl)

Bacigalupo et al. 1979). The fibres have certain features which distinguish them from vertebrate skeletal muscle fibres but which are characteristic of crustacean muscle. The fibres are invaginated by a branched system of clefts whose surface area is some 15 times that of the simple surface area (Selverston 1967), and which are filled with a mucopolysaccharide material. The clefts make up about 8% of the fibre volume, as judged from electron micrographs although lower values are suggested from $^{45}Ca^{2+}$ efflux experiments. (Ashley and Lea 1978). Excitatory tubules originate both from the sarcolemma both at the periphery and from the clefts, and they run not only transversely, but also spirally and longitudinally (they will however be referred to collectively as T tubules in this article). The tubules make contacts with large terminal cisternae of the SR at triads occurring at the A-I overlap zone and are able to release Ca^{2+} as located by image-intensifier techniques (Ashley et al. 1984; Timmerman et al. 1990). They also form diads, where only one cisternum contacts the tubule. "Feet" occur in the gap between the membrane of the T-tubule and the SR. The longitudinal SR is fenestrated, with numerous perforations, and was described by Hoyle et al. (1973) as surprisingly sparse, compared with other crustacean muscles, putting its contribution to the fibre volume as only 0.5%, as measured from electron microscope (EM) pictures. Unlike in vertebrate muscle, the triads and diads occur towards the outer part of an A band most commonly but other positions in the sarcomere have been observed. (Hoyle et al. 1973), the average length of which is 8–10 μm (Hoyle and Smyth 1963).

Barnacle SR can be isolated as a vesicular fraction (Hasselbach 1966; Garcia et al. 1975; Altamirano et al. 1988). The vesicles display a

Fig. 18. **A** Successive responses to 5 mM caffeine of barnacle myofibrillar bundles at two free [Ca^{2+}] 0.14 μM (*traces a,b*) in low relaxing medium (LR) at pH 7.1. In a exposure to 1.5 μM free Ca^{2+} (in presence of 0.1 mM ethylene glycol Bis (β-aminoethylether) N,N,N′,N′ tetra-acetic acid (EGTA) total) for 2 min results in the recovery of the caffeine response, which otherwise virtually disappears (b). Temp., 13°C–15°C. (From Lea and Ashley 1989). **B** Ryanodine-induced tension responses in the myofibrillar bundle from the barnacle. 5 mM caffeine gave a phasic contraction in the first bundle (1.5 mN in 15 s) in solution with 0.1 mM EGTA (pCa, 6.7). In a second bundle, ryanodine (100 μM) gave a delayed contraction (0.7 mN in 130 s) at pCa 6.7. This is compared with a contraction of 1.7 mN in 60 s by a pCa 5.8 solution finally (EGTA, 5 mM); temp., 14°C. (From Lea, unpubl). **C** Isometric tension records from a single barnacle myofibrillar bundle (diameter 140 μm) showing the effect of ryanodine on tension responses following the rapid photolysis of 0.1 mM nitr-5 (caged Ca^{2+}) in air at three initial pCa values. Responses to a 55 mJ laser pulse were obtained after equilibration at pCa 6.90 (A), 6.76 (B) and 6.65 (C). The bundle was then transferred to a relaxing solution containing 10^{-4} M ryanodine for 20 min and then a "low relaxing" solution. The protocol with a 55 mJ pulse was then repeated in B and C. *Upper traces* (i), pen recordings; *lower traces* (ii), oscilloscope traces at a faster time calibration. Records before and after ryanodine are *superimposed.* After response B, the SR was reloaded with Ca^{2+} using a pCa of 6.4 (5 mM EGTA solution) for 5 min; temp., 12°C. (From Lea and Ashley 1990)

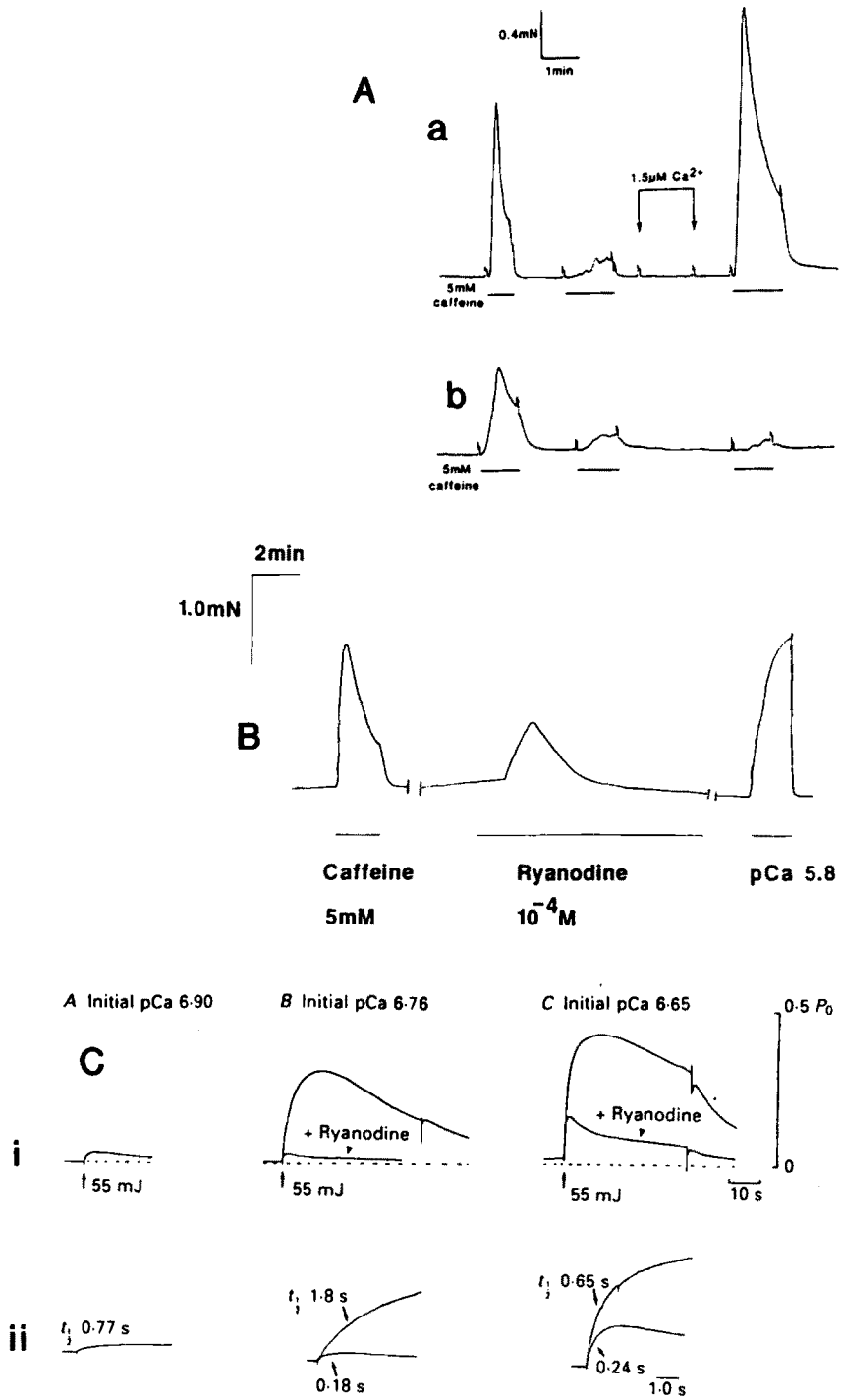

A
0.4mN
1min
a
1.5μM Ca2+
5mM caffeine
b
5mM caffeine
2min
1.0mN
B
Caffeine
5mM
Ryanodine
10-4M
pCa 5.8
A Initial pCa 6·90
B Initial pCa 6·76
C Initial pCa 6·65
0·5 P0
C
i
+ Ryanodine
55 mJ
+ Ryanodine
55 mJ
55 mJ
10 s
0
ii
t½ 0·77 s
t½ 1·8 s
0·18 s
t½ 0·65 s
0·24 s
1·0 s

Fig. 18A-C

Ca^{2+}-dependent ATPase activity and in the presence of ATP and oxalate take up Ca^{2+} (Fig. 17A,B). The major component of the SR protein has a mol.wt. of 106 kDa, as in rabbit muscle, where it has been identified as the Ca^{2+} transporter ATPase. No proteins similar to the 55 kDa calsequestrin of vertebrate muscle were found in barnacle (Fig. 17C).

Isolated myofibrillar bundles, which are analogous to skinned fibre preparations from vertebrate muscle, can be prepared by splitting single fibres longitudinally under paraffin oil, and pulling out bundles, of typically 100 μm diameter, with fine forceps (Ashley and Moisescu 1973a, 1977; Ashley et al. 1991b). These possess a caffeine-releasable Ca^{2+} store (Ashley et al. 1974d; Ashley and Moisescu 1975; Lea and Ashley 1981) which in vertebrate fibres is known to be the SR. This store can be replenished by Ca^{2+} loading at low free $[Ca^{2+}]$ (Fig. 18A; Lea 1986; Lea and Ashley 1989). Similarly calcium-induced calcium release (CICR), known to be mediated in vertebrate and cardiac muscle by the SR ryanodine-sensitive Ca^{2+} release channels (Fig. 18B,C) and shown to be present also in crustacean muscle (Seok et al. 1992; Olivares et al. 1992; Arispe et al. 1992), can be demonstrated in myofibrillar bundles using both aequorin and force development to assess the release of Ca^{2+} (Lea and Ashley 1989, 1990; see also Sect. 5.5). The caffeine-sensitive Ca^{2+} store can retain its Ca^{2+} for some time in intact fibres which have been immersed in Ca^{2+} -free saline, since caffeine injected into fibres still gave contractions after 6 h and for longer, providing Ca^{2+} loss was reduced by La^{3+} in the $[Ca^{2+}]_0$ salines (Ashley et al. 1977).

The involvement of release of Ca^{2+} from an intracellular Ca^{2+} store during an electrically stimulated contraction of a barnacle fibre is also indicated by measurements of depolarisation-induced uptake of radioactive $^{45}Ca^{2+}$. The rise in *total* calcium inside the fibre which was estimated to occur from I_{Ca} is sufficient to account for only a fraction of the observable tension in response to a depolarisation. This was shown from $^{45}Ca^{2+}$ influxes measured with an intracellular glass scintillator in non-perfused fibres intracellularly injected with 1 mM $EGTA_i$, bathed in artificial sea water (ASW; 10 mM $[Ca^{2+}]_0$; Ashley and Lea 1978; Van Wagoner et al. 1985; Fig. 19). From the extra Ca^{2+} influx per pulse of 31–95 pmol cm^{-2} (depolarisation +50 mV, 225 ms), the estimated increase in *total* calcium inside (not allowing for binding to TnC and other sites) is 1–4 μM kg^{-1} per pulse. Much larger $^{45}Ca^{2+}$ influxes were found with perfused fibres in 60 mM $[Ca^{2+}]_0$ by Atwater et al. (1974), using a method of counting the $^{45}Ca^{2+}$ which subsequently appeared in the perfusate. Their value was estimated to increase the *total* calcium inside by 11 μM kg^{-1} (500 ms pulse, +50 mV depolarisation). The major difference between the two experimental approaches was that the perfused fibres were inflated by the perfusion solution to nearly four times their normal volume (that is a doubling of

the surface area, if the radius also changes); it is not known to what extent this could account for the higher $^{45}Ca^{2+}$ entry but the technique employed reduced current oscillations and improved the radial voltage clamp. Atwater et al. (1974) argue that a correction should be employed to allow for inflation, giving a value of 33 μM as the increase in total Ca^{2+} during a 500 ms depolarisation. Even this value is considerably less than the reported value of 220 μM Ca^{2+} which is released in a frog fibre, presumably from the SR, during a simple twitch contraction (Miledi et al. 1982). If all the 33 μM Ca^{2+} in barnacle were to bind to the two calcium-specific (Collins et al. 1991) tension-generating sites on TnC (total Ca^{2+} bound 140 μM kg^{-1}), one model (Ashley and Moisescu 1972a) of tension development predicts only 2% of maximum tension (Ashley and Lea 1978).

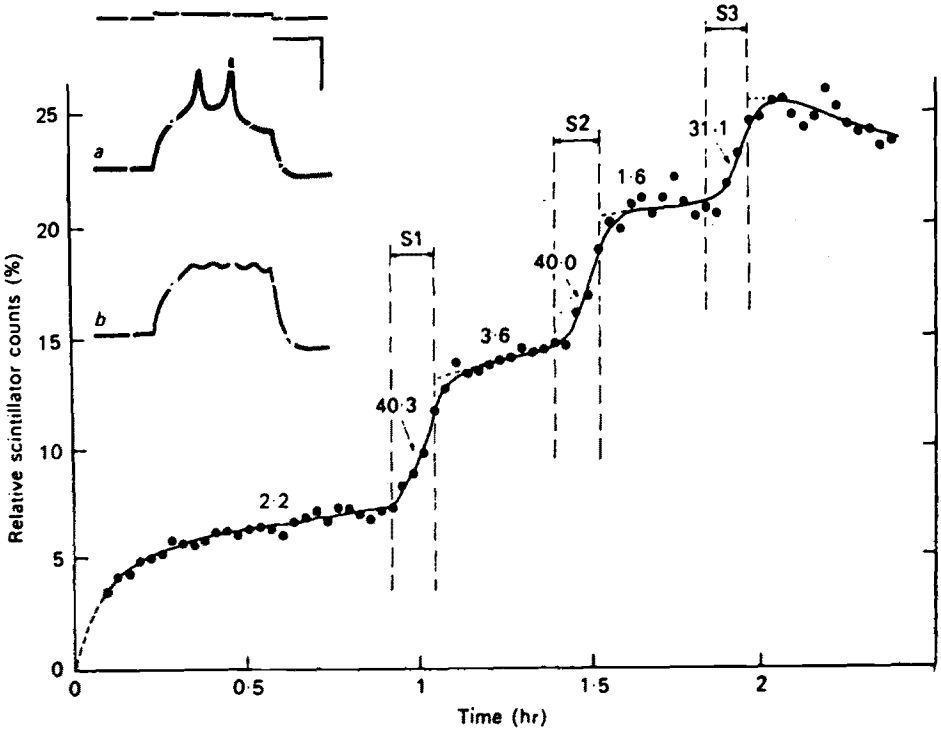


Fig. 19. The effects of membrane depolarisation on the Ca^{2+} influx measured by a scintillator probe in a fibre injected with ethylene glycol Bis (β-aminoethylether) N,N,N′,N′ tetra-acetic acid (EGTA; final concentration 16.0 mmol kg^{-1}). During the periods marked *S1*, *S2*, and *S5*, the fibre was stimulated with constant amplitude, rectangular current pulses (*inset top trace*), 250 ms in duration and at a frequency of 1.0 s^{-1}, to give depolarising membrane responses shown in the *inset* (a) at the start and (b) at the finish of each period. Mean E_m = −54 mV. Calibrations for a and b: *vertical*, top 300 μA, 20 mV; *horizontal*, 100 ms. Numbers give the apparent influx values in pmol. cm^{-2}. s^{-1}. Temp., 20°C. (From Ashley and Lea 1978)

5.4 Is There a Ryanodine Receptor – Ca^{2+} Release Channel in Barnacle SR Membranes?

Much recent progress has been made in the field of E-C coupling of both vertebrate skeletal muscle and vertebrate cardiac muscle and is associated with the isolation of the ryanodine receptor, its identification as the "foot" process seen in electron micrographs of the triadic junction, and the important demonstration that it acts as a Ca^{2+} channel when reconstituted into planar lipid bilayers (for reviews see Fleischer et al. 1985; Ma et al. 1988; Fleischer and Inui 1989; Lai and Meissner 1989; Ashley et al. 1991a).

Are similar Ca^{2+}-release channels present and are they associated with the SR membrane in barnacle and other crustacean striated muscle? All the available evidence suggests that they are, and although as yet the ryanodine receptor protein has not been isolated from this muscle, isolated SR membrane preparations show a major specific [^{3}H]ryanodine binding in the nanomolar range (Compagnon et al. 1989) as well as in lobster (Seok et al. 1992; Olivares et al. 1992; Arispe et al. 1992). Electron-dense foot processes can be seen in the triadic gap between the T tubule and the terminal cisternum (Fahrenbach 1965; Smith 1966; Hoyle et al. 1973). In transverse sections each foot is about 30 nm wide and its centre is separated from that of its neighbour by about 42 nm. Each foot appears to span about 75% of the triadic gap which totals 20–25 nm in this section. By comparison, in other striated muscles the feet are separated centre-to-centre by 37 nm in the frog twitch and about 30 nm in the crayfish fibres (Eastwood et al. 1982). At the triadic junctions of frog twitch muscle the width of the feet is 14–18 nm. Thus barnacle 'feet', from EM evidence, are similar in appearance and distribution to those of vertebrate skeletal muscle (Timmerman, unpublished).

An EM study of various invertebrate muscles including those of another crustacean, the crayfish, has conformed that the individual feet appear indistinguishable from those of vertebrate muscle, possessing the same four rounded subunits with a central depression (Castellani et al. 1989; Loesser et al. 1992). Furthermore, the ryanodine receptor has been isolated from crayfish muscle and appears in electron micrographs as a 25 nm square particle, has a molecular weight of 380–400 kDa subunit and can conduct Na^+ when incorporated into planar lipid bilayers (Formelova et al. 1990).

In freeze-fracture EM pictures of skeletal muscle of vertebrates the ryanodine receptors are closely associated with ovoid particles on the face of the transverse tubule, which have been identified as dihydropyridine (DHP) receptors (Block et al. 1988). These receptors seem to function as both L-type Ca^{2+} channels (Mikami et al. 1989) and as a voltage sensor

responsible for sensing the T-tubular potential during E-C coupling in vertebrate skeletal muscle (Rios and Brum 1987; Rios and Pizarro 1991; Rios and Gonzalez 1991). No such association between foot processes and T-tubule particles has been seen in crustacean muscle (Loesser et al. 1992), although even in vertebrate muscle the orderly arrangement is often lost as a result of the freeze-fracturing process (Franzini-Armstrong, personal communication). DHP binding in crayfish muscle is relatively low, with values similar to those from cardiac muscle, but less than those observed in vertebrate skeletal muscle (Kriszanova et al. 1990). This fact may simply reflect a low density of T tubules in crayfish muscle. The purified receptor protein had a molecular weight of 190 kDa and appeared as a 16 × 23 nm ovoid particle under electron microscopy, confirming its similarity with the vertebrate DHP receptor. A sensitivity of the voltage-gated I_{Ca} and of E-C coupling of barnacle muscle to DHP has not been demonstrated convincingly (Duchateau et al. unpublished; Rojas, E., personal communication). On the other hand, the voltage-dependent influx of ^{45}Ca in single barnacle fibres is completely blocked by 0.5 mM methoxyverapamil ((D600), Ashley and Lea 1978), as are calcium fluxes through DHP-sensitive L-type Ca^{2+} channels in other cells.

The difference between the Ca^{2+} sensitivities of E-C coupling of vertebrate skeletal and cardiac muscle appears to be due to an intrinsic difference in the respective DHP receptor structures (Tanabe et al. 1987; Takeshima et al. 1989) and in their calcium current kinetics (Tanabe et al. 1990, 1991). The determination of the structure of crustacean DHP receptors should help to clarify further the relation between I_{Ca} and excitation-contraction coupling in this muscle.

Ryanodine has a caffeine-like ability to release SR Ca^{2+} in isolated myofibrillar bundles from barnacle muscle: in weakly Ca^{2+}-buffered solutions (0.1 mM EGTA), 0.1 mM ryanodine caused a phasic contraction following a lag of 75 s at 14°C (Fig. 18B) (Hwang et al. 1987); this effect is similar to that reported on skinned fibres of frog twitch muscle (Lamb and Stephenson 1990) and is also seen in split fibres of crab muscle (Lea, unpublished). The CICR mechanism of barnacle muscle is inhibited by treatment with ryanodine (0.1 mM in a relaxing solution containing 5 mM EGTA for 20 min; Lea and Ashley 1990; see Fig 18B). Whether this is a result of Ca^{2+} depletion of the SR or is more a consequence of a specific inhibition of the Ca^{2+}-release channel cannot be decided at present. Both effects appear also to occur in vertebrate skeletal and cardiac muscle. Low concentrations of ryanodine (nanomolar) open the vertebrate Ca^{2+} release channel and lock it in a sub-conductance state, whereas concentrations above 10 μM close the channel (Lai and Meissner 1989).

5.5 How Is Ca^{2+} Released from the SR During Excitation-Contraction Coupling?

Several mechanisms have been proposed to explain how Ca^{2+} is released from the SR following depolarisation of fibres of skeletal and cardiac muscle (reviews: Endo 1977; Stephenson 1981; Fabiato 1983; Rios and Pizarro 1991; Rios and Gonzalez 1991; Ashley et al. 1991a). It is worthwhile examining their respective claims in relation to the contraction of crustacean muscle fibres.

5.5.1 The Charge Coupled or Mechanical Coupling Model

The charge coupled model was first proposed by Schneider and Chandler (1973) as a result of measurements from single frog fibres of very small currents, which were similar to the gating currents (non-linear charge movement) observed in nerve axons and which occurred in response to a membrane depolarisation. In its present form the model states that depolarisation of the T-tubule membrane causes a movement of charge within the membrane and that this movement is communicated to the SR Ca^{2+} release channel, resulting in its opening. The fact that in vertebrate skeletal muscle part of the charge movement appears to come from the DHP receptors (Lamb 1986; Rios and Brum 1987) and part from the SR release (Garcia et al. 1990), and that the DHP receptor in the T-tubules is located close to the SR Ca^{2+} channel ryanodine receptor make this protein complex an attractive possibility for the site of the transduction mechanism in vertebrate skeletal muscle.

Analogous charge movements have been recorded from barnacle muscle fibres in a preliminary study (Ashley et al. 1986a). The nonlinear component of membrane capacitative current was determined by addition of control and test current transients from a single fibre which was bathed in saline containing 4-AP^+ to reduce the delayed inward potassium current (I_K). Both 'on' and 'off' charge movements were detected for subthreshold depolarisation steps from minus 40 mV to minus 10 mV, below the threshold for I_{Ca}. The integrated 'on' and 'off' gating currents were equal for a given pulse. For voltage pulses up to +20 mV, the 'off' current, obtained upon returning to minus 40 mV, was fast enough to be separated from the slower inward ionic current tail, and this was found to increase sigmoidally with voltage (Fig. 20). These properties are similar to those of the nonlinear membrane charge movements seen in vertebrate skeletal muscle (Chandler et al. 1976).

The source of these charge movement current in barnacle fibres is unknown at present. Perhaps the biggest objection to a vertebrate-type charge movement mechanism operating in E-C coupling in crustacean muscle is the absence of a morphological association at the triadic (and diadic) junction between the foot and the DHP receptor (Franzini-Armstrong, personal communication).

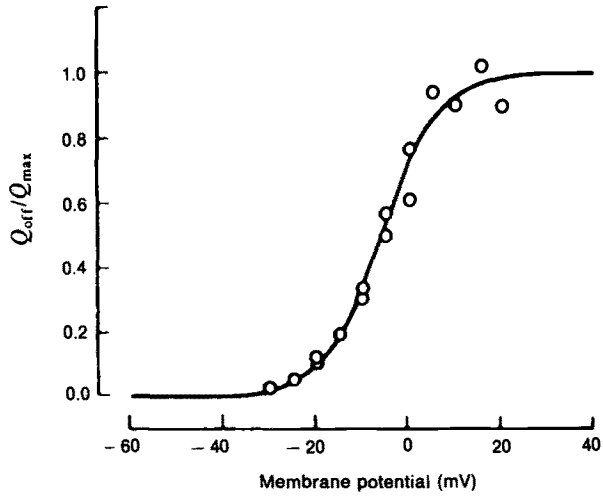


Fig. 20. Voltage dependence of 'off' gating current. Each data point represents the normalised value of 16 signal-averaged charge movement measurements. The *solid line* was obtained by least-squares fit to a two-state Boltzmann distribution function in which the midpoint voltage was about minus 6 mV and the e-fold steepness was 6.3. Temp., 6–8°C. (From Ashley et al. 1986a)

5.5.2 Ca^{2+}-Induced Ca^{2+} Release

Several features of excitation-contraction coupling of barnacle striated muscle are consistent with the primary Ca^{2+} releasing mechanism of the SR being CICR. This process, which was first shown in skinned fibres of frog muscle (Endo et al. 1970; Ford and Podolsky 1970), has since been studied in detail in cardiac muscle (Fabiato 1983). It is basically a process whereby a concentration of free Ca^{2+}, too low to cause significant tension generation by direct activation of the myofibrils, is able to release stored Ca^{2+} from the SR and thus cause maximum tension as a result of this subsequently released stored Ca^{2+}. This type of SR Ca^{2+} release has been shown to be a property of the ryanodine-binding Ca^{2+} channel complex in both vertebrate skeletal muscle (Lai et al. 1988; Smith et al. 1988) and in cardiac muscle (Williams and Ashley 1989). The depolarisation-induced I_{Ca} which enters across the sarcolemma and presumably the cleft and T tubule membranes, would seem to be an ideal source of trigger Ca^{2+} for this release mechanism to occur from the SR of barnacle striated muscle fibres.

Isolated myofibrillar bundles of barnacle muscle fibres have been shown to release SR Ca^{2+} through CICR (Stephenson and Williams 1980; Lea and Ashley 1989, 1990). The resulting rise in myoplasmic [Ca^{2+}] has been monitored by both tension development and by the free Ca^{2+} indicator aequorin (Fig. 21A) under conditions of low Ca^{2+} buffering by the bathing solution (0.1 mM EGTA). A phasic rise in myoplasmic Ca^{2+}, induced by a

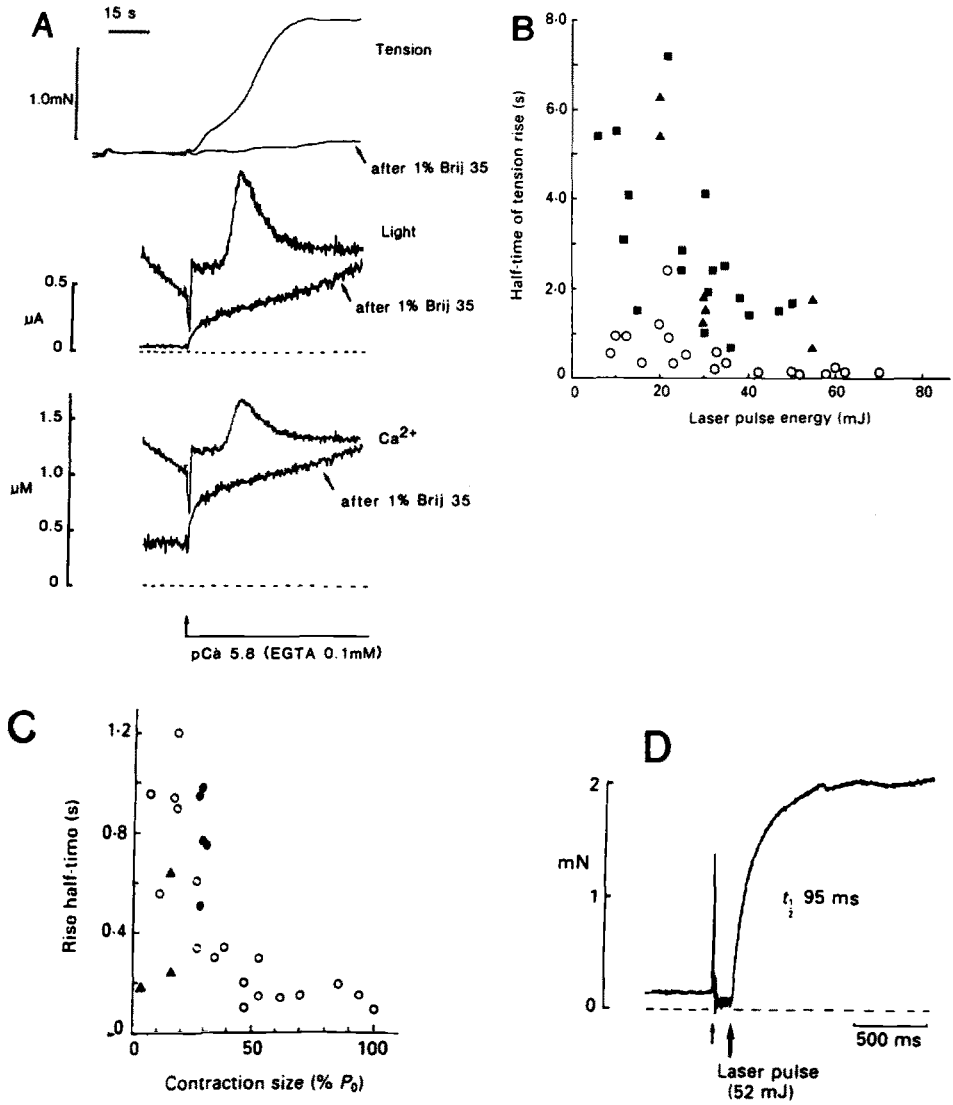


Fig. 21. **A** Isometric tension, aequorin light and free Ca^{2+} from a barnacle myofibrillar bundles with superimposed traces from before and after inactivation of the sarcoplasmic reticulum (SR) by Brij and caffeine; temp., 14°C. (From Lea and Ashley 1989). **B** The half-times for the rise in tension in a barnacle myofibrillar bundle following the photolysis of 0.1 mM nitr-5 at initial pCa 6.8–6.6 (■ and ▲) and of 2.0 mM nitr-5 at initial pCa 6.4 (○), plotted against laser pulse energy (millijoules). Combined data from 20 myofibrillar bundles: each point represents a single measure. For the 0.1 mM nitr-5 data, the bundle (with intact SR) was either in the air (▲) or in solution in the trough (■) at the time of the laser pulse. The 2.0 mM nitr-5 data are from either bundles with intact SR or bundles after treatment with Triton X-100; temp., 12°C. (From Lea and Ashley 1990). **C** The half-times for the rise in tension of contractions, which were attributed to direct activation of the myofibrils by the Ca^{2+} released from photolysis of nitr-5, plotted as a function of contraction amplitude (%P_0). The values for contractions with 2.0 mM nitr-5,

pCa 5.8 solution, could be abolished by destruction of the SR membranes by detergents (Triton X100 or Brij), and also by increasing the Ca^{2+} buffering (EGTA concentration) of the solution.

The rate of contraction is relatively slow in these experiments because of the time taken for the trigger Ca^{2+} to diffuse from the bathing solution to the SR within the myofibrillar bundle (see Fig. 25A). This delay has been avoided in some recent work, in which the photorelease of Ca^{2+} from the photo-labile caged calcium molecule, nitr-5, has been used as a trigger for CICR (Lea and Ashley 1990). In this method, a laser pulse of UV light was focussed on a myofibrillar bundle equilibrated in a solution containing 0.1 mM nitr-5 at an initial pCa which caused little release of Ca^{2+} from the SR. The release of Ca^{2+} within 1 ms after photolysis of Ca^{2+}-nitr-5 initiated a rapid phasic contraction, which could be inhibited by pretreatment with ryanodine (Fig. 18C), confirming its SR origin. The time to half amplitude (half rise-time) was as short as 0.7 s at 12°C, but the mean value of 2.3 s (for contractions larger than 40% P_o) was significantly slower than the rise of tetanic force in intact muscle fibres, (177 ms at 12°C, Griffiths et al. 1990). Isolated myofibrillar bundles could be made to contract as fast as this when the photorelease of Ca^{2+} (2 mM nitr-5) was large enough to activate directly the contractile proteins (mean half rise-time 164 ms at 12°C; Fig. 21B, C, D). The disparity in times of the CICR responses and the physiological contraction may be taken as evidence that CICR is not the sole physiological release mechanism. However, in cardiac muscle, where the case for physiological CICR is strong, CICR-initiated contractions, generated in skinned trabeculae by photolysis of nitr-5, were also slower than the physiological contractions of intact trabeculae (Kentish et al. 1990).

Calculation indicates that the likely rise in sarcoplasmic $[Ca^{2+}]$ which results from I_{Ca} is within the necessary range in which the free Ca^{2+} can initiated CICR in vitro. This point can be deduced from Caputo and Dipolo's tension-voltage relation in barnacle in the presence of the SR Ca^{2+} release inhibitor, procaine (Fig. 15B). By assuming that the tension response at +50 mV is due solely to externally derived Ca^{2+} (via I_{Ca}) binding directly to the force generating sites on TnC, an approximate value for the maximum rise in sarcoplasmic $[Ca^{2+}]$ brought about by this process is to a pCa of 6.2. This pCa value is certainly sufficient to initiate a significant CICR

Fig. 21 (*cont.*). pCa 6.4 (○), are from **B** and are replotted on an expanded vertical scale. The values for 0.1 mM nitr-5 are from myofibrillar bundles with the SR inactivated either by ryanodine (▲) or Triton X-100 (●); temp., 12°C. **D** The tension trace from a barnacle myofibrillar bundle shows a typical fast activation at 2 mM nitr-5 pCa 6.4 of a 100 μm diameter bundle, which was suspended in air (first arrow) before illumination by a 52 mJ laser pulse. Contraction amplitude 251 kN m^{-2}. (From Lea and Ashley 1990)

from isolated myofibrillar bundles retaining functional SR (Lea and Ashley 1990).

The sigmoidal shape of the tension-voltage relationship for intact muscle fibres of *Balanus* is not consistent with SR Ca^{2+} release working through CICR alone, however, because tension is generated at large positive membrane potentials at which I_{Ca} should be zero. As described earlier (Sect. 5.2), TEA^+ and the perfusion of fibres with Na^+-free solutions converted the tension-voltage relation into a bell-shaped curve, indicating a much closer influence of I_{Ca} (Hidalgo et al. 1979). Further work is required to see whether tension generation at large positive potentials under normal circumstances is perhaps the result of a second I_{Ca}-independent mechanism.

One feature of the Ca^{2+} transients which have been recorded from cannulated fibres of *Balanus* is that for a +25-mV depolarising pulse (current clamp), the Ca^{2+} transient continued to increase for up to 50 ms after the end of 50- to 100-ms-duration pulse (Ashley and Ridgway 1968, 1970). Just such an effect is to be expected if the increased sarcoplasmic Ca^{2+} at the end of the depolarisation continues to stimulate a release of Ca^{2+} from the SR through the CICR mechanism until it is attenuated by the activity of SR pump. Ca^{2+} transients from frog muscle fibres show similar behaviour when CICR is stimulated by the presence of low concentrations of caffeine (Simon et al. 1989), but is not seen in the absence of caffeine.

The rate of Ca^{2+} release from the SR during a depolarisation-induced contraction of a barnacle muscle fibre has been computed from the time courses of the tension change and the free Ca^{2+} transients, using a model in which the free Ca^{2+} in the sarcoplasm at any moment is determined by Ca^{2+} binding to the myofibrils and by Ca^{2+} movements across the SR membrane (Ashley and Moisescu 1972a,b, 1973b; Ashley et al. 1974c). The rate of Ca^{2+} release from the SR is given by:

$$(dCa/dt)_{SR\ release} = a\ [Ca^{2+}]_{free} + b$$

where the variables (a) and (b) depend on the amplitude of the depolarising pulse. This model makes no assumptions about the primary mechanism of Ca^{2+} release from the SR, but during the Ca^{2+} transient it displays positive feedback so that the SR Ca^{2+} release is a regenerative process. An analysis of the Ca^{2+} transient resulting from a long depolarising pulse (500 ms) indicated such a relationship. How the rise in sarcoplasmic Ca^{2+} is prevented from becoming an all or nothing process is not known; in cardiac muscle the E-C coupling mechanism may be explained by a time-dependent inactivation of CICR (Fabiato 1985). In barnacle muscle, the model included a 'lag' between the rise in free Ca^{2+} and the *increase in the rate* of Ca^{2+} uptake by the SR; the rate of SR uptake by the pump is assumed to be slow, requiring the binding of two calciums to sites on the

SR for activation. At the end of the depolarising pulse the release rate is assumed to fall quasi-exponentially, within about 10 ms at 10°–12°C.

5.5.3 Inositol Trisphosphate?

The important role of inositol 1,4,5-trisphosphate ($InsP_3$) as a second messenger in a variety of calls by mobilising intracellular Ca^{2+} stores (Berridge and Irvine 1984) has led to the suggestion that $InsP_3$ plays a similar role in E-C coupling of skeletal muscle (Vergara et al. 1985). In such a scheme, following membrane depolarisation, $InsP_3$ is released from a precursor, phosphatidylinositol bisphosphate (PIP_2), in the T tubule membrane and then diffuses across the triadic gap (20 nm) to the SR membrane where it binds to a receptor (ryanodine receptor?) and opens Ca^{2+} release channels in the cisternal membrane. This idea has been the source of considerable debate, with some workers claiming that $InsP_3$ can release SR Ca^{2+} in both intact and in skinned muscle fibres, and also gate Ca^{2+} channels in isolated SR membranes. Others have failed to find any such effects (reviewed by Hidalgo and Jaimovich 1989; Jaimovich 1991).

Barnacle muscle fibres have not escaped the $InsP_3$ controversy. The contradictory results from the myofibrillar bundle preparation have typified the general debate which $InsP_3$ has generated concerning vertebrate skeletal muscle. Rojas et al. (1987) and Rojas and Hidalgo (1990) reported that $InsP_3$ caused a transient release of Ca^{2+} within myofibrillar bundles provided the SR was preloaded with Ca^{2+}. Maximum release was obtained at $InsP_3$ concentrations below 10 μM. In contrast in an earlier study, Lea et al. (1986) found virtually no effect of up to 500 μM $InsP_3$ under a variety of ionic conditions, using isometric tension to detect SR Ca^{2+} release. How can such different results be obtained from the same preparation? One difference between the experimental protocols of the two groups was the presence of 100 μM EGTA in the bathing solutions of Lea et al. (1986) and its omission in the solutions of Rojas et al. (1987). It is conceivable that 100 μM EGTA was sufficient to buffer an $InsP_3$-induced Ca^{2+} release and any additional regenerative release, thus preventing a detectable rise in tension. A relevant question here is: how does the Ca^{2+} buffering of 100 μM EGTA compare with the total Ca^{2+} buffering in the sarcoplasm of barnacle muscle fibres provided by TnC, amino acids and other endogenous chelators (e.g. parvalbumins in frog muscle)? The concentration of STnC Ca^{2+} regulatory (T) sites in frog is certainly of the same order (140 μM) as the EGTA concentration.

Rojas et al. (1987) also found that $InsP_3$, when injected into intact single muscle fibres, caused a contracture, the maximum recorded tension of 1.6 kg cm^{-2} (over 25% of maximum achievable tension, P_{max}) being obtained by injection of 8 nmol $InsP_3$ into a 50 mg fibre. Furthermore,

Compagnon et al. (1989) have detected significant concentrations of the phospholipid components of the $InsP_3$ cycle in barnacle muscle, notably PIP_2. They also reported that electrical stimulation reduced the incorporation of labelled precursors into these components.

There are indications therefore that $InsP_3$ may have a function in controlling the sarcoplasmic $[Ca^{2+}]$ of barnacle muscle fibres, perhaps as in nonmuscle cells acting as a second messenger in response to hormones and neurotransmitters (proctolin?). Whether $InsP_3$ acts as an essential link in E-C coupling is still, however, open to question, since barnacle muscle has recently been shown to posses the DHP/ryanodine receptor complex typical of vertebrate skeletal muscle (Sect. 5.4).

5.5.4 Protons

As discussed in detail in Sect. 6, CO_2 has been shown to release SR Ca^{2+} from both intact muscle fibres and the isolated myofibrillar bundles of barnacle muscle (Lea and Ashley 1978, 1981). One mechanism to account for this is that CO_2 causes a fall in the pH inside the SR lumen, which by some mechanism, opens SR Ca^{2+} channels. This posed the question: Could physiological Ca^{2+} release be through H^+ gating of the SR Ca^{2+} channels? The lack of an effect of protonophores in releasing SR Ca^{2+} in myofibrillar bundle experiments apparently ruled out the existence of a resting H^+ gradient across the SR membrane which could be utilised in such a gating mechanism (Lea 1986). The ryanodine receptor SR Ca^{2+} channel of vertebrate skeletal muscle, when incorporated into planar lipid bilayers, can be gated by an *increase* in pH on either the sarcoplasmic or luminal sides of the membrane (Ma et al. 1988). Thus this result offers no support for the acidification mechanism idea in order to explain the effect of CO_2 on barnacle muscle: vertebrate muscle does not however possess such a CO_2-induced Ca^{2+} release under normal conditions. Could SR Ca^{2+} channels be gated by an *increase* in sarcoplasmic pH in E-C coupling? The answer is probably no for barnacle muscle, since a rise in solution pH from 7.0 to 7.5 failed to release SR Ca^{2+} at resting free Ca^{2+} levels in myofibrillar bundle experiments (Lea 1986). Direct experiments of pH changes on the isolated barnacle ryanodine receptor have not yet been reported.

6 Perturbations of the Free $[Ca^{2+}]$: Na^+ Gradients and pH

6.1 pH

Other interventions, which are able to perturb the steady state as reflected by the changes in the resting free $[Ca^{2+}]$, are the effects of acidification

as well as alterations in the sodium concentration gradient across the cell membrane. In the experiments, referred to in Sect. 5.5.4 where the effect of acidification upon the contractility of fibres was investigated, there was a marked increase in the resting free [Ca^{2+}] associated with a gradual increase in the baseline force of the barnacle muscle fibre. This could be brought about by the application of permeant species such as CO_2, or phthalate (Fig. 22a). This effect has been also demonstrated in cardiac muscle, as well as directly and indirectly in other muscle and nonmuscle cells. The opposite effect, a decrease in the internal free calcium concentration, was observed upon application of ammonium chloride (Fig. 22a).

It was not clear from these experiments performed on intact muscle fibres whether the increase in free Ca^{2+} was brought about either by the internal change in sarcoplasmic pH (pH_i), which is of the order of 1 pH unit lower when the CO_2 concentration is 100% (Caldwell 1958; Aickin and Thomas 1975; Boron 1977), or by an effect on or a pH change within an internal organelle. One way of investigating this problem was to use mechanically skinned (SR functional) muscle fibres (see Sect. 5.5.4). By increasing the concentration of bicarbonate in the medium surrounding the myofibrillar bundle, the external pH can be maintained virtually constant while, at the same time, increasing the CO_2 concentration to 100%. In bundles of myofibrils exposed to this protocol, CO_2 increased the free calcium concentration in the myofibrillar (external) space, as indicated by an increase in aequorin light and this produced a force response from the myofibrillar bundle preparation (Fig. 22b).

The increase in free calcium appears to be the result of an effect of CO_2/HCO_3^- upon the SR, and is blocked by procaine which inhibits the CICR (Fig. 22c) and also by caffeine which depletes the SR Ca^{2+} store (Fig. 22b). This result, together with the finding that the increase in free calcium is also partially prevented by weak bases, such as amantidine, led to the suggestion that the Ca^{2+}-release mechanism may be a process initiated by a change in the pH *within the lumen* of the SR: a H^+-mediated calcium-release process (see Sect. 5.5.4 for details).

Fig. 22. **a** The effect of externally applied salines containing either CO_2 or NH_4Cl upon the aequorin light emission from a single barnacle muscle fibre. E_m, −50 mV. (Calibration: *vertical*, 200 nA; *horizontal*, 15 min.). Temp., 17°–23°C. (Lea and Ashley, unpublished observations). **b** Caffeine and CO_2 responses from barnacle myofibrillar bundles. i, prior exposure to 20 mM caffeine in low-relaxing solution with Cl^- as the anion (LR-Cl) abolishes the response to CO_2 + HCO_3^- solution; ii, A (CO_2 + HCO_3^-)-solution induced contraction reduces the size of the subsequent caffeine response. Successive experiments on two separate bundles of diameter 250–300 μm, taken from the same *Balanus nubilus* muscle fibre. Note the residual caffeine response at the end of each trace. Temp.,

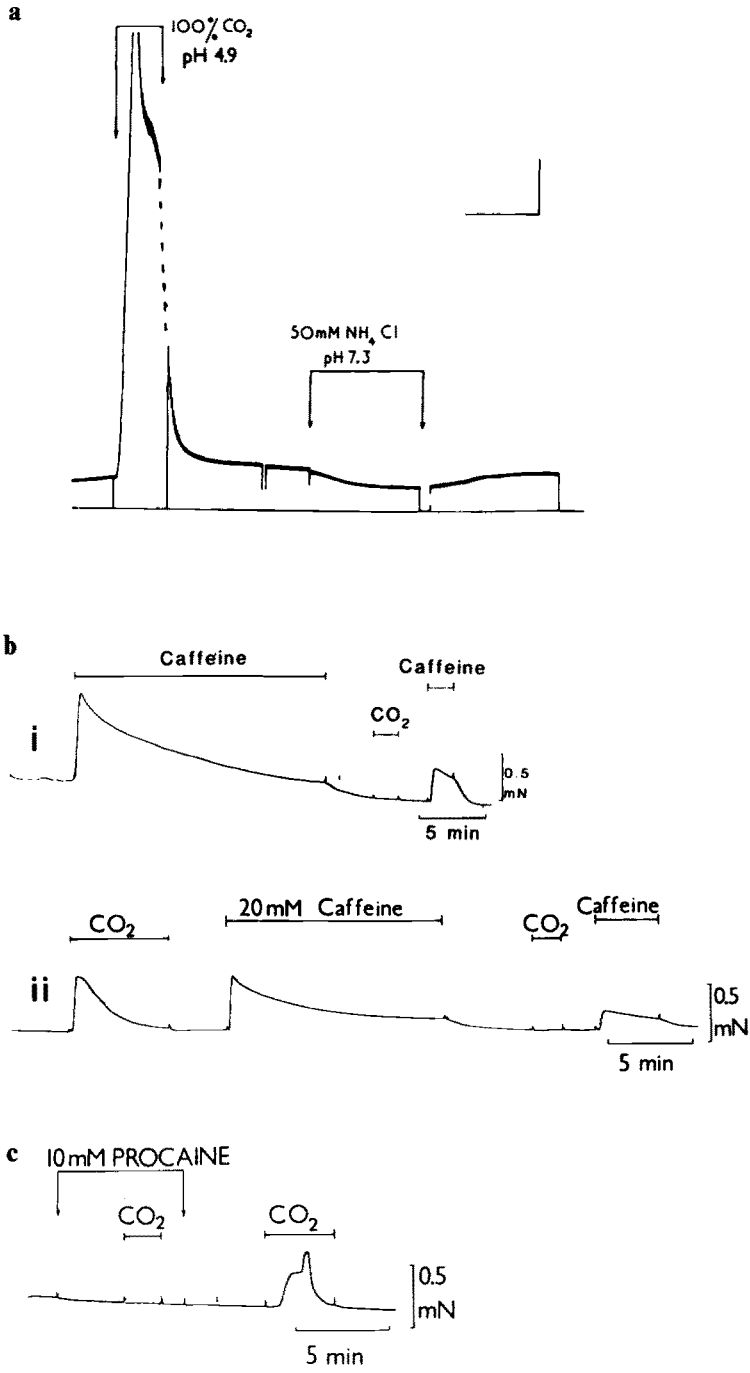


Fig. 22 (*cont.*). 14°–16°C. (From Lea and Ashley 1981). **c** CO_2 + HCO_3^- solution fails to produce a response in the presence of 10 mM procaine, but does so after its removal (*B. nubilus* myofibrillar bundle). Temp., 14°–16°C. (From Lea and Ashley 1981)

6.2 Sodium-Calcium Exchange

The first indication that a Na^+:Ca^{2+} exchange transport mechanism operates across the sarcolemma of barnacle striated muscle fibres was the observation that the replacement of extracellular Na^+ by Li^+ caused an increase in the unidirectional Ca^{2+} influx (Dipolo 1973) and a decrease in the unidirectional Ca^{2+} efflux (Ashley et al. 1974a; Russell and Blaustein 1974, 1975; Nelson and Blaustein 1981). The rise in sarcoplasmic free [Ca^{2+}] which resulted from the net $I_{Ca(Na-Ca)}$ could be detected readily with the photoprotein aequorin (Ashley et al. 1974a; Fig. 23A). Since then investigators have addressed the following questions: (1) what proportion of the resting Ca^{2+} fluxes are mediated by Na^+-Ca^{2+} exchange? (2) To what extent does Na^+-Ca^{2+} exchange participate in the transient increase in sarcoplasmic free Ca^{2+} during E-C coupling? (3) What are the kinetics and stoichiometry of the exchange mechanism?

In a detailed study in Blaustein's laboratory of the unidirectional $^{45}Ca^{2+}$ influx and ^{22}Na efflux in perfused muscle fibres, an exchange stoichiometry of 3 Na^+: 1 Ca^{2+} has been demonstrated (Rasgado-Flores et al. 1989; Blaustein et al. 1991). In these experiments, the Na^+ pump was inhibited by ouabain, intracellular Ca^{2+} sequestration was inhibited by caffeine and the protonophore FCCP, and the $I_{Ca(Na-Ca)}$ maximised by replacing extracellular Na^+ by Li^+. When the [Na^+] in the perfusate was varied between 6 and 106 mM the ratio of $[Ca^{2+}]_o$-dependent Na^+ efflux to the $[Na^+]_i$-dependent $I_{Ca(Na-Ca)}$ influx was found to give a mean value of 3.1, with the maximum values for Na^+ efflux and Ca^{2+} influx being 75 and 25 pmol cm^{-2} s^{-1} respectively. As expected for an exchange mechanism, the $I_{Ca(Na-Ca)}$ influx increased with $[Na^+]_i$ while the Na^+ efflux increased with $[Ca^{2+}]_o$. Somewhat unexpectedly, an increase in internal Ca^{2+} also increased both of these fluxes: the $[Ca^{2+}]_o$-dependent Na^+ efflux was half-maximally activated by an intracellular [Ca^{2+}] of 0.3–0.7 μM (Fig. 23B,C). This activating [Ca^{2+}] does not appear to be transported by the exchanger.

In resting muscle fibres these results imply that the Na^+-Ca^{2+} exchanger operates at a low rate with most of the resting Ca^{2+} efflux (60%) being mediated by an ATP-dependent pump, as seems to be the case in squid axons. When the muscle fibre is activated by depolarisation of the sarcolemma, the rise in $[Ca^{2+}]_i$ will act to stimulate the Na^+-Ca^{2+} exchanger, moving Ca^{2+} into the fibre and Na^+ out of it. There will be an additional stimulation of the exchanger due to the membrane depolarisation, since the exchange is electrogenic (voltage-sensitive), moving one positive charge out of the fibre for each exchanger turnover (one Ca^{2+} with three Na^+). In other cells the $I_{Ca(Na-Ca)}$ is increased as the membrane potential is made more positive inside [squid giant axons, Allen and Baker (1986); cardiac cells,

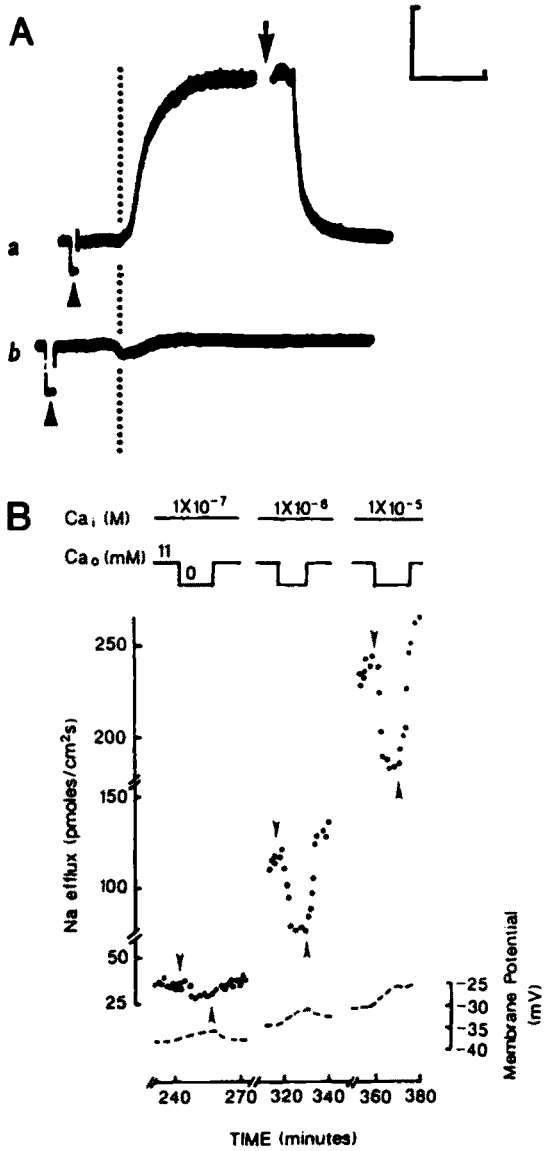


Fig. 23. **A** The effect of Na^+-free (Li^+-replaced) saline on the rate of light emission from an aequorin injected single *Maia* (crab) muscle fibre. (**a**) 10 mM Ca_o^{2+}; (**b**) 0 mM Ca_o^{2+}. *Initial* arrow indicates magnitude of ambient and resting fibre light emission. The *dotted line* indicates the time of application of the test saline and the *second arrow* the time of reapplication of Na^+-free and Ca^{2+}-free saline. Calibration: *horizontal*, 10 s, *vertical*, 20 nA. Temp., ca. 20°C; resting potential minus 62 mV. (From Ashley et al. 1974a). **B** $[Ca^{2+}]_o$-dependent Na^+ efflux measured at various $[Ca^{2+}]_i$. The external solution was (Na^+-free) Tris-ASW (saline) containing 0.1 mM ouabain; the internal perfusion fluid contained 46 mM Na^+ and various $[Ca^{2+}]_i$ obtained with a calcium-ethylene glycol Bis (β-aminoethylether)N,N,N',N' tetra-acetic acid (EGTA) buffer solutions containing 8 mM $EGTA_{total}$. *Downward arrowheads* denote times when external Ca^{2+} (11 mM) was

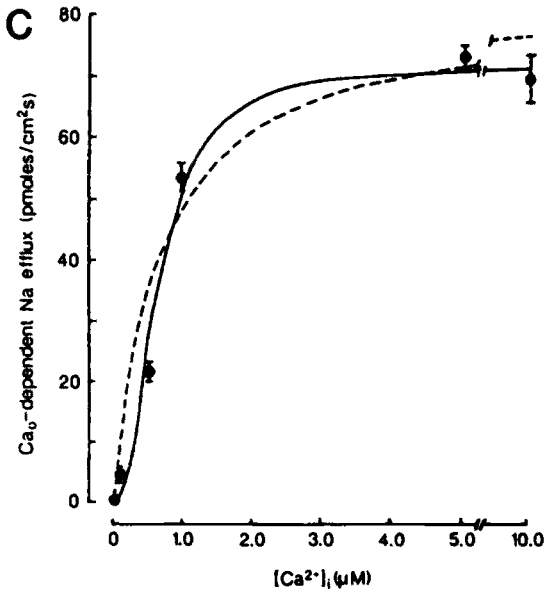


Fig. 23 (*cont.*). replaced by Mg^{2+}; *upward arrowheads* indicate when Ca^{2+} was restored. The various $[Ca^{2+}]_i$ levels are shown at the *top* of the figure. The *abscissa* indicates the time after starting perfusion with tracer ^{22}Na; temp., 16°C. (From Rasgado-Flores et al. 1989). C $[Ca^{2+}]_o$-dependent Na^+ efflux graphed as a function of $[Ca^{2+}]_i$, with $Tris^+$ as the predominant external cation. These data are from the experiment in B and four similar experiments; *closed circles* (•) indicate mean ± standard error of mean (SEM). The *continuous* and *discontinuous lines* are the best-fit solutions to the Hill equation and the Michaelis-Menten equation, respectively. The calculated parameters are: for the Hill equation K_{Ca_i}, 0.7±0.1 μM; $J_{Na(max)}$, 72±3 pmol cm^{-2} s^{-1}; Hill coefficient, 2.2±0.5; and for the Michaelis-Menten equation, K_{Ca_i}, 0.7±0.2 μM and $J_{Na(max)}$, 81±6 pmol cm^{-2} s^{-1}. Note that if the $[Ca^{2+}]_i$ just under the sarcolemma was underestimated at nominally 1.0 μM, and was actually 1.5 or 2.0 μM because of the large Ca^{2+} influx, the K_{Ca_i} would be increased to 0.8–1.0 μM, and the Hill coefficient would be reduced to 1.6 or 1.3 respectively. Temp., 16°C. (From Rasgado-Flores et al. 1989)

Beuckelmann and Wier (1989)]. Upon repolarisation of the fibre membrane, the exchanger would help to move Ca^{2+} out of the fibre.

In resting muscle fibres (as opposed to electrically stimulated fibres) the maximum $I_{Ca(Na-Ca)}$ was measured to be 25 pmol cm^{-2} s^{-1} (Rasgado-Flores et al. 1989) at 16°C. Given the 3:1 Na^+-Ca^{2+} electrogenic exchange, it can be calculated that this will produce an exchange outward current of 2.5 μA cm^{-2}. It is not known if this maximum rate of exchange is exceeded when the fibre membrane is depolarised. As it stands, this expected outward current is relatively small compared with the outward current "bumps" of 50–500 μA cm^{-2} which were measured in untreated muscle fibres by Caputo and Dipolo (1978) and these are thought to be carried mainly by K^+. It is apparent therefore that the detection of the Na^+-Ca^{2+} exchange current can only be carried out in the absence of an outward K^+ current.

How does the value of the $I_{Ca(Na-Ca)}$ compare with I_{Ca} through the voltage-dependent Ca^{2+} channels? In muscle fibres which had been first injected with EGTA, a 250 ms, 50-mV depolarising pulse resulted in an extra I_{Ca} of 80 pmol cm^{-2} (Ashley and Lea 1978). Even assuming that the Na^+-Ca^{2+} exchange is working maximally during the pulse, its contribution to the I_{Ca} of the depolarising pulse could be no more than $25 \times 0.25 = 6.25$ pmol cm^{-2} per 250 ms pulse at 20°C. However, it is not known how the Na^+-Ca^{2+} exchange functions when the membrane is depolarised. Evidence from squid giant axons and cardiac cells shows that $I_{Ca(Na-Ca)}$ increases in amplitude as E_m is made more positive (Allen and Baker 1986; Beuckelmann and Wier 1989). For example, the exchange of I_{Ca} influx increased by 140% when the axon membrane was depolarised from minus 40 mV to +40 mV.

The extent to which $I_{Ca(Na-Ca)}$ contributes to the intracellular Ca^{2+} transient in E-C coupling of barnacle muscle fibres is further discussed in Sect. 5.2.

In summary, perturbations of the calcium transporting system, whether across the cell membrane, or involving a disturbance in the steady state of an intracellular organelle by inhibiting the uptake or stimulating the release of calcium, will be reflected by changes in the free Ca^{2+}. This section emphasises the importance of the balance of calcium fluxes throughout the muscle cell and indicates that, in the resting state, the steady-state free $[Ca^{2+}]$ is finely controlled by the careful balancing of the resting fluxes. This latter point is indicated by the result in Fig. 6A, which demonstrates the effect of simply altering the external calcium concentration upon the resting free calcium concentration. It seems clear that, upon lowering the external calcium concentration, there is a decrease in the surface calcium flux, which is reflected in a decrease in the resting calcium concentration as detected by aequorin. Under these conditions, the SR and plasma membrane are presumably able to maintain the lower free Ca^{2+} in the sarcoplasm.

7 Relaxation: Effect of Depolarisation, Parvalbumin, Chelators and Intracellular pH

7.1 Single Fibres

Single muscle fibres, previously injected axially by displacement (Hodgkin and Keynes 1956; Caldwell and Walster 1963) with the photoprotein aequorin were stimulated by a step depolarising pulse, the fibre was repolarised to either the initial holding potential (Fig. 24a, panels A & B) or to a membrane potential more positive (Fig. 24a, panel C) or negative (Fig. 24a, panel D) than this holding potential. The repolarisation was accompanied

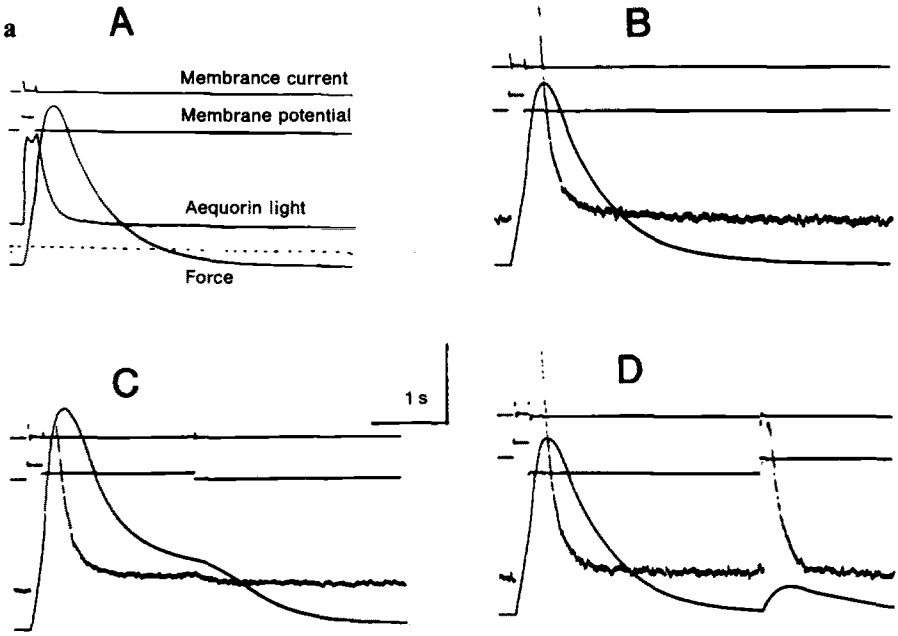


Fig. 24. **a** Aequorin light and tension responses of a single (cannulated) barnacle muscle to a 19 mV voltage clamp depolarising step (200 ms duration) and subsequent repolarisation to different membrane potentials. Panels **A** and **B** show the responses to repolarisation back to the holding potential (minus 49 mV), with the aequorin light signal shown on a higher gain in **B**. Panels **C** and **D** show the responses to repolarisation to a membrane potential 8 mV *positive* and 20 mV *negative* to the holding potential, respectively. The elevation of the aequorin light signal in **C** during relaxation appears sufficient to significantly reduce the rate of force decline, but would be virtually undetectable on a trace recorded with a light sensitivity equivalent to that in A. The *dashed line* in A indicates zero light level. *Vertical calibration bar*; membrane potential 200 mV, membrane current 200 μA; force 135 mg; aequorin light 200 nA **A**, 20 nA **B,D**, 40 nA **C**. Temp., 13°C.

by both a fall in aequorin luminescence and mechanical relaxation. When the membrane potential was repolarised to a voltage more negative than the holding potential, neither the rate of decline of the aequorin light signal (free calcium) nor that of mechanical relaxation was significantly altered, compared to the control. However, if the membrane potential is returned rapidly to values more positive than the holding potential (Fig. 24a, panel C), there is a marked slowing of the aequorin luminescence decline, which is also reflected in a slowing in the rate of tension decay.

It has previously been reported that relaxation of aequorin-injected barnacle muscle fibres under voltage clamp control is accompanied by a small elevation of the aequorin signals throughout the relaxation (Ashley and Lignon 1981). This "tail" to the aequorin luminescence response is unaffected by the hyperpolarising step change in membrane potential

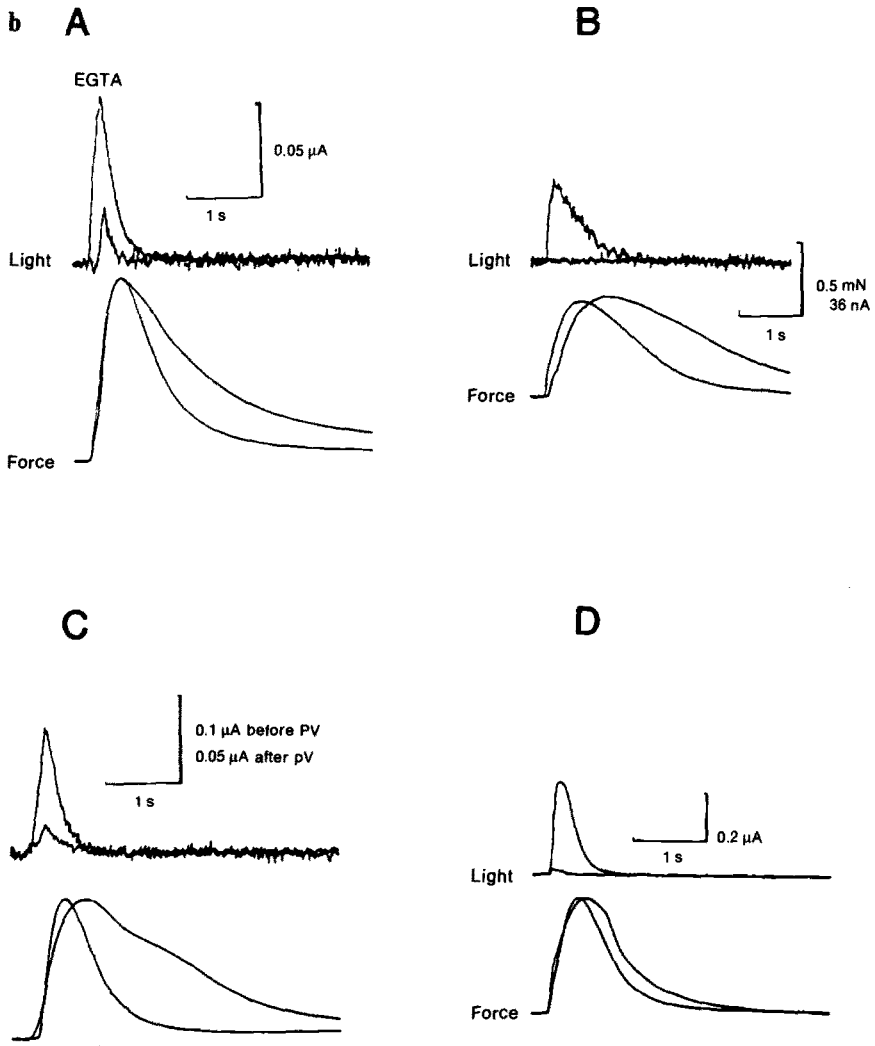


Fig. 24 (*cont.*). **b** Aequorin light records and tension from a single (cannulated) barnacle muscle fibre under voltage clamp control before and after injection of 'Ca^{2+} buffers'. **A** ethylene glycol Bis (β-aminoethylether) N,N,N′,N′ tetra-acetic acid (EGTA) ($[EGTA]_i$ = 3.6 mM); **B** 1,2-bis (o-aminophenoxy) ethane N,N,N′,N′ tetra-acetic acid (BAPTA) ($[BAPTA]_i$ = 1 mM); **C** rabbit parvalbumin ($[PV]_i$) = 1.2 mM; **D** perch II parvalbumin ($[PV]_i$) = 1.3 mM). In order to simplify comparison of force records, tensions have been scaled to the same amplitude, and as far as possible similar amplitude force responses have been chosen. In order to achieve this, much larger depolarisations were required after injection of chelators. The ratio of actual force amplitude after injection to that before were **A** 0.33, **B** 1.0, **C** 1.6 and **D** 0.94. In each case the force record showing the slower relaxation and smaller calcium transient was obtained after Ca^{2+} buffer injection. Temp., 13°C. (From Griffiths et al. 1990)

(Fig. 24a, panel D), which suggests that it is not a membrane current related phenomenon. A similar tail to the aequorin response is also seen in non-cannulated fibres (shell-attached) and this is made more obvious by the extremely low resting aequorin luminescence in these cells (Fig. 13). The slow decline in aequorin luminescence lasts as long as force is elevated above resting values and this suggests that the phenomenon is related to Ca^{2+} leaving the TnC sites. A similar tail to the aequorin transient has been observed in aequorin-injected single frog muscle fibres (Cannell and Allen 1984; Cannell 1986), which may be due in part to calcium loss from TnC, but probably also reflects parvalbumins present in high (millimolar) concentrations in frog.

It has been shown that the time course of relaxation of frog muscle fibres is greatly prolonged when it occurs under truly isometric conditions (sarcomere clamp) (Huxley and Simmons 1973; Ford et al. 1977), and the rapid relaxation observed in the absence of sarcomere length clamping in frog results from inhomogeneity of sarcomere lengths along the fibre, as rapidly relaxing regions are stretched by neighbouring fibre sections which are still highly activated. But the slowing of relaxation in Fig. 24a (panel C) produced by a depolarised membrane during relaxation shows that it should not be assumed that the rate of relaxation is totally independent of the free $[Ca^{2+}]$ even in the absence of truly isometric conditions. In addition to this effect of a small depolarisation upon the relaxation rate, in fibres injected with "calcium buffers" such as BAPTA (1,2-bis(2-aminophenoxy)ethane N,N,N′,N′-tetra acetic acid), EGTA and parvalbumin which are likely to load with Ca^{2+} during activation, the rate of mechanical relaxation is also slowed, presumably by a small elevation of free calcium above resting levels during relaxation brought about this 'extra' Ca^{2+}-loaded buffer in the cell. This is illustrated in Fig. 24b, where panels A and B show that the injection of both BAPTA and EGTA caused a marked slowing of relaxation, similar to the effects observed with parvalbumins (Fig. 24b, panels C and D). It is unlikely that either the presence of a small maintained depolarisation during relaxation or the presence of intracellular calcium buffers can affect the contractile apparatus directly, and it seems more reasonable to suppose that they affect the rate of calcium regulation and translocation in order to produce these effects (Gillis, 1985). Barnacle fibres do not contain significant amounts of native parvalbumins or calcium-binding proteins and so these agents cannot be a complicating factor here ($< 6\ \mu M$; Stein, personal communication; Ashley and Griffiths 1983; Griffiths et al. 1990; see Fig. 5).

7.2 Skinned Fibres

Intracellular acidification of intact muscle cells by CO_2 leads to a prolongation of the relaxation phase of force and similar effects are observed

with the permeant anion, phthalate (Ashley et al. 1979; Ashley 1983). This prolongation of relaxation could arise by a direct effect of H^+ upon the contractile proteins, for example, either by influencing the on or off rate for calcium from the troponin complex, or by directly affecting the cross-bridge and ATP kinetics and some evidence for this has recently been established, both in frog and barnacle fibres using the caged calcium chelator, diazo 2 (Palmer et al. 1991). One likely action in intact cells is, however, by changing the Ca^{2+} accumulating activity of the SR Ca^{2+} pump.

A possible *direct* effect of H^+ upon the contractile proteins was investigated in bundles of myofibrils (80–100 μm diameter). The results obtained (Lea and Ashley 1982) make it unlikely that the *very prolonged* relaxation observed in intact muscle fibres exposed to 100% CO_2 can be attributed to a direct effect of acidification on the Ca^{2+}_{off} kinetics of the myofibrillar proteins, since the range of pH values studied in these myofibrillar experiments (i.e. 7.1–6.0) was similar to the range of pH_i values of muscle fibres exposed to 100% CO_2, and these only produced modest-changes in myofibrillar bundle relaxation rates. A more likely cause of the effect of CO_2 on intact fibres is an impairment of the SR Ca^{2+} pump as a result of the decrease in pH_i, or possibly the increase in intracellular CO_2 and HCO_3^- concentrations. Certainly Garcia et al. (1975) have shown a narrow pH, as well as pCa optimum for the SR pump ATPase in barnacle (Fig. 17a). The Ca^{2+} pump had virtually no activity at either pH 6.5 or pH 8.0 and demonstrated an optimum pumping activity at pH 7.4, with 17°C being more efficacious than 27°C. The narrow pCa effect may explain the elevated free Ca^{2+} during relaxation.

8 Calcium and the Contractility of Myofibrils

8.1 Introduction

Manual mechanical removal of the cell membrane under mineral oil, as first described by Natori (1954), is the most effective method of "skinning a fibre" if the surface membrane is not required, but the SR membranes are to be retained. Other methods available for the complete or partial removal of the surface membrane include treatment with glycerol as well as chemical skinning with agents such as the detergents Brij, Triton, deoxycholate (DOC) and saponin, also inclusion of agents such as polyvinyl pyrolidene (PVP) or dextran to prevent the myofilament lattice expansion observed upon removal of the surface membrane. Saponin is more selective as a permeabilising agent, as it renders the surface membrane relatively more permeant to ions than the membranes of the SR, and these remain functional (Iino and Endo 1980). Large muscle cells are certainly amenable to

the mechanical skinning methods, and also to the preparation of myofibrillar bundles of whatever diameter is suitable, whereas, for smaller cells, such as those from vertebrate smooth muscle, saponin has been used. Alternatively, mechanical separation of the cells by homogenisation, followed by manual skinning, has also been successfully applied, particularly for small cardiac cells (Fabiato 1983, 1985).

8.2 pCa Clamp Method

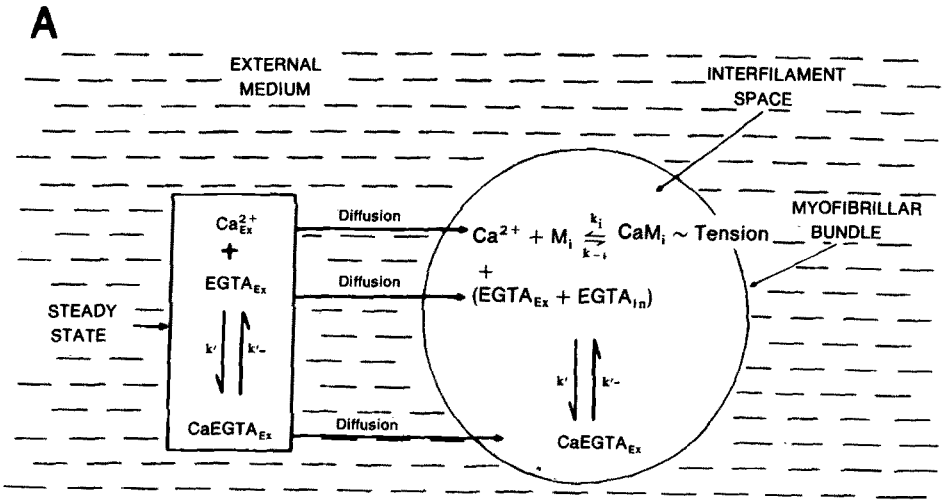
One of the puzzling observations noted when mechanically skinned fibres from frog were activated by calcium was the relatively slow development of isometric force (Hellam and Podolsky 1969; Ashley and Moisescu 1973a; Endo 1973), even when the calcium concentrations used were in the range of those encountered in intact cells. In examining the possible reasons for this effect, one must take into account those components still functionally intact, existing within the myofibrillar preparation, and capable of accumulating calcium from the intermyofilament space. These components have already been discussed (see Fig. 7). Hellam and Podolsky (1969) were the first to examine the influence of the SR upon the rate of development of tension in a mechanically skinned frog muscle preparation when challenged with an activating solution containing a high free $[Ca^{2+}]$ (low pCa value). (pCa is now defined on page 5). The rate of development of force, in preparations, where the elements of SR remained intact, was compared with those that had subsequently been treated with detergent to remove, or at least inactivate, the SR and mitochondrial Ca^{2+} uptake mechanisms. Although the half-time for force development was now considerably faster than with the SR still functioning, the rate was still much slower than that observed in vivo. Endo (1973) observed a similar slow rate of force development, and indicated that this did not appear to be related to the change in fibre volume, and hence to the accompanying change in lattice spacing that occurs upon skinning. Perhaps, in the process of skinning, other geometrical or biochemical changes occur that no longer permit force development to happen as rapidly or as fully as that encountered in vivo. Also diffusible factors such as the calmodulins or the enzymes involved in myosin light chain phosphorylation processes may well be lost in the skinning and subsequent equilibration in aqueous media. Thus, a demonstration that the skinned fibre preparation could react rapidly to changes in free Ca^{2+} was important if this preparation was to be used as a reliable model for processes that occur within intact fibres upon activation. Previous authors employed the same concentration of $EGTA_{total}$ in both the relaxing as well as in the activating solution, and this was in the range of 1–5 mM. Endo (1973) observed an increase in the rate of force development in frog skinned fibres when

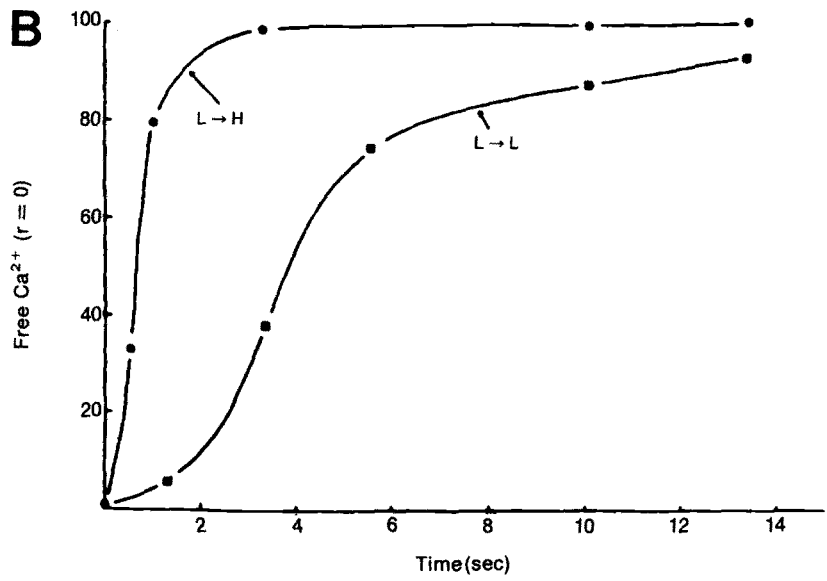
$EGTA_{total}$ and, hence, the concentration of the Ca-$EGTA^{2-}$ complex in the activating solution was increased, although here the total EGTA (mainly H_2EGTA^{2-}) concentration in the relaxing solution was also increased. The rate of force development observed under these circumstances was as fast as that witnessed when the calcium accumulating ability of the SR was reduced or destroyed by the presence of detergent (Brij-58, 0.5% w/v, $t_{1/2}$ ca. 1 sec at 10°C). Nevertheless, the rate was still far slower than that observed in vivo ($t_{1/2}$ ca. 50 ms at 10°C).

Ford and Podolsky (1972) had also observed that, in myofibrillar preparations where the SR was intact, the delay in force development was dependent upon the concentration of the Ca-$EGTA^{2-}$ complex in the activating solution. The detailed interpretation of the force transients was complicated by the presence of the SR and by the presence of the regenerative Ca^{2+} release mechanism (CICR), but the delay in force development was attributed at least partly to the time taken to fill the elements of the SR with calcium.

After analysing the way free Ca^{2+} concentration changes can affect isometric tension in intact barnacle muscle fibres (Ashley and Moisescu 1972a,b), it was apparent that the presence within the skinned fibre bundle of both diffusible (EGTA) and fixed calcium-binding sites (such as troponin) could well modify the rate at which the externally applied calcium buffer system reached an equilibrium [Ca^{2+}], particularly as the H_2EGTA^{2-} to $EGTA^{4-}$ reaction is fairly slow (Fig. 25A). One way to test this idea was to alter the protocol by which activation of the myofibrillar bundle was achieved, particularly in the absence of an effective SR (Ashley and Moisescu 1973a,b). The concentration of free EGTA in the relaxing solution was reduced to a value some 200 times smaller than the total

Fig. 25. **A** Schematic diagram for the activation of myofibrillar bundles with a calcium buffer such as ethylene glycol Bis-(β-aminoethylether) N,N,N′,N′ tetra-acetic acid (EGTA). The concentration of Ca-$EGTA^{2-}$ within the bundle *before* activation is considered negligible in this scheme (From Ashley 1983). **B** Estimate of the free [Ca^{2+}] change at axis of myofibrillar bundle when r = 0 as a function of time; (diam. 175 μm). In L → L, Ca-$EGTA^{2-}$ + H_2EGTA^{2-}) is 3 mM in relaxing and activating solutions; in L → H it is 0.1 mM in the relaxing and 20 mM in the activating. Free [Ca^{2+}] in activating solution 8.7×10^{-8} M. Apparent diffusion coefficient for EGTA = 4.6×10^{-6} cm^2 s^{-1} No account is taken here of the kinetics of Ca^{2+} binding to and H^+ dissociation from H_2EGTA^{2-} which is slow also ($k_{Ca-off-EGTA} = 0.3$ s^{-1}). Here L (low *total* EGTA in solution) and H (high concentration of *total* EGTA in solution). (From Ashley and Moisescu 1975; Ashley 1983). **C** Isometric force response from a bundle of mechanically skinned barnacle muscle fibres initially *A* in LR (low *total* EGTA concentration in 'relaxing' solution) (pCa 8.0) and subsequently transferred to HA (high *total* EGTA concentration in 'activating' solution) (pCa 4.3): *'pCa clamp' activation* method. Details in caption. $t_{1/2}$ for force 1.7 s at 13°C. (From Griffiths and Ashley, unpubl observations)


A
EXTERNAL MEDIUM
INTERFILAMENT SPACE
MYOFIBRILLAR BUNDLE
STEADY STATE
Diffusion
Diffusion
Diffusion
Ca2+Ex + EGTAEx
CaEGTAEx
Ca2+ + Mi ⇌ CaMi ~ Tension
+
(EGTAEx + EGTAin)
CaEGTAEx


B
Free Ca2+ (r = 0)
100
80
60
40
20
L → H
L → L
2
4
6
8
10
12
14
Time(sec)

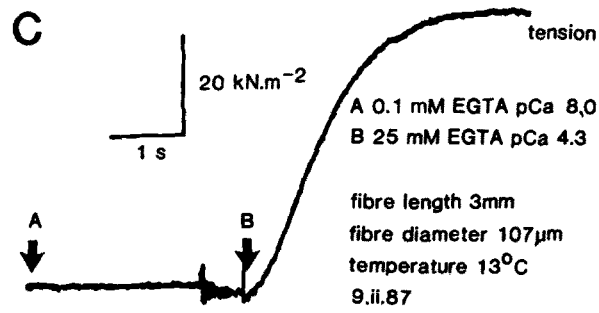

C
20 kN.m−2
1 s
tension
A 0.1 mM EGTA pCa 8,0
B 25 mM EGTA pCa 4.3
fibre length 3mm
fibre diameter 107µm
temperature 13°C
9.ii.87
A
B

Fig. 25A-C

EGTA concentration in the activating solution, while maintaining the same ionic strength in the two solutions by ionic replacement, using the EGTA substitute HDTA (hexamethylene diamine N, N, N′, N′ tetra acetic acid) which has a very low affinity for divalent ions but has the same net ionic charge as EGTA at pH 7.0 (Moisescu 1976). Now the relatively low concentration of free H_2EGTA^{2-} in the relaxing solution present within the myofibrillar preparation should not appreciably disturb the rate at which the externally applied calcium buffer system achieves equilibrium and establishes a $[Ca^{2+}]$ value, as it diffuses into the bundle. The free Ca^{2+} should be determined essentially by the rate at which Ca-$EGTA^{2-}$ diffuses and is thus faster the higher the concentration of this component: the free $[Ca^{2+}]$ changes rapidly in the bundle under these circumstances, and hence the term 'pCa clamp' has been applied to this activating protocol (see Fig. 25B).

When the preparation is activated by solutions containing the same concentration of EGTA as in the relaxing solution, the rate of tension development is slow. If, however, the newer activation protocol was employed (pCa clamp) by reducing the free H_2EGTA^{2-} in the relaxing solution and increasing the Ca-$EGTA^{2-}$ concentration in the activating but without a significant change in the ionic strength between the relaxing and activating solutions, the rate of tension development is considerably faster (Fig. 25C). The kinetic situation during activation can be approximated to the problem of the diffusion of a molecular species into an empty cylinder, of radius r, containing a finite concentration of material (C_1). The interfilament free calcium concentration, produced as a result of the low $EGTA_{total}$ (L) to high $EGTA_{total}$ (H) activating protocol, reaches some 90% of the external concentration within 1–2 s using the experimentally determined apparent diffusion coefficient for H_2EGTA^{2-} of $4.0–4.6 \times 10^{-6}$ cm^2 s^{-1} for relaxed myofibrils at 20°C (Ashley and Moisescu 1975). When the activating protocol is used, where the $EGTA_{total}$ concentration is the same in the activating as in the relaxing solution (L to L), the free calcium concentration within the bundle only approaches 90% of the external concentration after some 13 s (Fig. 25B). In these calculations no account was taken of the dissociation rate of the Ca-$EGTA^{2-}$ complex (*ca* 10^2 s^{-1}), but the results for the L to H activating protocol are essentially unchanged, even when this and other factors (such as an unstirred layer surrounding the skinned preparation) are also taken into consideration.

Although previous calculations suggest that the free $[Ca^{2+}]$ within the bundle resulting from the L to H activation protocol should equilibrate with that externally within 1–2 s (Fig. 25B), it was important to determine whether this was so experimentally to explain the rapid force event

(Fig. 25C). The calcium-sensitive photoprotein aequorin was employed (Ashley et al. 1974b,c; Ashley and Moisescu 1975), and the time course of light emission from the aequorin within the bundle was used as an index of the mean free [Ca^{2+}] within the myofilament space. It was initially necessary to have an accurate estimate of the apparent diffusion coefficient of aequorin within the myofibrillar preparation, so as to be certain that the major fraction of the light response observed upon activation was from aequorin actually within the barnacle bundle, rather than from aequorin that had diffused out of the bundle into the external medium during the activation period (ca. 10 s).

Bundles of barnacle myofibrils were transferred for known periods of time into a loading solution containing aequorin, rinsed briefly in an aequorin-free relaxing solution, and then activated in an aequorin-free solution at a much higher free [Ca^{2+}]. This protocol was repeated for the same skinned fibre bundle, but for increasing loading times. The area of each light emission response following Ca^{2+} activation represented the complete utilisation of all the aequorin molecules in the preparation, and hence was directly proportional to the number of aequorin molecules that had penetrated the bundle in the particular loading time. The area reached a constant value for loading times greater than 6 min for bundles of diameter ca. 160 μm (Fig. 26A), suggesting that diffusion at this time was virtually complete into the space available to the photoprotein. The result suggested an apparent diffusion coefficient of ca. 1×10^{-7} cm^2 s^{-1} at 20°C, at an ionic strength of 0.13 M, assuming aequorin was able to diffuse into an empty cylinder. Thus, for a bundle whose radius is 75 μm, 90% loading with aequorin occurs in about 5 min. This apparent diffusion coefficient for aequorin is some 15–20 times slower than the diffusion coefficient for aequorin in free solution (Shimomura and Johnson 1969, 1979), and some four times slower (Ashley et al. 1974b,c, 1975) than for the radial diffusion of the photoprotein in large axons (Baker et al. 1971).

In frog muscle, a slightly smaller value was observed for the longitudinal diffusion of aequorin of 0.5×10^{-7} cm^2 s^{-1} at 15°C, but the major point is that the diffusion of aequorin was some 40 times slower than that of H_2EGTA^{2-} (or Ca-$EGTA^{2-}$).

Calculations based upon this value for the apparent diffusion coefficient indicate that 80% of the light signal would be due to aequorin still located within the myofibrillar preparation after some 10 s in an aequorin-free solution, when the bundle was initially fully "loaded" with photoprotein. The photoprotein, therefore, could be used as an effective measure of the free Ca^{2+} within the bundle and hence give an index of the rapidity with which the Ca^{2+} achieved equilibration, compared to force development.

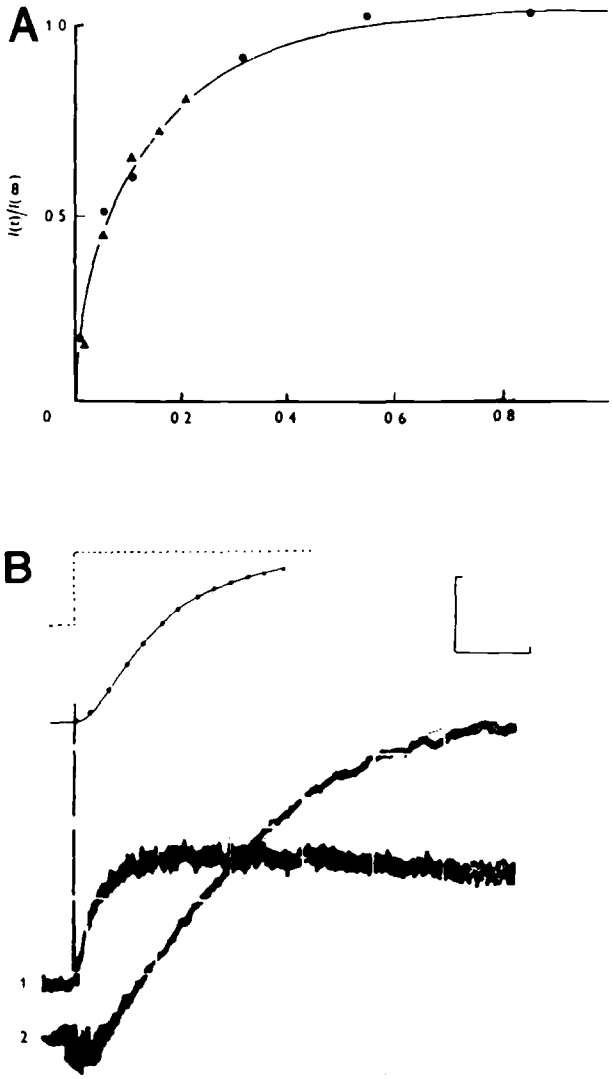


Fig. 26 *(p. 208–210).* **A** Determination of the diffusion of aequorin in an isolated bundle of myofibrils (*Balanus nubilus*). Two sets of experimental results (•, ▲), both bundles with radii, r = 78 μm; temp., 20°C. The *line* is the predicted diffusion of aequorin fitted by an apparent diffusion coefficient of 1×10^{-7} cm^2 s^{-1} (ionic strength 0.13 M). To convert the abscissa to time units, multiply by 10(min). The light intensity I(t) is proportional to the aequorin concentration within the bundle at time t, and I(∞) is proportional to the aequorin concentration at steady state (i.e., time ∞), so that $I(t)/I(\infty) = 1-4\Sigma_{n=1}^{\infty} 1/\mu_n^2 \exp[-\mu_n^2(D'_{Aeq}t/r^2)]$, where D'_{Aeq} is the *apparent* diffusion coefficient for aequorin, r is the radius of the bundle, and μ_n is the solution of the Bessel function of order zero ($F_o(\mu) = 0$). (From Ashley and Moisescu 1975). **B** Activation of a myofibrillar bundle (diam. 175 μm) (*B. nubilus*) after preloading with aequorin. Aequorin-light

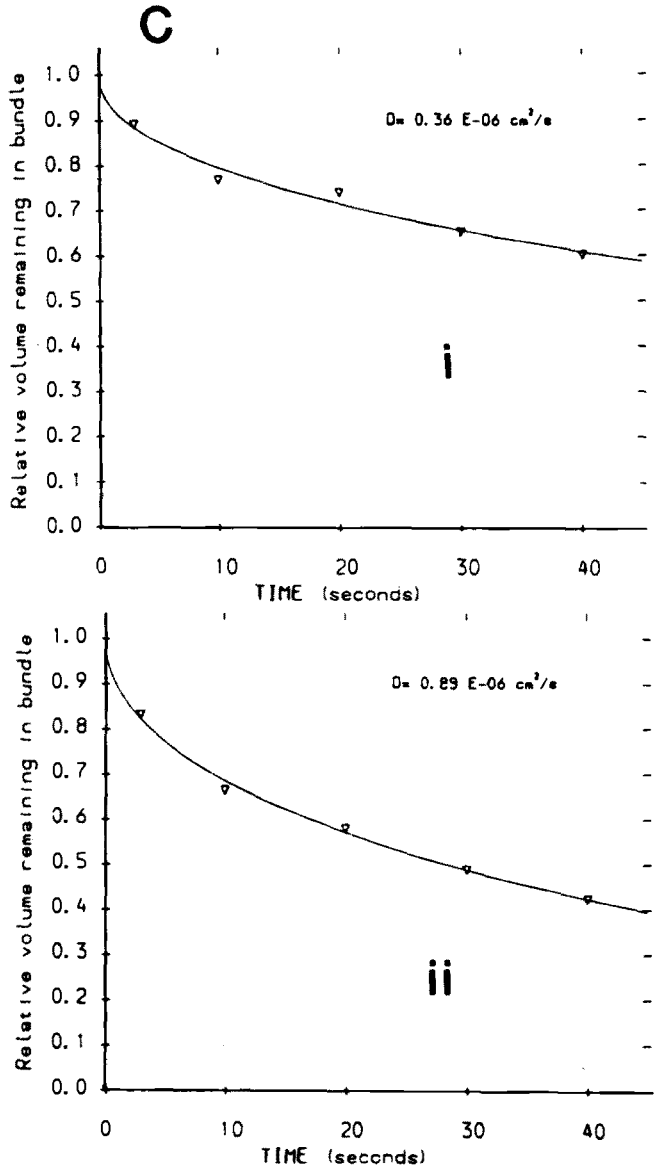


Fig. 26 (*cont.*). (*trace 1*) and force (*trace 2*) responses activated externally with Ca-EGTA^{2-} buffer solution (free [Ca^{2+}] 8.7 × 10^{-8} M). Initially, the preparation was in a high-total EGTA relaxing solution (HR) (10 min), followed by a low-total EGTA relaxing solution (LR) (5 min). Aequorin, in 'low relaxing' (LR), was applied uniformly along the segment while immersed under mineral oil (3 mm length). After 6 min the bundle was washed in low relaxing (2–3 s). The '*blip*' on *trace 1* is electrostatic and indicates the moment of immersion in the activating solution. *Insert*: theoretical force points (solid dots) calculated from the following equation assuming a step increase in calcium (*dotted line*).

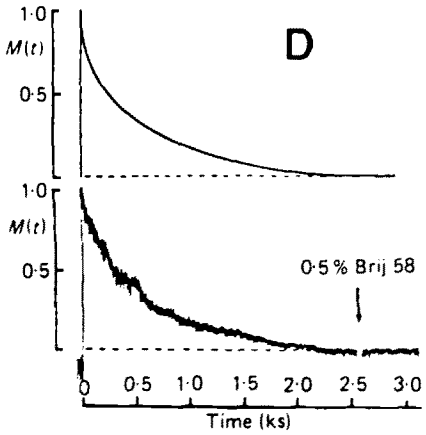


Fig. 26 (*cont.*). The *solid line* represents the force in *trace 2* (scale reduced by half). $P/P_\infty = 1 + (\gamma_2/\gamma_1 - \gamma_2)\exp(-\gamma_1 t) + (\gamma_1/\gamma_2 - \gamma_1)\exp(-\gamma_2 t)$, where P/P_∞ is the ratio between the instaneous value of the tension and its steady state value; the parameters $\gamma_1 = 0.38\ s^{-1}$ and $\gamma_2 = 0.48\ s^{-1}$ depend upon the rate constants and free $[Ca^{2+}]$ in the 'consecutive scheme' (Ashley and Moisescu 1972a Ashley and Moisescu, 1977). Composition of solutions: high total EGTA relaxing (free $[Ca^{2+}] < 10^{-9}$ M), 20 mM EGTA; low total EGTA relaxing (free $Ca^{2+} \sim 10^{-9}$ M), 0.1 mM EGTA, 20 mM K_2SO_4; activating (free $Ca^{2+} = 8.7 \times 10^{-8}$ M), total EGTA, 20 mM. All solutions contained in addition: 40 mM KCl, 10 mM TES, 0.1 mM Mg^{2+}, 4 mM ATP (total), A23187 2.5 μg ml^{-1}, buffered with KOH to 7.1 ± 0.01. Ionic strength 0.13 M, temp., 20°C. Calibration: *vertical*, 100 nA (1), 0.15 mN (2); *horizontal*, 2 s. (From Ashley, Moisescu and Rose 1974b; Moisescu 1975). **C** Fura-2 and $[^{14}C]$ glycine: Efflux of fura-2 (i) and $[^{14}C]$glycine (ii) from a barnacle myofibrillar bundle with time. The *solid lines* are fitted to the data points; where the diffusing substance (M) entering or leaving a cylinder of radius a (in this case the radius of the myofibrillar bundle) in time t. $M/M_\infty = 1 - \Sigma_{n=1}^{\infty}[(4/a^2\alpha_n^2)\exp(-D\alpha_n^2 t)]$ where M_∞ is the amount after infinite time and the α_n are the positive roots of the Bessel function of order zero F_0 ($a\ \alpha_n$) = 0. No significant difference was found between the fit using this equation and that obtained by use of the solution of the diffusion equation obtained by use of the Laplace transform. Tem., 20°C. (From Timmerman and Ashley 1986). **D** Barnacle TnC_{2DANZ} diffusion: *Top*, fitted curve of the data shown in *lower panel*. M(t), amount of solute remaining in the fibre after time t. *Bottom*, fluorescence of a myofibrillar bundle from a fibre injected with $BTnC_{2DANZ}$ 24 h previously. M(t), the normalised fluorescence of the fibre at time t (excitation 325 nm, emission 520 nm, temp., 11°C, r = 250 μm). $[BTnC_{2DANZ}] = 80\ \mu M$. (From Ashley et al. 1988b)

The experimental light and tension records obtained are illustrated in Fig. 26B for a barnacle myofibrillar bundle, loaded with aequorin (see legend) and activated by the 'pCa clamp' protocol. The aequorin light response (trace 1), and hence the free calcium, equilibrates rapidly rising to a relatively steady value within 1–2 s, as predicted by the previous calculations (Fig. 25B). Force, however, does not reach a steady value at this free $[Ca^{2+}]$ until some 10 sec later, suggesting that the free $[Ca^{2+}]$ equilibrates in the bundle more rapidly than subsequent reactions that lead to

force production. This work, using the pCa clamp method, adds support to the ideas on Ca^{2+} activation derived from single barnacle muscle fibres (see Sect. 1.2). A similar phenomenon was observed with frog myofibrillar bundles (Ashley and Moisescu 1975; Moisescu 1976) and has subsequently been confirmed using caged Ca^{2+} (Sect. 8.3).

This work on isolated myofbrils did not clarify the nature, or exact location, of the rate-limiting steps that lead to force production in skeletal muscle. In addition, rate limiting steps in the pre-steady state development of isometric tetanic force may not be rate limiting once a steady state has been achieved and is being maintained.

More recently the use of photolabile compounds which either release or remove Ca^{2+} rapidly have provided further information on the pre-steady state reactions.

8.3 Caged Ca^{2+} Method

One method of circumventing the problem of the diffusion delay in skinned fibres is to allow the compounds to diffuse into the fibres in an inactive or relatively inactive form and then to convert them to an active form *in situ*. Such a technique became possible with the introduction of photolabile "caged" compounds (Engels and Schlaeger 1977; Kaplan et al. 1978). The first photolabile compound to be used extensively in muscle research was caged ATP introduced by Kaplan (Kaplan 1990) and this has been used extensively to probe the kinetics of the force and the actomyosin ATPase cycle (Goldman et al. 1982).

The experiments with caged ATP have demonstrated the elegant way in which the technique of flash photolysis of caged compounds can be applied to study the reaction rates of processes within skinned muscle fibres. The application of similar techniques to the study of the activation of muscle by Ca^{2+} had to await the development of a suitable compound. This required the synthesis of a calcium chelator whose affinity for calcium would fall upon photolysis. Several such photosensitive chelators have now been synthesised (Adams et al. 1986, 1988; Kaplan and Ellis-Davies 1988; Ellis-Davies and Kaplan 1988); the nitr series is based upon the parent Ca^{2+} chelator BAPTA (Tsien 1980).

Tsien and co-workers (Adams et al. 1986, 1988) considered the properties that would be required by an ideal caged calcium molecule for physiological investigations. A caged Ca^{2+} should have the following properties: (1) bind Ca^{2+} prior to photolysis with a K_d of 0.1–0.3 μM under physiological conditions (pH 7.0, ionic strength ca. 0.1 M), as this is the concentration of Ca^{2+} in resting cells (Ashley and Campbell 1979), otherwise

the chelator would not have a significant amount of Ca^{2+} bound to it before photolysis; (2) have a K_d for Mg^{2+} greater than 1 mM, so that there would not be a significant amount of Mg^{2+} bound to the compound prior to photolysis and therefore importantly no great change in the free Mg^{2+} concentration following photolysis; (3) after photolysis the chelator should have a K_d for Ca^{2+} greater than 1 μM; (4) the release of Ca^{2+} following photolysis should be fast (< 1 ms), to be useful in examining the kinetics of Ca^{2+}-activated processes in cells (for example the k_{on} Ca^{2+} for STnC,

Fig. 27. **A** Direct activation by nitr-5 photolysis of a barnacle myofibrillar bundle (diam. 88 μm), which was first exposed to Triton X-100 (1%) for 20 min to remove the sarcoplasmic reticulum (SR). For each of the three superimposed records the bundle was first equilibrated in a solution containing 2.0 mM nitr-5 plus Ca^{2+} (initial pCa 6.4) and then briefly lifted into the air for the laser pulse (50 ns duration). Temp., 12°C. **B** Conventional activation (*not 'pCa clamp'*) of the same bundle as in *A* by activating solutions (5 mM $EGTA_{total}$) at pCa values 6.2, 6.0 and 5.8. For each contraction the bundle was first equilibrated in 'low total EGTA relaxing' (LR) (5 mM EGTA) solution; 'high relaxing' (high total EGTA) (HR) solution was used to relax the bundle afterwards. Temp., 12°C. **C** The time course of the rise in free $[Ca^{2+}]$ following partial photolysis of Ca^{2+}-loaded caged calcium (nitr-5): (i), 0.1 mM nitr-5 (initial pCa 6.8); and (ii), 2.0 mM nitr-5 (initial pCa 6.5), as predicted by computer simulation. At t = 0 ms the effect of the laser pulse was simulated to produce 60% photolysis of Ca^{2+} -nitr-5 and Mg^{2+} -nitr-5 and 30% of free nitr-5. Myofibrillar binding sites were either ignored or included at 140 μM (STnC site I and II; T sites) with a K_d of 1.3 μM. The nitr-5 was assumed to be homogeneously distributed in the myofibrillar bundle. The effects of the SR on the free Ca^2-are ignored. The time course of the laser-induced rise in free $[Ca^{2+}]$ within a myofibrillar bundle, equilibrated with a nitr-5 solution, was computed using a NAG (Numerical Algorithm YM Group, Oxford) library routine. Using the Gear method, this solved a set of differential equations for the rate of concentration change of each ligand species involved in the generation of the Ca^{2+} jump at 20°C. The binding of metal ion M to ligand L was described by Eqs 1,2:

$$M + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} ML \qquad \ldots(1)$$

$$d[ML]/dt = k_{on}[M][L] - k_{off}[ML] \qquad \ldots(2)$$

The dissociation constants (K_d) and forward and back rate constants for both divalent ion species (k_{on}, k_{off} respectively) from both the unphotolysed and the photolysed nitr-5 species and also to and from skeletal troponin C (STnC) T sites (calcium-specific) are from Lea and Ashley (1990). The model did not take into account Ca^{2+} binding to the Ca^{2+}-Mg^{2+} sites of TnC ("P" sites), as this process is considered much slower than for the Ca^{2+}-specific sites (Robertson et al. 1981; Gillis et al. 1982), nor did it include Ca^{2+} uptake and release across the SR membrane.

The photolysis reaction was assumed to be instantaneous at t = 0, although an intermediate is first formed which then changes with a time constant of 2500 s^{-1} to the nitrosobenzophenone (photolysed nitr-5; Adams et al. 1988). For a given laser pulse energy, the percentage photolysis of Ca^{2+} -nitr-5 (and Mg^{2+} -nitr-5) was taken as twice as that of the free nitr-5; this relation has been found using high-pressure liquid chromatography separation of the photolysed and unphotolysed nitr-5 (Barsotti and Ferenczi, unpublished). (From Lea and Ashley 1990; Ashley et al. 1991a)

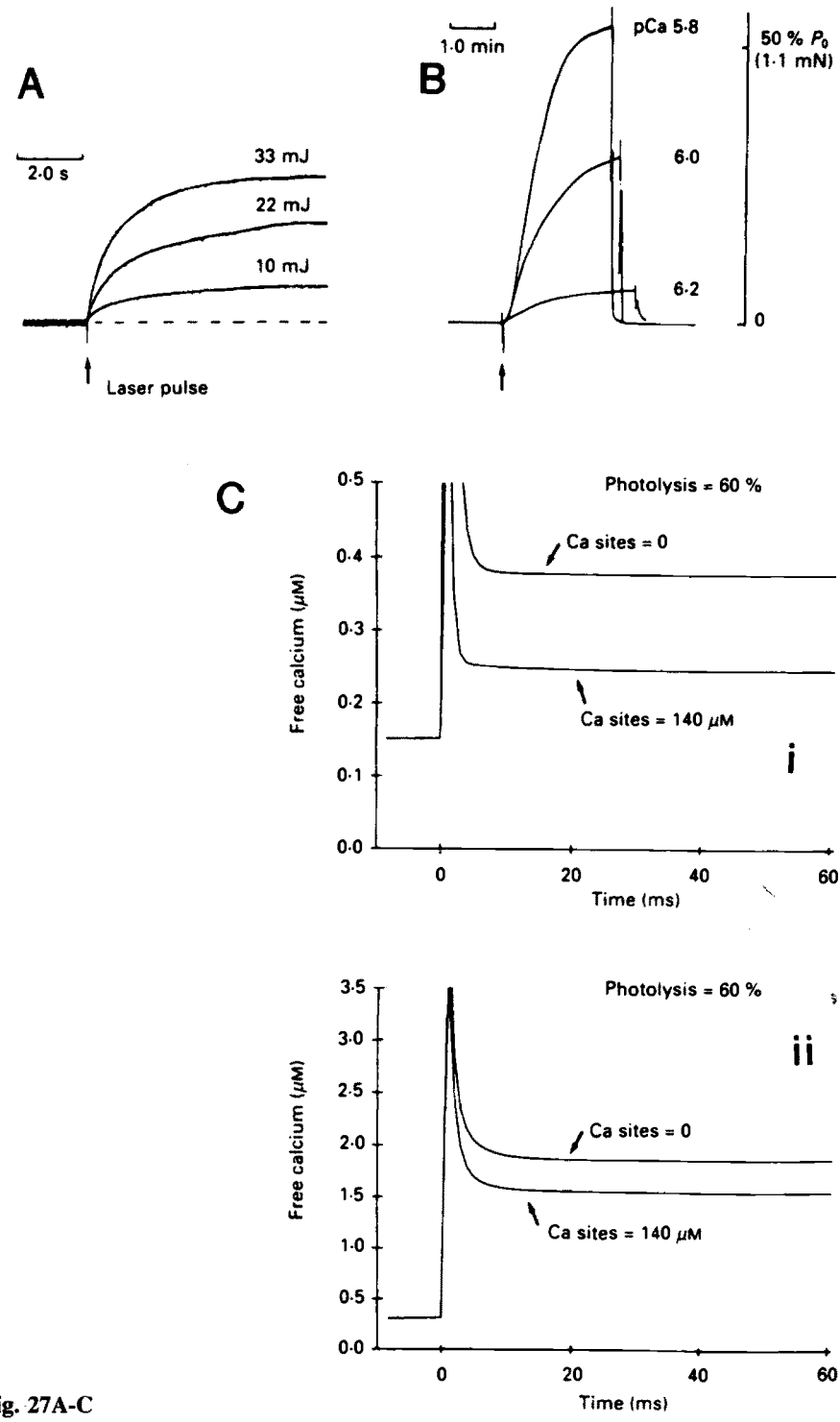

A
2·0 s
33 mJ
22 mJ
10 mJ
Laser pulse
B
1·0 min
pCa 5·8
6·0
6·2
50 % P_0
(1·1 mN)
0
C
Photolysis = 60 %
Ca sites = 0
Ca sites = 140 μM
i
Free calcium (μM)
0·5
0·4
0·3
0·2
0·1
0·0
0
20
40
60
Time (ms)
Photolysis = 60 %
ii
Ca sites = 0
Ca sites = 140 μM
Free calcium (μM)
3·5
3·0
2·5
2·0
1·5
1·0
0·5
0·0
0
20
40
60
Time (ms)

Fig. 27A-C

ca. 10^8 M^{-1} s^{-1} and at 1 μM Ca^{2+} have a $t_{1/2}$ ca. 3.5 ms/site); (5) have non-toxic photolysis products; and (6) be relatively pH insensitive over the physiological range.

The compound nitr-5 now meets most physiological criteria and has been successfully used in both neurophysiological studies (Gurney et al. 1987; Tsien and Zucker 1986) as well as in studies of muscle kinetics (Ashley et al. 1987a,b, 1988a, 1989b; Lea et al. 1990). Its K_d prior to photolysis is 270 nM (12°C; ionic strength (I) of 0.2 M) which changes to 12 μM after photolysis. The quantum efficiency has been measured (Adams et al. 1988) as 0.012 for calcium-free nitr-5 and 0.035 for calcium-saturated nitr-5 ($\epsilon = 5500$ M^{-1} cm^{-1} at 355–365 nm prior to photolysis).

The delay imposed by diffusion in earlier pCa-clamp experiments can be overcome by rapidly (< 1 ms) increasing the free Ca^{2+} concentration within the myofibrillar space of a skinned muscle fibre using flash photolysis of nitr-5. The photolysis of nitr-5 can produce increases in free [Ca^{2+}] (Ca^{2+} jumps) of various sizes within barnacle (and frog) myofibrillar bundles and considerably faster than can be produced by simple diffusion of Ca^{2+} and Ca-EGTA^{2-} from bathing solutions even using the 'pCa clamp' protocol (Sect. 8.2) (Fig. 27A c.f. 27B).

For example, the photorelease of Ca^{2+} from 0.1 mM nitr-5 (50% Ca loaded) triggered CICR from the intact barnacle SR from a mechanical skinned fibre and resulted in a phasic contraction, which had an amplitude of up to 100% maximum tension and a rise half-time as short as 0.65 s, although the mean value for contractions over 40% P_o was 2.3 s at 12°C (Fig. 21B,C). Treatment of the bundles with either ryanodine or with Triton X-100, to inactivate SR Ca^{2+} release and uptake, abolished most of this phasic response, leaving only a small residual component which had a faster rate of rise than the major CICR component and which is attributed to direct activation of the myofibrils by the Ca^{2+} released from nitr-5 photolysis. The use of caffeine to deplete the SR Ca^{2+} store was also found to abolish this phasic component in a similar way to ryanodine (Ashley et al. 1989a).

However much shorter rise half-times (mean 164 ms for tension greater than 40% P_o) were observed by photolysis of 2.0 mM (50% Ca^{2+} loaded) nitr-5 (pCa 6.4) which gives bigger free Ca^{2+} changes than for 0.1 mM nitr-5. Figs. 21C,D demonstrate clearly that myofibrillar bundles can contract just as fast as intact fibres (Griffiths et al. 1990) when the Ca^{2+} jump is sufficient and is not diffusion limited. These activation rates are 1–2 orders of magnitude faster than were obtained at the same temperature with the conventional 'pCa clamp' method where a pCa 4.3 solution with 25 mM $EGTA_{total}$, produced a maximal contraction with a half rise time of only 1–2 s (Fig. 25C).

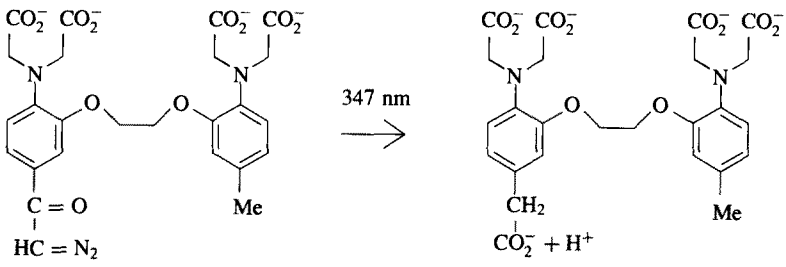
Finally, it can be seen from Fig. 27A that the half-times of contraction for the direct myofibrillar activation (2.0 mM nitr-5) are significantly shorter than those of the CICR-induced ones (0.1 mM nitr) over the laser energy range, varying from 2.4 s at 22 mJ to 92 ms at 70 mJ. These values are replotted, on an expanded scale in Fig. 21C, versus the amplitude of the contraction. The longer times clearly occur with the smaller contractions, while above 50% P_o the half-times are fairly constant at around 200 ms. Significantly, the majority of half-times of contractions using 0.1 mM nitr-5 with bundles treated to remove CICR by either ryanodine (Fig. 18C) or Triton X-100 treatment, lie within the values seen for direct activation responses in Figs. 21B,D and 27A. This is consistent with this residual response remaining after SR inactivation, being due to the direct activation of the myofibrils by Ca^{2+} released from nitr-5.

8.4 Caged Chelators: Diazo-2

Diazo-2 (Adams et al. 1989) a photolabile derivative of BAPTA. Upon photolysis, diazo-2 is rapidly (>2000 s^{-1}) converted from a chelator with a low affinity for Ca^{2+} (K_d = 2.2 μM) to one with a high affinity for Ca^{2+} (K_d = 0.073 μM) (Fig. 28 Inset). The spectrum obtained when 20 μM diazo-2 either calcium-free (a) or calcium-loaded (b) was photolysed in a cuvette. After one flash of the frequency-doubled ruby laser, exposure of these two samples to a further series of 10 flashes reduced the absorbance of calcium-free diazo-2 at 370 nm to 4%. of its initial value and the absorbance of calcium-loaded diazo-2 at 330 nm to 15% of its initial value.

In single frog (chemically) skinned fibres (Fig. 28a) or mechanically skinned barnacle fibres (Fig. 28b) photolysis of the caged chelator can promote rates of relaxation which are at least as fast as those seen in vivo, whilst the fall in free calcium is likely to be much faster ($t_{1/2}$ < 1 ms) (Mulligan and Ashley 1989; Ashley et al. 1990; Mulligan et al. 1990; Palmer et al. 1991) than even the fast phase of the relaxation in vivo at 12°C ($t_{1/2}$ = 70–80 ms). The relaxation in both barnacle and frog can be clearly divided into an initial fast and later slow exponential phases (frog *ca.* 42 s^{-1}, 12 s^{-1} and barnacle *ca.* 5 s^{-1} and 1 s^{-1} at 12°C). In mysoin-regulated scallop striated muscle the rates achieved are far faster than intact muscle (Palmer et al. 1990; Fig. 28c).

It is now possible to use this method to study how rapidly the TnC-bound Ca^{2+} falls, using fluorescently labelled derivatives such as TnC_{DANZ} (Sect. 3) and to determine which steps limit relaxation 'in vivo' in both actin and myosin regulated species.



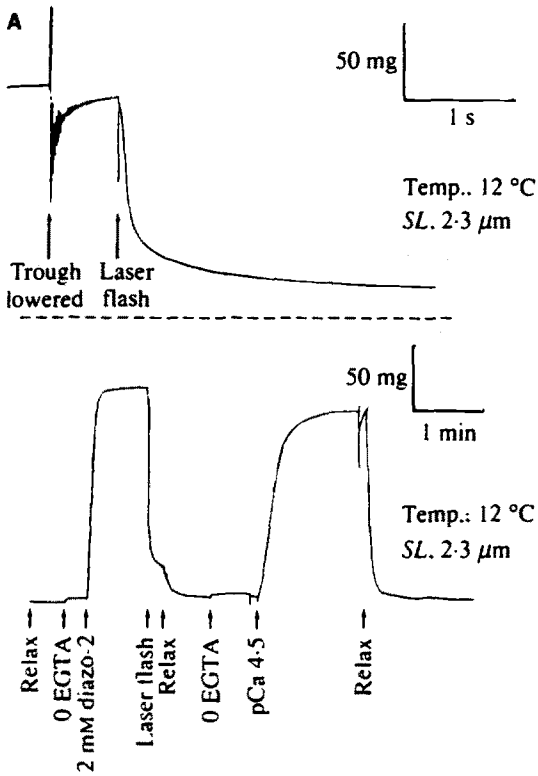
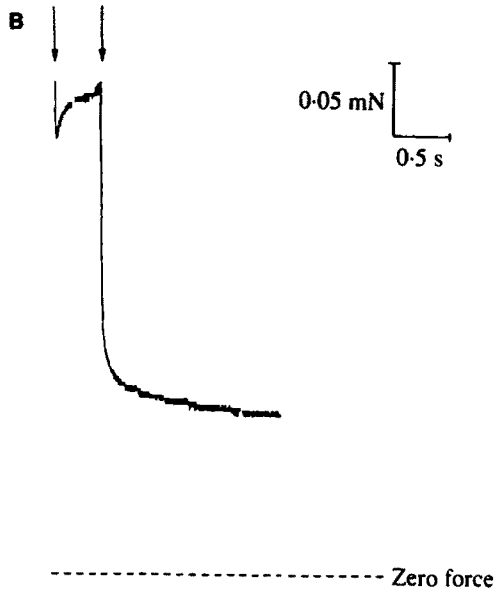


Fig. 28. Schematic diagram of diazo-2 reaction (above). (From Adams et al. 1989) and contractile responses (next page). **A** *The upper* panel shows the tension of a single frog skinned (1% Triton X-100) muscle fibre which has been equilibrated in 2 mM diazo-2 and then exposed to a brief flash (50 ns) of light from a frequency-doubled ruby laser (347 nm). The *lower* panel shows the same tension recording on a slower time scale allowing the development of tension in diazo-2 to be seen and compared with the tension developed in an Ca-EGTA^{2-} activating solution, pCa 4.5, d = 120 μm; temp., 12°C. (From Mulligan and Ashley 1989 with permission). **B** Force relaxation of bundle of skinned (1% Triton X-100) (myosin-regulated) scallop striated muscle bundle upon



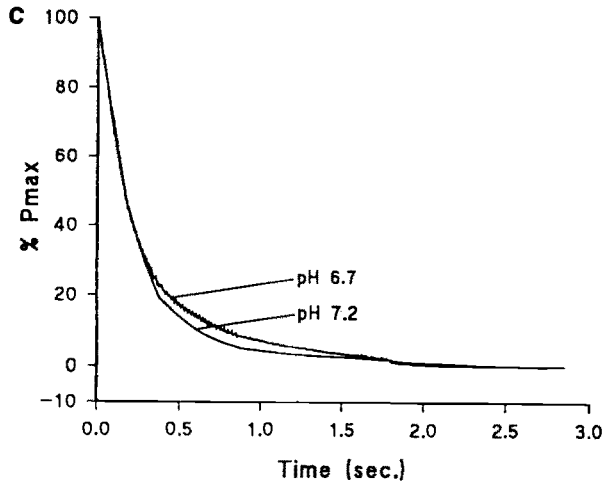


Fig. 28 (*cont.*). laser flash photolysis of diazo-2. d, 200 μm; length, 3 mm; P_{max}, 0.34 mN; temp., 11°C, $t_{1/2}$ relax, 14 ms; diazo-2, 2 mM; pCa-activating solution, >5.7 (not Ca^{2+} buffered); $[Mg^{2+}]_{free}$, 1 mM, after photolysis pCa >6.5. (From Palmer et al. 1990, and unpublished experiments) First arrow, removal from trough into air, second arrow, laser flash. C Force relaxation of bundle of mechanically skinned barnacle myfibrillar bundle upon flash photolysis of diazo 2. d, 100 μm, length 2.5 mm, t 12°C. [diazo 2] 2 mM, pre-activating solution pCa >5.7 (not Ca^{2+} buffered), $[Mg^{2+}]_{free}$ 1 mM. $t_{1/2}$ 170 ms at pH 7.2 (n = 30) and pH 6.7 (n = 17). (Palmer and Ashley, unpubl)

9 Mechanics of Barnacle Skinned Fibres

9.1 Rapid Mechanical Transients

Rapid mechanical transients have given, in frog muscle, detailed insight into the basic steps in force production by cross-bridges (Huxley and Simmons, 1973). Examination of the mechanics of barnacle myofibrillar bundles has revealed a similar series of steps in force production as in frog.

Myofibrillar bundles from barnacle, are activated by initial exposure to a Ca^{2+} solution of pCa 5.2 in which force developed slowly over a period of about 1 min. During the development of tension, the sarcomere pattern deteriorated due to an increased background intensity, however the first-order reflection was usually still discernible, although higher-order reflections were lost. The maximum tension developed was 108 ± 52 kN m^{-2} ($n = 18$) at 5°C (some 20% maximum of the tetanic force of intact fibres). When the activation reached a plateau, rapid length steps were imposed on the preparation. Rapid stretch or release of barnacle myofibrillar bundles produced tension transients (Fig. 29B) similar to those reported in single frog muscle fibres, that is, an initial rapid drop in tension, a rapid phase of tension recovery and subsequently a slow phase of tension recovery (Fig. 29E) (Huxley and Simmons 1973). From such experiments it was possible to construct a T_1 curve (expressing the initial rapid change in tension following a rapid mechanical step) of peak force at the end of a length change against the size of the length step. An example is shown in Fig. 29A,C. By extrapolation of the linear portion of the curve obtained for stretches to zero tension, one obtains y_0, the size of an instantaneous release necessary to just discharge tension, in the absence of inertia. This yields a value of $0.48 \pm 0.04\%$ l_0 (initial sarcomere length) from six bundles in the barnacle, while experiments performed on intact frog fibres gave a value of 1% l_0. A similar value of 1% l_0 was obtained by plotting the force-extension relation for a single step, i.e. plotting the increment in force against the increment in length throughout the length

Fig. 29 *(p. 218–220).* Mechanics of barnacle myofibrils: **A** Extent of a rapid a mechanical transient from full tetanic force (T_1 curve) from six myofibrillar bundles from *Balanus nubilus*. Sarcomere length 9 μm.; temp., 5°C. **B** Experimental force responses to an imposed slow length change (600 μs) applied to barnacle myofibrillar bundle under same conditions as A. **C** Force-extension curve resulting from type of record presented in B resulting from slower length change (600 μs). Temp., 5°C. **D** Dependence of reciprocal half-time of the quick recovery of tension following a *very rapid* (250 μs) step length change as a function of step size for two myofibrillar preparations. Curve fitted by eye. Temp., 5°C. **E** Quick recovery phase (from a very rapid 250 μs length step) observed in a barnacle myofibrillar bundle with a particularly linear T_1 curve. Temp., 5°C; sarcomere length 8.5 μm.

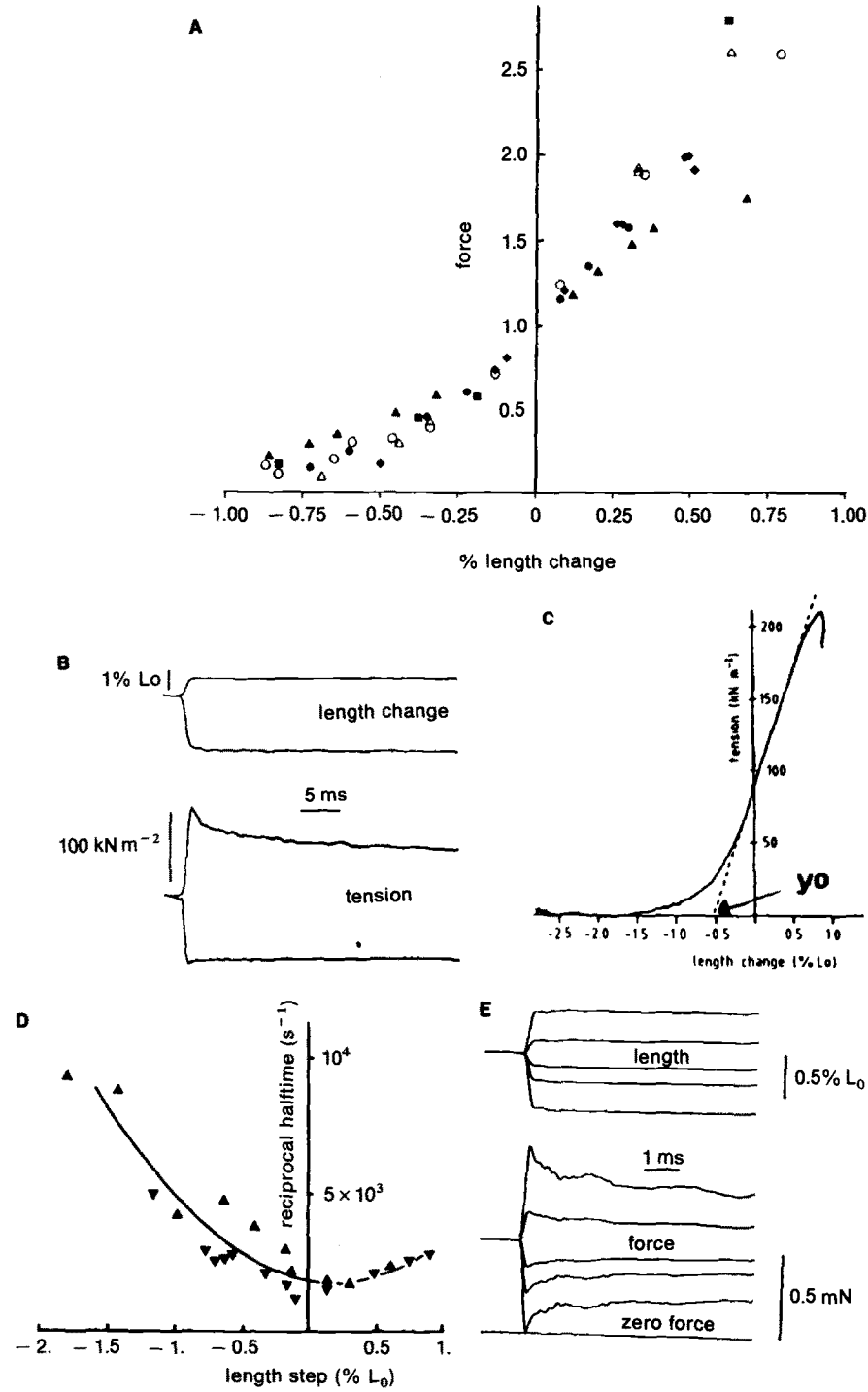

A
force
% length change
B
1% Lo
length change
5 ms
100 kN m⁻²
tension
C
tension (kN m⁻²)
yo
length change (% Lo)
D
reciprocal halftime (s⁻¹)
length step (% L0)
E
length
0.5% L0
1 ms
force
zero force
0.5 mN

Fig. 29A-D

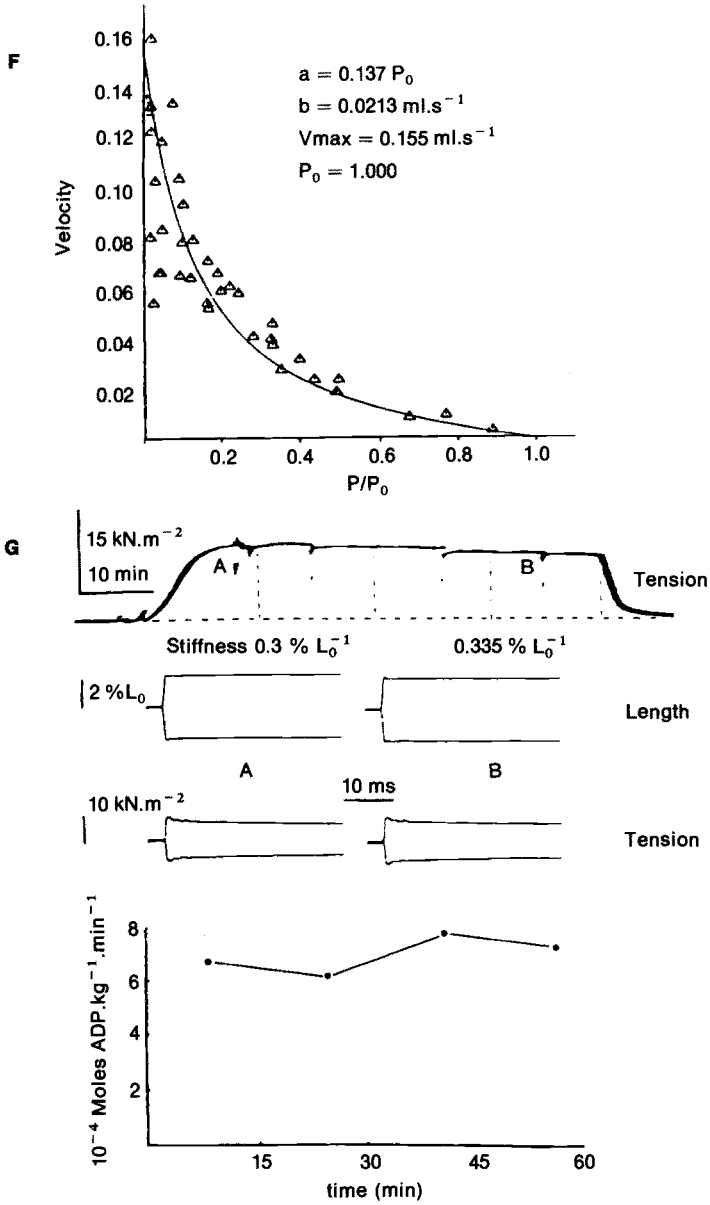


Fig. 29 (*cont.*). **F** Force-velocity curve obtained from seven barnacle myofibrillar bundles at 8°C. Tangential fitting procedure. Linear fitting for force yielded values of 0.172 P_o for a, 0.023 muscle lengths per second for b, 0.14 muscle lengths per second for V_{max}, and 1.041 for P_o. Sarcomere length 8 μm. **G** Time course of force responses and myofibrillar ATPase (ADP production) during a prolonged contraction. *Top panel*, force; middle panel: force responses to a rapid length change; *bottom panel*, ATPase activity. The initial departure from resting tension is followed by a rapid fall in force, then a slow recovery. This represents the point at which the activated fibre was allowed to shorten from a sarcomere length of 8 μm to 4.5 μm. Temp., 16°C. (From Griffiths et al. 1990)

change. This is shown in Fig. 29C. The same fibres as were used to obtain a $T_1 y_o$ value gave a mean y_o of 0.40 ± 0.15% l_o from their force-extension curves.

The quick recovery phase after release of a barnacle myofibrillar bundle occurred very rapidly indeed. In our initial experiments where length steps were completed in 600 μs, this phase appeared to be completely absent. This was due to its having reached virtual completion within the time course of the length change. Subsequent refinements to the stretcher permitted steps to be completed within 250 μs, which revealed a clear quick recovery phase that began to occur before the step was completed. The dependence of the half-time of the quick recovery on size of length step is shown in Fig. 29D. It is apparent that the point of intersection of the ordinate occurs at about 1 ms, which is similar to the values obtained from intact frog fibres (Ford et al. 1977; see Lombardi et al. 1992). For stretches, the quick recovery occurs more slowly than for releases, and its time course is therefore better resolved. When comparable sizes of stretch (i.e. stretches giving the same relative change in force) were used in frog and barnacle myofibrillar bundles, it was found that the two preparations had similar half-times for the quick recovery phase. In frog fibres, stretches of sufficient amplitude to yield T_1 tensions of 1.1–1.5 times isometric force had quick phase half-times of 270–770 μs, in barnacle fibres the same range produced half-times of 200–650 μs, both at 5°–7°C.

Some fibres were subjected to very large releases sufficient to completely discharge isometric tension and exceed the range of tension recovery by the mechanisms responsible for the quick phase. In this case, the recovery of tension occurs partially as a result of the loss of bridges under negative strain, partially through the cycling of bridges which have become attached at new actin sites, and partially through lattice constriction (Cecchi et al. 1990) and, corresponds to phase 4 of the scheme of Huxley and Simmons (1973). The half-time of this tension recovery was dependent upon the size of the release applied, being faster for smaller releases. The mean half-time for recovery from a release just reaching zero tension was 120–150 ms at 13°C (Fig. 33 D).

9.2 Force-Velocity Relations

The force-velocity curve was obtained by applying releases in the form of a ramp to a myofibrillar bundle following rapid maximal activation. During the ramp, fibre length shortened at a constant velocity, which should correspond to a constant tension according to the force-velocity relationship (A.V.Hill 1938). However, the appropriate tension is not reached instantaneously, so the ramp was preceded by an abrupt release sufficient to

discharge the tension to about the appropriate level for that velocity of shortening. The size of the initial step was adjusted until the force level attained a stable value for a period of 100 ms after the step. This was then taken as the appropriate force level. At high velocities, force tended to decline after this period even though the velocity remained constant. An example of a force-velocity curve obtained in this way is shown in Fig. 29F. This method was applied to seven myofibrillar bundles and the mean maximum shortening velocity (V_{max}) was 0.155 muscle lengths per second at 8°C, as predicted by curve fitting to the Hill force-velocity relation. This fitting procedure also permitted an estimate of the constants a, P_o and b, which yielded values of 0.137 and 2.13×10^{-2} muscle lengths per second respectively. The value of V_{max} was checked by use of the alternative "slack length method", in which myofibrillar bundles were subjected to a release large enough to discharge isometric tension completely. The point at which recovery of tension began was then determined and plotted against the size of the release. The gradient of the line so obtained yields V_{max}. From five fibres we obtained a mean value of 0.161 ± 0.092 muscle lengths per second at 8°C.

9.3 ATPase Activity

Mean isometric ATPase activity was measured from four myofibrillar bundles. Immediately after tension began to develop, the preparations were allowed to shorten to 4.5–5 μm sarcomere length while ATPase activity was determined with reduced nicotinamide adenine dinucleotide (NADH) assay (Griffiths et al. 1990). The ATPase activity was found to be 0.018 ± 0.006 mmol kg wet weight^{-1} s^{-1} (ten measurements) at 16°C. During a prolonged contraction, adenosine diphosphate (ADP) production could be measured several times without an intervening period of relaxation between samples. It was found that the ATPase activity was largely independent of the incubation time, and appeared not to decline significantly during the course of a contraction. This is shown in Fig. 29G, in which a bundle was incubated for 1 h, taking incubation solutions at 15-min intervals. Unfortunately ADP accumulation was too slow to permit measurements at shorter intervals to be performed. The time course of the quick recovery and the stiffness of the muscle did not change significantly during the 1-h contraction, which implies that fibres from the white depressor muscles do not enter a "catch"-like state during a prolonged contraction. However, there are reports of a state akin to 'catch' in the white adductor fibres when the external pH is altered and where relaxation is slowed (Hoyle and Smyth 1963; Iwamoto et al. 1990). It is not clear whether this phenomena is a direct effect on the myofibrils or via a slowing of Ca^{2+} accumulation by the SR Ca^{2+} ATPase (see Section 7.2).

Application of the method of sinusoidal analysis to a crustacean muscle preparation, the walking leg muscle from the crayfish, has been described by Kawai et al. (1977). The analysis of tension responses to sinusoidal length changes can be modelled in terms of three rate constants, 2 πa, 2 πb and 2 πc. The first two rate constants are too slow to be involved in the quick recovery of tension from a length step, and have been proposed to be associated with the phase 4 recovery stage of Huxley and Simmons (1973). The third rate constant is fast enough to contribute to the quick recovery. Kawai and colleagues (1977) obtained a value for this rate constant of 503 s^{-1} for intact crayfish fibres at 20°C. In that work, a comparable value of about 220 s^{-1} can be deduced for intact frog semitendinosus muscle at 8°C. Subsequent work making use of the same technique of data handling produced a value of 590 s^{-1} for rate constant 2 πc in chemically skinned rabbit psoas fibres (Kawai et al. 1987). In agreement with our findings it would appear that the cross-bridge events associated with the 2 πc rate constant occur at relatively similar rates in the crustacean and amphibian preparations that have been studied. The estimates of y_o from these two papers are somewhat different from those reported here. For crayfish a y_o of 1% l_o was found, while for rabbit a value of 1.2% l_o was reported. Since crayfish fibres are similar to barnacle muscle in having a relatively long sarcomere length (8–10-μm), it might be expected that if compliance were located primarily in the cross-bridges, then y_o would be inversely proportional to sarcomere length. However, the presence of such long sarcomeres causes the relative importance of any series compliance in the crustacean preparations to be increased, resulting in a relatively greater possible error in the y_o estimates. If the compliances associated with the cross-bridges are the same for both long and short sarcomere length preparations, and are extended by the same amount by a cross-bridge "power stroke", then it is apparent that the length-step change required to discharge the cross-bridge compliance is much smaller in the preparation containing longer sarcomeres (which, having a greater number of bridges acting in parallel, will also develop more tension). It follows that if the series elastic components at the ends of each preparation are equal, then for a given length step, a greater proportion of the stretch or release will occur at the series compliance in the preparation with long sarcomeres. Unless a sarcomere length clamp of some sort is used, this will lead to a greater error in estimates of y_o for the long sarcomere (i.e. crustacean) preparation. In addition, the sinusoidal analysis did not examine frequencies above 250 Hz. Therefore, additional components of the quick recovery phase, having characteristic frequencies higher than 150 Hz would not be detected, and would thus not contribute to y_o displacement of 15 nm per half sarcomere between actin and myosin filaments. This is in fact larger than the value obtained

from intact frog muscle (Ford et al. 1977), now believed to be 4 nm per half sarcomere. However, a number of groups have shown that y_0 is much longer in skinned fibre preparations from the frog (Griffiths et al. 1979; Goldman and Simmons 1984). y_0 only approaches the value of intact fibres when measured under conditions of sarcomere length control, where lattice shrinkage with high-molecular-weight polymers has taken place and where correction for truncation of the elastic response by the quick recovery has been performed (Goldman and Simmons 1986). When determined without adoption of any of these measures to reduce its value, the y_0 values in the literature are in agreement with that reported here for intact frog muscle, i.e. 1% l_0.

Estimates of the maximum tension developed by a crustacean fibre give values much larger than those found in vertebrate preparations. This may be explained in terms of the increased myofilament length causing greater numbers of cross-bridges to be acting in parallel in each half sarcomere, thus increasing the maximum tension that can be exerted. However, a complete explanation of this form must take into account the structure of the myosin filament, the arrangement of the filament lattice and the proportion of the cross-sectional area of a fibre devoted to the mechanical apparatus. Hoyle et al. (1973) described a wide range of sarcomere lengths in barnacle muscle (3.9–20.3 μm) with accompanying variations in filament length, particularly for the actin filaments. This might be expected to cause great disorder during isometric contractions, where long sarcomeres should shorten at the expense of shorter ones. But this is not observed when we activate intact fibres, where the first order laser diffraction band remains prominent and relatively constant in position. Although we detect a higher passive stiffness in relaxed barnacle muscle than frog, this is too small to account for the maintenance of sarcomere length. The large series-elastic component (14% of rest length) observed in intact barnacle fibres (Hoyle and Abbott 1967) when allowed to shorten was not observed in the skinned fibre preparation (Griffiths et al. 1990). It may be that this series-elasticity in the intact fibre preparation resides in the tendons, despite claims that they are "virtually inelastic" (Hoyle and Abbott 1967): if not, then its origin is uncertain. Since we detect only one population of sarcomere lengths in both myofibrillar experiments and intact fibres (Fig. 14A), it seems likely that the large majority of sarcomeres have a resting length of about 8 μm, while a small population deviate from this value with a very large range. Given the predominance of sarcomeres with a resting length of about 8 μm, our findings show qualitatively the result expected from the independent force generator model of muscular contraction (A.F. Huxley 1957), and the stiffness model of Huxley and Simmons (1973). That is, stiffness is located principally in the cross-bridge in an activated barnacle muscle.

9.4 ATPase Activity, Force-Velocity Curve and Mechanics

We estimate that the ATPase activity of activated barnacle muscle (myofibrils) is 0.018 mmol kg^{-1} s^{-1} at 16°C (Fig. 29G). This value is 10- to 20-fold higher than the ATPase activity we measure in relaxed myofibrils. The ATPase activity of single skinned frog muscle fibres has also been measured by a number of groups. Levy et al. (1976) reported a value of 0.115 μmol ADP mg $protein^{-1}$ min^{-1} at 4°C for frog semitendinosus fibres, which yields a value of 0.244 mmol kg^{-1} s^{-1} if a fibre diameter of 100 μm is assumed. Griffiths et al. (1982) reported a value of 0.1 mmol kg^{-1} s^{-1} for frog sartorius muscle using a skinning method and an ADP assay technique similar to that described here and at the same temperature as the earlier work. Since both Griffiths et al. (1982) and the present work used microscopic observation of the fibre to compute the fibre volume and swelling of the filament lattice is known to occur on mechanical skinning of frog muscle fibres (Matsubara and Elliott 1972), both ATPase values are probably underestimates of the true ATPase activity. The mean isometric tension for nine intact barnacle fibres was 395 kN m^{-2} at 12°C. For frog muscle at this temperature, a tension of about 250 kN m^{-2} would be expected. Although the barnacle develops more tension than the frog (Hoyle and Smyth 1963; Hoyle and Abbott 1967), the value is about 0.44 of that expected for a strict inverse proportionality between sarcomere length and isometric tension. This is unlikely to represent a different distribution of cross-bridge states during isometric contraction compared to frog muscle, since the ratio of fibre stiffness during maximal calcium activation to that in rigor is similar to the ratio observed in frog muscle (Stoneham and Griffiths, unpublished observations). It would seem plausible that the number of cycling bridges per unit fibre volume may be smaller in barnacle muscle than in frog, even under optimum conditions for tension development. Taking into account the effects of reduced cross-bridge concentration, fibre swelling and the short sarcomere length chosen for these experiments, our findings are in reasonable agreement with those of Hasselbach (1966), who reported ATPase activities of 0.53 and 0.1 μmol mg $protein^{-1}$ min^{-1} for frog and barnacle myofibrillar ATPase activity respectively at 25°C, i.e. a slower ATPase activity in barnacle than frog by at least a factor of 5. Similarly, the V_{max} of barnacle muscle lies in the range 0.155–0.161 muscle lengths s^{-1}. V_{max} has been estimated from a number of species and a good selection is quoted by Woledge et al. (1985). Taking the values of Cecchi et al. (1978) for V_{max} and its temperature dependence in intact frog fibres, one would expect a V_{max} at 7°C of about 3.0 muscle lengths per second. Since the sarcomere length of barnacle fibres used in these experiments was 3.6 times longer than that used in frog muscle experiments. Therefore, we should multiply by this value in order to compare V_{max} values in terms

of cross-bridge kinetics. This gives a barnacle V_{max} of about 0.19 of that in frog muscle. Although this ratio is somewhat higher than observed for the isometric ATPase activities of the two preparations, it still supports the view that cross-bridge kinetics are considerably slower in barnacle than in frog. The rise of isometric tetanus tension in intact barnacle fibres occurs

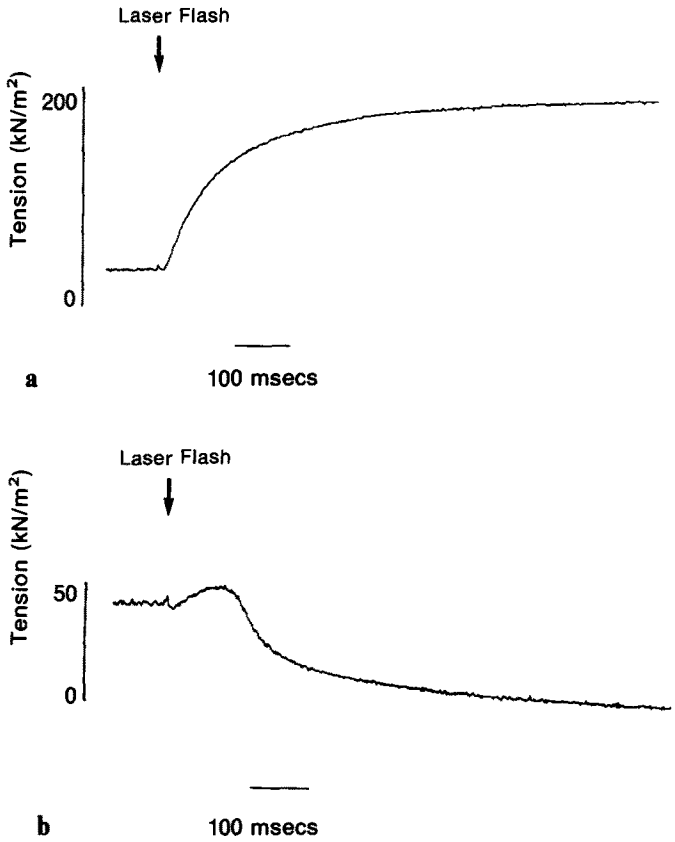


Fig. 30. **a** Tension transient in barnacle myofibrillar bundle following the photolysis of caged ATP (cATP) in the presence of calcium (pCa 4.5). Tension increased to at least 90% of the level resulting from activation in a solution containing calcium (pCa 3.8) and Mg-ATP (2 mM). The rise in tension was exponential with a rate constant of ca. 7 s^{-1}. Solution: cATP 4 mM, TES 60 mM, $EGTA_{total}$ 10 mM, Mg^{2+} 2 mM (free), Ca^{2+} 32 μM (free), glutathione 30 mM, K^+ 130 mM and propionate 50 mM; pH 7.0; ionic strength 150 mM. temp., 12.3°C. **b** Tension transient as in *A* following the photolysis of cATP, but in the absence of calcium (pCa < 8.0), tension first slightly decreased, then rose to a maximum and finally declined to the relaxed level. This result suggests that protein cooperativity (rigor bridges) allow the transient development of a force producing state. Solution: TES 60 mM, EGTA 10 mM, Mg^{2+} 2 mM (free), glutathione 30 mM, K^+ 130 mM and propionate 50 mM; pH 7.0; ionic strength 150 mM. Temp., 12.3°C. (Mulligan and Ashley, unpubl)

with a half-time of 177 ms, similar to the rate at which myofibrillar bundles are activated by caged Ca^{2+} (164 ms at 12°C; Lea and Ashley 1990) (Figs. 21B,C,D) or force re-develops after a shortening (Fig. 33D).

For a single tibialis anterior fibre from *Rana temporaria* at 15°C, the half-time of the tetanus rise is 36.9 ms (Griffiths and Taylor, unpublished observations), similar to that observed in frog Triton-skinned semitendinosus fibres at the same temperature activated with caged calcium (Ashley et al. 1987a, 1991a). Thus frog muscle is about 4–5-fold faster than barnacle muscle, whether it be the intact or chemically skinned fibre preparations. The recent report of caged ATP activation of mechanically skinned barnacle muscle (Mulligan et al. 1987) indicates a somewhat shorter half-time of rise of tension at 12°C from the rigor state in the prescence of Ca^{2+} 7 s^{-1} (i.e. $t_{1/2}$ 100 ms) compared to caged calcium activation from the relaxed state at 12°C $t_{1/2}$ 164 ms). (Sect. 9.5 and Fig. 30a), as with frog.

As can be seen in Fig. 13, the free calcium concentration increases relatively slowly in intact barnacle fibres compared with frog muscle and this phenomenon might by itself cause a slowing of the force rise in these crustacean muscle. The caged Ca^{2+} experiments (Fig. 21C,D) tend to refute this notion, however and overall it seems that the steps of the cross-bridge cycle prior to tension development, but post-Ca^{2+} binding as STn_{on} rate for Ca^{2+} is fast, are somewhat slower, by a factor of 5, than comparable steps in frog. The 'quick' phase (phase 2 of Huxley and Simmons 1973) of tension recovery from a step release is similar in the two preparations. Thus, if it is assumed that the time course of the quick phase is related to the kinetics of the tension-generating step of the cross-bridge cycle (Lombardi et al. 1992), then this result implies that the slow step responsible for the difference in ATPase activity and V_{max} is *not* the tension-generating step per se. These findings suggest that there is a step which is subsequent to tension generation, but occurring before detachment of the 'rigor complex' that has a rate constant of about 0.18 s^{-1}, and is rate limiting under isometric conditions.

9.5 Caged ATP Experiments

Rapid changes in ATP concentration can provide detailed information on the actomyosin ATPase cycle and when combined with the myofibrilar preparation gives insight into the relation between fibre mechanics and the ATPase reaction compared for example to the cuvette kinetics of the actomyosin ATPase (Goldman et al. 1982).

Skinned myofibrillar bundles from barnacle muscle were placed in rigor by removing ATP from the bathing solution and replacing it with caged ATP in the absence of Ca^{2+} (+EGTA). The complete removal of ATP from

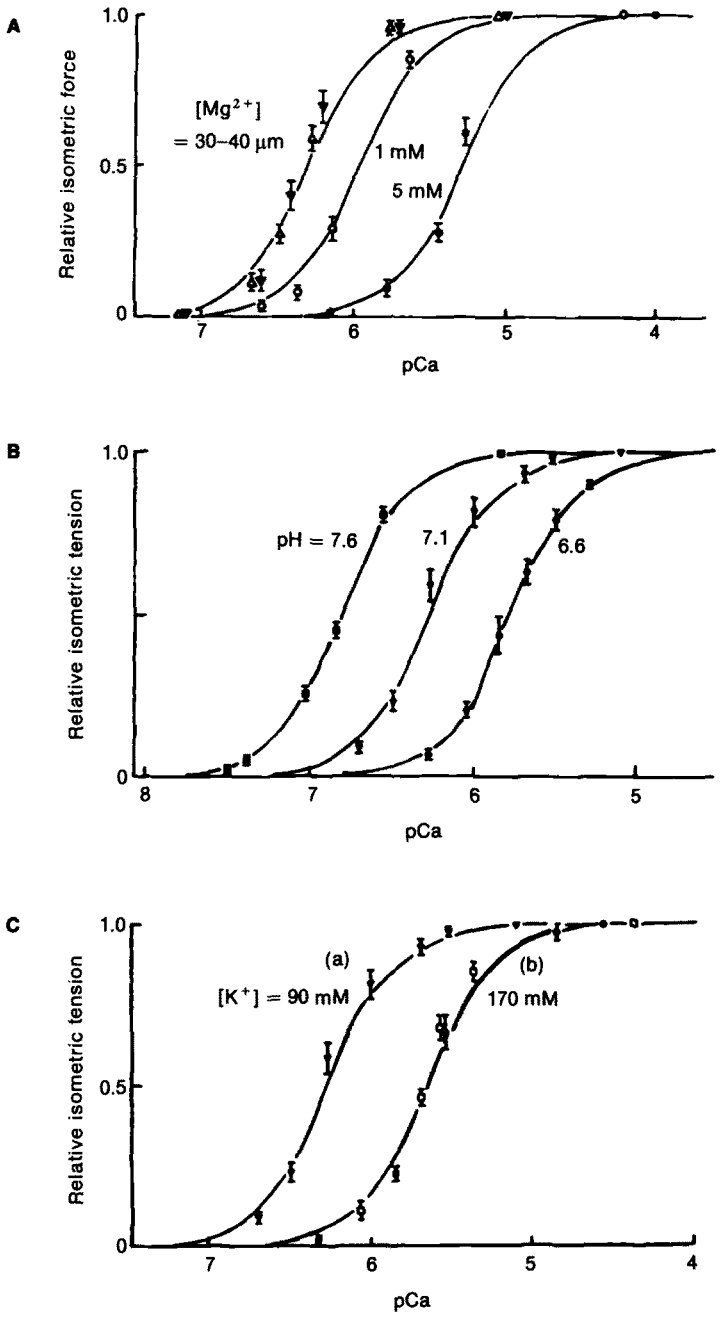


Fig. 31. A Relative isometric tension-pCa relationship in barnacle myofibrillar preparations. Free $[Mg^{2+}]$ 30 μM (△), 40 μM (▲), 1 mM (○) and 5 mM (●). Temp., 20 ± 1°C (△), (○), (●) and 4 ± 1°C (▼). The max. absolute tension is not significantly affected by the variation in $[Mg^{2+}]$. However, the drop in temperature from 20°C (△) to 4°C (▼) resulted in a diminished (less than 50%) absolute tension response. The *continuous*

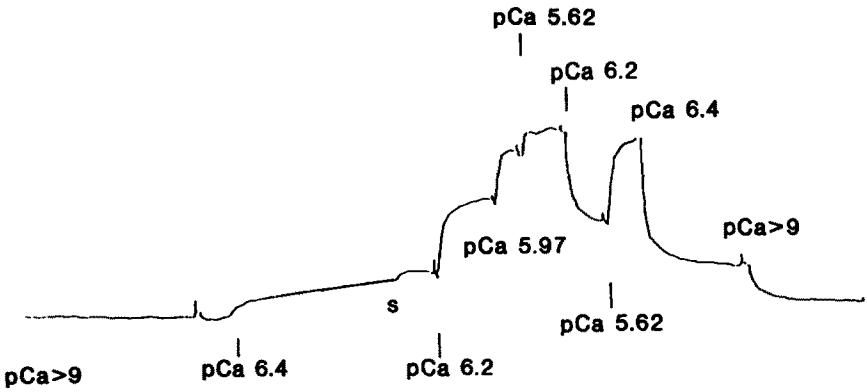


Fig. 31 (*cont.*). *lines* are theoretical predictions for $K_1^{app} < K_2^{app}$ and $K_1^{app} \times K_2^{app}$: 4.2×10^{12} M^{-2} (△, ▼); 8.6×10^{11} M^{-2} (○); and 4×10^{10} M^{-2} (●). 20 mM caffeine present throughout to reduce activity of sarcoplasmic reticulum (SR). Where K_1^{app} and K_2^{app} express the apparent Ca^{2+} binding affinity of the functional unit for tension, having two sites, M_1 and M_2 $Ca^{2+} + (M_1^* - M_2) \overset{K_1^{app}}{\rightleftharpoons} (CaM_1 - M_2^*) + Ca^{2+} \overset{K_2^{app}}{\rightleftharpoons} (CaM_1 - M_2Ca)\alpha$ Force, and the askerisk indicates a site able to bind calcium (From Ashley and Moisescu 1972a, 1977). **B** The influence of pH on the relative isometric tension-pCa relationship in barnacle myofibrillar preparations. The sets of activating solutions had the following pH values, respectively: 6.60 ± 0.01 (●), 7.10 ± 0.01 (▼), and 7.60 ± 0.01 (■). Temp., 20 ± 1°C. The continuous curves are theoretical predictions for $K_1^{app} < K_2^{app}$ and $K_1^{app} \times K_2^{app}$: 4.2×10^{13} M^{-2} (■); 4×10^{12} M^{-2} (▼); and 4.2×10^{11} M^{-2} (●). 20 mM caffeine present throughout. **C** The effect of increasing $[K^+]$ in the activating solutions from 90 mM (▼) to 170 mM (□, ●) on the relationship between relative isometric tension response and pCa in barnacle myofibrils. Temp., 20 ± 1°C. The max. force response for (▼) was about 30% larger than for (□), and ca. 40% higher than for (●). The ionic strength for (●) was *ca.* 25 mM higher than for (□). The continuous lines are theoretical predictions for $K_1^{app} < K_2^{app}$ and $K_1^{app} \times K_2^{app}$: 4.2×10^{12} M^{-2} (▼) and 2.1×10^{11} M^{-2} (□) and (●). 20 mM caffeine present throughout. (A-C from Ashley and Moisescu 1977) **D** Isometric tension responses from a bundle of barnacle myofibrils when cycled through a series of varying pCa solutions. Solution composition: ethylene glycol Bis (β-aminoethylether) N,N,N′,N′ tetra-acetic) (EGTA) 30 mM, (N-tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid) (TES) 60 mM, ATP 5 mM, $MgCl_2$ 1 mM, CP 10 mM, caffeine 20 mM, Cr Kinase 20 units ml^{-1}, pH 7.10, temp., 20°C. There is little hysteresis of force: compare when increasing the pCa (force decrease) with when pCa is decreased (force increase). S, stir. (From Ashley and Moisescu, unpubl)

myofibrils and the development of rigor tension requires an ATP-utilising system to be added e.g. hexokinase plus glucose (Goldman et al. 1982, 1984). ATP was released relatively rapidly (approximately 100 s^{-1}) following a brief pulse of light from a frequency doubled ruby laser (<200 ns pulse at 347 nm). After a short delay, partly due to the time taken for ATP to be released ($t_{1/2}$ *ca.* 7 ms), tension decreased with a rate constant of 10 s^{-1}. The amount of ATP liberated after each flash was varied by changing the energy of each laser flash. The record of tension showed an initial rise in

tension immediately after the laser flash provided the pre-photolysis tension was less than 50 mN m^{-2}, this rise was followed by a fall in tension to the relaxed level (Fig. 30b). If the ATP concentration was reduced, the initial peak in tension was delayed, the amplitude of the tension rise increased and the final relaxation was slower. The overall rate constant for the detachment phase, measured either from the peak of the tension transient or by a subtraction technique was approximately 2×10^5 M^{-1} s^{-1}. This is similar to the ATP-dependent rate for the dissociation of acto heavy meromyosin at an ionic strength of 0.1 M of 1×10^5 M^{-1} s^{-1} at 0.5 M ionic strength (White and Taylor 1976).

The *initial* rise in tension has no counterpart in solution cuvette studies, but can easily be demonstrated in skinned muscle fibres when a fibre in rigor is transferred to a solution containing ATP and no Ca^{2+}. One explanation for this observation could be that some of the cross-bridges bind ATP and, instead of entering the relaxed state, enter the force generating part of the cross-bridge cycle. This might be possible if, as has been suggested (Bremel and Weber 1972), the presence of some rigor (AM) cross-bridges activate the thin filament by a co-operative mechanism, although recent work in frog (Ferenzi, unpubl.) suggests that it is the AM.ADP.P. state which is responsible for maintaining the thin filament in an on-state since the initial rise is abolished by apyrase. Thus in Fig. 30b in the absence of Ca^{2+} (pCa $<$ 8.0), tension first decreased, then rose to a maximum and finally declined to the relaxed level, while in the presence of Ca^{2+} (pCa 4.5), tension increased to at least 90% of the level resulting from activation in a solution containing Ca^{2+} (pCa 3.8) and Mg-ATP (2 mM). The rise in tension was exponential with a rate constant of ca. 7 s^{-1} (Fig. 31A) at 12°C.

9.6 Steady-State Force: pCa Relations

By employing the rapid equilibrating solutions based upon the pCa clamp protocol (Ashley and Moisescu 1973a, 1975, 1977; Moisescu 1976), the effects of varying local anesthetics (Ashley and DeClerck 1981), free Mg^{2+} and pH_i upon the force-pCa relation can investigated.

9.6.1 Effect of $[Mg^{2+}]$

The influence of $[Mg^{2+}]$ upon the steady-state isometric tension-pCa relationship for barnacle myofibrils is illustrated in Fig. 31A. The free Mg^{2+} and Ca^{2+} concentrations were controlled in a manner such that the ionic strength and K^+ concentration were essentially constant for all three curves. The main effect of increasing $[Mg^{2+}]$ is to shift the curve relating isometric tension to pCa along the pCa axis towards higher $[Ca^{2+}]$. This effect seems

unlikely to be due to an increase in the concentration of MgATP since for 1 mM [Mg^{2+}] and 5 mM [Mg^{2+}] the MgATP concentration has increased only slightly from ca. 4.4 to ca. 4.8 mM, respectively, while the curves have been shifted by some 0.66 pCa units. In contrast to this, the curve for 1 mM [Mg^{2+}] was shifted by only 0.3 pCa u. in comparison with the curve for 34 μM, and here the MgATP concentration has changed from ca. 4.4 to ca. 1 mM, respectively. Other experiments performed in the absence of an ATP regenerative system, which is normally present, (Ashley and Moisescu 1974) indicated that an increase in [MgATP] from 1 to 5 mM did not affect significantly the relative tension-pCa relation for this preparation.

The effect of temperature on the relationship between relative isometric tension and [Ca^{2+}] has been investigated by cooling the set of standard solutions to 4 $\pm$1°C. After lowering the temperature, the pH was re-adjusted with HC1 to 7.10$\pm$0.01, so that no significant change in the ionic strength, [Mg^{2+}], or [MgATP] occurred. There was, however, a slightly higher [Ca^{2+}] than that at room temperature, since the apparent binding constant of Ca^{2+} to EGTA decreases by some 20%. The results from this series of tension responses performed at 4 $\pm$ 1°C overlap those obtained at 25°C. This result indicates that the apparent affinity of the tension sites for Ca^{2+} and thin filament cooperativity is not significantly affected by changes in temperature over this range. In contrast to the relative tension, the absolute tension response at 4°C was < 50% of that at 20°C.

9.6.2 Effect of pH

The effect of varying the pH between 6.6 and 7.6 on the relation between tension and pCa is illustrated in Fig. 31B. The results show that a change of pH has a marked effect on the curves; a change of 0.5 pH units. produces a shift in the curve by ca. 0.5 log units, the relation becoming less sensitive to Ca^{2+} as the [H^+] is increased. The absolute maximum values for tension were not, however, affected by changes in pH over this range. Results similar to these were described previously with this preparation under conditions where the ATP-regenerating system was not present in the bathing solutions (Ashley and Moisescu 1974).

9.6.3 Effect of Varying the [K^+]/Ionic Strength

It was observed that changes in the [K^+] in the activating solutions over the range of 90–170 mM, at an essentially constant [MgATP] and [Mg^{2+}], had a marked effect on the relative tension-pCa relation. An increase in [K^+] produced a shift in the relative isometric tension response towards higher [Ca^{2+}] (ca. 0.6 log units over the above-mentioned range of [K^+]). The replacement of the ATP-regenerating system with 10 mM ATP, at low

$[Mg^{2+}]$ and constant $[K^+]$, did not produce a significant change in the relation (Fig. 31C), although the ionic strength and osmolarity were appreciably changed. This latter observation indicates also that a change in the ionic strength of ca. 25 mM has no significant effect in itself on the relative tension-pCa relation in barnacle, and suggests that the shift observed is due mainly to a specific effect of K^+. As judged by the similarity of the two sets of results it appears that the presence of 15 mM ATP in the activating solutions was as efficient as was an ATP-regenerating system at maintaining the [Mg-ATP]. In addition, the presence of 20 mM caffeine in Fig. 31C strongly suggests that this agent does not effect either P_{max}, or the relative position of the force-pCa curve in barnacle myofibrils.

9.6.4 Hysteresis

In the course of these experiments, force cycles were produced by changing the pCa (Fig. 31D). We were not able to confirm the extreme hysteresis phenomenon of the type recorded by other workers (Ridgway et al. 1983) in this preparation. In these experiments, single fibres were mechanically divided and myofibrillar bundles removed and placed in relaxation solution to which 20 mM caffeine was included to reduce SR effects. This in itself has no effect either upon the relative pCa-force relation or upon maximum force (Fig. 31C). Perhaps the hysteresis reported by others is related either to the chemical skinning procedures (intact fibres skinned in 1% Triton X-100, Ridgway et al. 1983) or to gross changes in sarcomere length occurring during the repeated force cycling.

9.6.5 TnC Replacement ($BTnC_1$ and $BTnC_2$)

One way of assessing the underlying basis of the changes in apparent affinity of the myofibrils is to examine the nature of the calcium-sensor molecule located on the thin filament, TnC. Recently, the two isoforms of barnacle TnC ($BTnC_1$ and $BTnC_2$) have been isolated and sequenced (Potter et al. 1987; Francois et al. 1990; Collins et al. 1991), and the cDNAs can be expressed in E. coli. The evidence is that $BTnC_1$ and $BTnC_2$ are products from different genes. Both contain the four ancestral Ca^{2+}-binding regions (I to IV from the N-terminus). Functional Ca^{2+} binding sites are only present in sites II and IV as judged by the sequence data. Where site IV is predicted to be high affinity in both isoforms (0.2 μM $[K_{dCa}]$, while site II is lower affinity (K_{dCa} 2 μM $BTnC_2$ and 20 μM $BTnC_1$; Collins et al. 1991).

Both isoforms can restore the Ca^{2+} sensitivity and allow force production in native BTnC-depleted (EDTA-extracted) bundles of barnacle myofibrils (Fig. 32A,B); (Ashley et al. 1987d, 1991b). This and the evidence that there is no residual force in the absence of BTnC (EDTA extracted

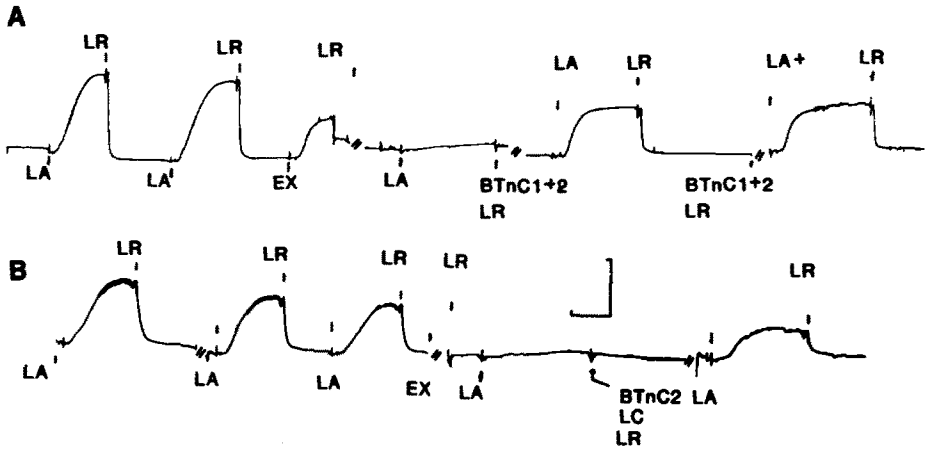


Fig. 32. A Isometric force records before, during extraction, and following the reincorporation of the barnacle troponin C isoforms ($BTnC_1$ and $BTnC_2$). Extraction of native TnC in low ionic strength, ethylenediaminetetra-acetate (EDTA) solution pH 7.6 (Ex) was for 30 min. Reincorporation of TnC in LR (low total EGTA relaxing solution) containing $BTnC_1$ and $BTnC_2$ (0.2mg ml^{-1}) for 30 min. Calibration: vertical, 100 kN m^{-2}; horizontal, 30 s; temp., 20°C, LA+ (low total EGTA activating solution) with $BTnC_1$ and $BTnC_2$ (0.2 mg ml^{-1}); LA, pCa 3.8, LR, pCa >7.0. Bundle diameter *ca.* 120 μm. **B** As for A but for a different barnacle mechanically skinned myofibrillar bundle, diam. *ca.* 100 μm. [$BTnC_2$] 0.2 mg ml^{-1}, LC, lobster light chains (1 mg ml^{-1}). Leupeptin (protease inhibitor) (1 mM) present in all solutions. There are 60 min between the first LA contraction and the second. (From Ashley et al. 1991b)

system) suggests these fibres are solely an actin regulated system (Ashley et al. 1991b; Dubyak 1986). Reported changes in the Ca^{2+} sensitivity of the fibres occurring with time from fibre isolation have been reported and were suggested to be due to myosin regulation (Stephenson and Williams 1980). The underlying reasons for these effects are unclear but are not evidently due to a myosin regulated system, as judged from the present biochemistry.

The $BTnC_2$ isoform (but not $BTnC_1$) can be fluorescently labelled with the agent danzylaziridine and this has the potential of providing information as to the state of Ca^{2+} binding to the thin filament of these fibres, when native $BTnC_2$ is replaced or exchanged, as with the rabbit fibres experiments exchanged with (rabbit skeletal) $STnC_{DANZ}$ (Zot et al. 1986; Guth and Potter 1987).

In the cuvette, $BTnC_{2DANZ}$ clearly shows a small shift when the free [Mg^{2+}] is increased from 0 to 5 mM. This is in the same direction as the shift in the force-pCa curve with a similar change in free [Mg^{2+}] and would support the view that at least part of the Mg^{2+} effect lies with a change in relative affinity of $BTnC_2$ for Ca^{2+}. Recently another label for BTnC calcium occupancy, namely $BTnC_{2\ acrylodan}$ has been described,

and has been used successfully in intact fibres and here the time course of the fluorescence change is very similar to that of force (Fig. 9c c.f. to Fig. 9a).

9.6.6 Cooperativity

The steady-state isometric tension results presented here all show a sigmoidal relationship with free [Ca^{2+}] when plotted semi-logarithmically (Fig. 31). The steepness suggested by the experimental results is significantly greater than can be predicted by the cooperative action of two Ca^{2+} in an 'independent' scheme (Ashley and Moisescu 1972a, 1977) and the curves are better fitted by the 'consecutive' alternative.

However, the steepness of the pCa-tension relation in various skinned muscle preparations has been reported as varying from a Hill coefficient (n_H; A.V. Hill 1910) of 2 to 6 (Shiner and Solaro 1984; Brandt et al. 1980). T.L. Hill (1985) suggests that n_H represents the extreme limit of positive cooperativity for an equilibrium binding model. That is a model in which the sites on each unit are either all occupied or all empty. He therefore concludes that n_H derived from the pCa-tension relation is only an *operational index* of cooperativity, rather than a direct measurement of the number of interacting binding sites. The n_H does represent, however, a lower limit to the number of sites which must interact in an equilibrium binding model to produce the degree of cooperativity observed.

In an equilibrium situation (no energy fluxes) n_H may not be greater than the number of interacting sites; there are only two low-affinity or regulatory sites for Ca^{2+} binding on each skeletal TnC molecule and hence it is difficult to understand at first sight how n_H can be greater than 2, unless other calcium-binding sites are involved in muscle regulation. This idea has been challenged by Shiner and Solaro (1984). They point out that a muscle fibre is not in the strict sense ever at thermodynamic equilibrium (except perhaps in rigor), but instead approaches a stationary non-equilibrium steady state with a constant energy flux due to ATP hydrolysis and cross-bridge cycling. Whitehead (1979) showed that the number of interacting sites is not an upper limit for n_H of a non-equilibrium process.

The second point made by Shiner and Solaro (1982, 1984) is that the functional unit may not be limited to one troponin, one tropomyosin and seven G actins, but may extend along the thin filament to include adjacent units and may even involve the whole thin actin filament (Brandt et al. 1987). Such a mechanism was postulated by Grabarek et al. (1983) and Grabarek and Gergely (1983) to explain the cooperative binding of Ca^{2+} to the low-affinity sites, when TnC was incorporated into actin filaments. Brandt et al. (1987) showed that the degree of cooperativity between Ca^{2+} binding and force could be reduced by the extraction of *only* a small

proportion of the troponin on the thin filament. They therefore proposed that all 26 troponin-tropomyosin complexes attached to one rabbit actin filament form a highly cooperative system which could be modelled with the concerted-transition formalism (Monod et al. 1965).

In order to explain this steep dependence of force development on free $[Ca^{2+}]$ T.L. Hill (1983) proposed a model with two underlying hypotheses: (1) Ca^{2+} binds more strongly to the regulatory sites on TnC if myosin is already attached to actin; (2) there are end-to-end interactions between tropomyosin molecules along the thin actin filament, so that a tropomyosin molecule is more likely to disinhibit this interaction.

9.6.7 Rapid Mechanics of Single Fibres: TnC Affinity Changes

The balance of evidence certainly strongly suggests that rigor cross-bridges enhance the affinity of Ca^{2+} binding to the regulatory site or sites on TnC even though there are a number of contradictory reports. More interesting is the possibility that actively cycling cross-bridges may have a similar effect. Guth and Potter (1987) observed an apparent ten-fold increase in the affinity of these sites when cross-bridges were cycling based upon $STnC_{DANZ}$ fluorescence changes. However, radioisotope studies of calcium binding (Pan and Solaro 1987 in cardiac muscle; Fuchs 1985 in rabbit skeletal; and Wnuk et al. 1984 in crayfish, have not shown any measurable increase in the Ca^{2+} affinity of these sites in the presence of cycling cross-bridges, unlike the rigor state where increases in ^{45}Ca binding are readily detected. More recently however, Hofman and Fuchs (1987) have reported a force-dependent component of Ca^{2+} binding to ox heart muscle. In contrast to the majority of these steady-state measurements, several groups (Housmans et al. 1983; Ridgway and Gordon 1984; Gordon and Ridgway 1976, 1978, 1987, 1989, 1990; Fig. 33A,C) have observed that a rapid shortening (length change) in both cardiac and barnacle muscle, a perturbation which is likely to decrease the number of attached cross-bridges, is associated with an increase in the free $[Ca^{2+}]$ from aequorin-injected fibres. The free $[Ca^{2+}]$ change occurs later than the length change. This response has been interpreted as, and is consistent with, a release of Ca^{2+} from the regulatory sites of TnC on the thin filament following a decrease in the Ca^{2+} affinity when the myosin heads (cross-bridges) detach (see below). Fuchs (1985) has pointed out that rapid shortening is a state where there is a rapid detachment of bridges and caution must be exercised in extending inferences from a transient state (shortening) to the steady force state. It is likely that shortening produces a redistribution of cross-bridges and it is quite possible that this redistribution causes a transient, reduced affinity of the thin filament regulatory sites for Ca^{2+}, which subsequently recovers when the steady isometric force

state is re-established. Certainly the ^{45}Ca isotope studies have not the time resolution to detect these transient changes in thin filament (TnC) affinity.

It is clear that a rapid mechanical perturbation does produce an increase in free Ca^{2+}, albeit delayed. In Fig. 33A when force is at the plateau value and presumably the net number of attached bridges is constant, the extra light (and Ca^{2+}) seen on rapid shortening declines. The extra Ca^{2+} cannot therefore be related *solely* to the numbers of bridges but must also in some way be related to the *free Ca^{2+} transient* which drives the Ca^{2+} binding event initially.

Calculation of the time course of the free Ca^{2+} during the rising phase of force emphasises the fact that the extra Ca^{2+} produced by the brief mechanical perturbation is delayed from the time course of the free Ca^{2+} event, but nevertheless still precedes the tension response (Fig. 33A). This finding could imply that the extra light is not related simply to the strongly attached high force state; for example in Fig. 33A, there is extra light at a moment when there is no detectable force. Perhaps strongly attached low force bridges, or even weakly attached bridges, exist at that moment (Ashley et al. 1987c, 1988c) and contributes, to the extra light phenomenon and is perhaps akin, in time course, to that of the (A2) first actin layer line (Fig. 14Bb).

If the *extra* light is dependent entirely upon affinity changes brought about by cross-bridge binding, and yet the process is itself fast, then the time course of the extra Ca^{2+} events should be close to that of force development (as seen, for example, with the $BTnC_2$ acrylodan fluorescence signal, Fig. 9C), when corrections are made for internal length changes. If the event is dependent upon cross-bridge binding and the resulting enhanced Ca^{2+} attachment is slow, then the time course of the extra Ca^{2+} may well be slower than the time course of force.

There also seems to be a non-equivalence of the extra light with force. The *extra* light at the beginning and end of the aequorin light trace are the same, but the forces are some 100% different. This might suggest that extra light is governed by cross-bridges when they attach (during force upstroke), but is unrelated during steady force. Certainly in force relaxation there is a switch to a strongly-attached, low-force state when the free Ca^{2+} declines, at least in frog muscle (Cecchi et al. 1991). Also it seems clear that the decline in the extra light accompanies the fall in the free Ca^{2+} transient, despite a maintained steady state high force level. This suggests that force maintenance and cross-bridge cycling occurs in the steady state without any major net change in thin filament Ca^{2+} affinity.

It is possible that the extra Ca^{2+} is derived not from the myofilaments but is in fact related to mechanical enhancement of the Ca^{2+} release from the SR, so that as long as Ca^{2+} is elevated, Ca^{2+} release (CICR) is operational

and stretch dependent: CICR is certainly present in barnacle fibres (Lea and Ashley 1990)? Finally, the fact that the extra light event seems correlated with force recovery and that the rate of force recovery (Fig. 33B) from a rapid release becomes slower as relaxation proceeds might also be the cause of the smaller extra light later in relaxation. Other experiments need to be performed where the velocity of force redevelopment is kept constant and the effect of this upon the size of the extra light component determined.

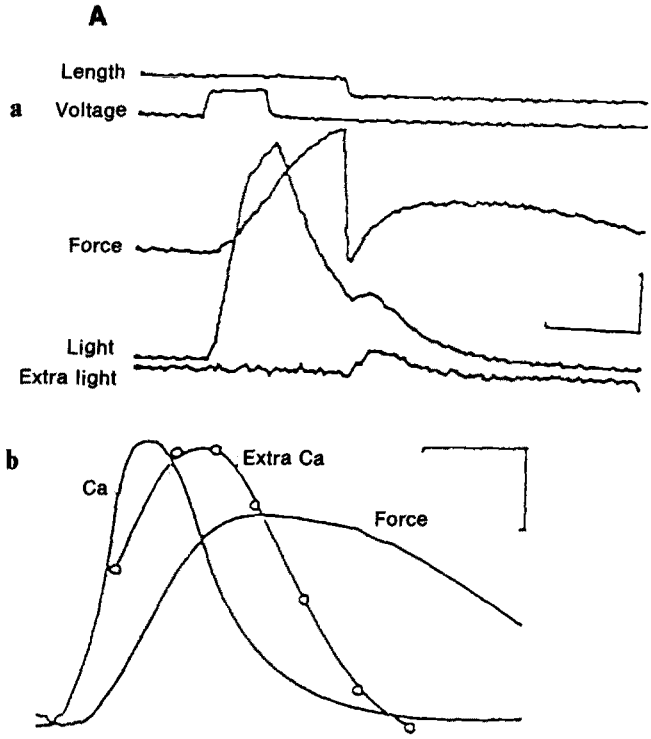


Fig. 33 (*p. 237-239*). **A** Effect of a shortening step applied to a cannulated barnacle fibre and made during the declining phase of the calcium transient shortening step. **a**. *Traces*: membrane VOLTAGE, 160 mV cal^{-1}; isometric FORCE 9 g cal^{-1}; fibre LENGTH, 8 mm cal^{-1}; aequorin LIGHT signal (calcium transient), 200 nA cal^{-1}; EXTRA LIGHT, 200 nA cal^{-1}. EXTRA LIGHT is computed by subtracting the control LIGHT signal when there is no length change from the LIGHT signal in response to the shortening step. Horizontal sweep, 400 ms cal^{-1}; temp., 8°C; fibre length, 27 mm; fibre weight, 80 mg; resting glow, 37 nA. **b**. Extra calcium seen after the shortening step made at different times during the calcium transient. CALCIUM transient, relative units; isometric FORCE, 4.5 grams cal^{-1}. EXTRA CALCIUM observed with the shortening step of 5% of the initial fibre length imposed at different times during the calcium transient plotted as occuring at the time of the shortening step. FORCE solid line and EXTRA calcium (0), calcium and extra calcium were calculated as in Ridgway and Gordon (1984). Horizontal sweep, 400 ms cal^{-1}; fibre weight, 38 mg; fibre length, 20 mm; temp., 9°C. (From Ridgway and Gordon 1984; Gordon et al. 1988).

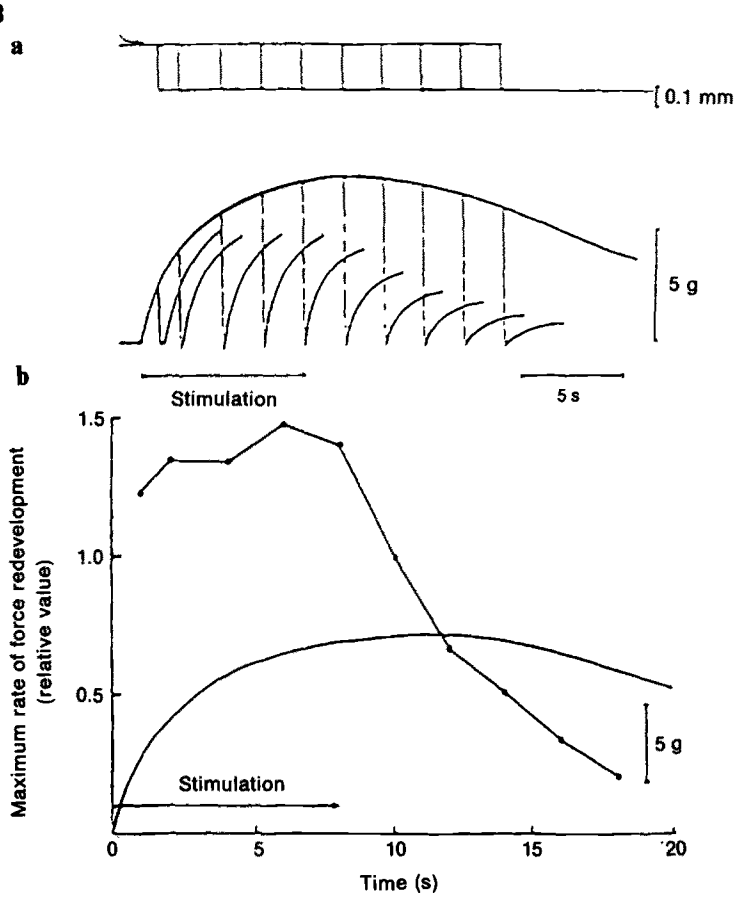


Fig. 33 (*cont.*). **B** Changes in the rate of force redevelopment during the mechanical response to 8 s of electrical stimulation applied to a shell-attached bundle of intact barnacle fibres from adductor muscle. **a** Superimposed length and force records when quick releases (about 2% of l_o) were applied at various times during the mechanical response to 8 s of electrical stimulation to reduce the force just to zero. After each release, the time course of force redevelopment for the first 2 s is shown. **b** Relations between the max. rate of force development and the time after the onset of stimulation, together with the time course of the mechanical response. The rates are expressed relative to the max. rate of isometric force development in response to stimulation; temp., 5°C. (From Iwamoto et al. 1990). **C** Force and aequorin light signals from shell-attached intact single barnacle muscle fibre which undergoes unloaded shortening at the peak of an electrically evoked contraction. Sarcomere length 8.2 μm, temp., 12°C. Two superimposed traces from the same fibre stimulated identically. (From Ashley and Griffiths, unpubl). **D** Isometric tension recovery in barnacle myofibrillar bundle following a large release (shortening) from P_o. The length step (3.5% l_o) (l_o, initial length of preparation) was sufficient to reduce the force to <10% P_o and break the majority of cross-bridges. Recovery of force ($t_{1/2}$ *ca.* 122 ms) represents mainly cross-bridge re-attachment. Barnacle myofibrillar bundle activated throughout in HA (high total EGTA activating solution) (pCa 3.8); other details in caption. Temp., 13°C. (From Ashley and Griffiths, unpubl)

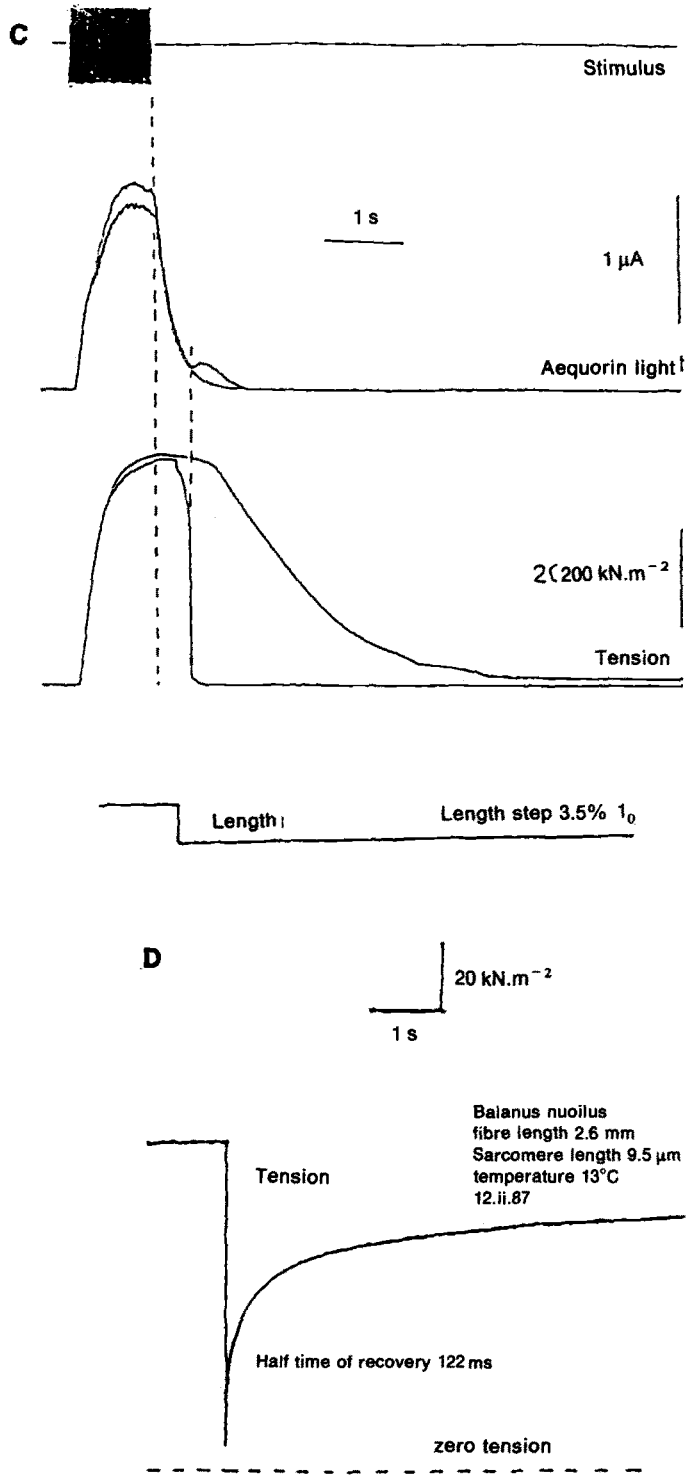

C
Stimulus
1 s
1 μA
Aequorin light
200 kN.m⁻²
Tension
Length
Length step 3.5% l0
D
20 kN.m⁻²
1 s
Balanus nuoilus
fibre length 2.6 mm
Sarcomere length 9.5 μm
temperature 13°C
12.ii.87
Tension
Half time of recovery 122 ms
zero tension

Fig. 33 (*cont.*).

9.6.8 Potentiation of Contraction

9.6.8.1 With Sulmazol (AR-L-115 BS). The cardiac ionotropic drug (AR-L-115 BS), or Verdax increases the sensitivity of cardiac myofibrils to Ca^{2+} (Hertzig et al. 1981; Solaro and Ruegg 1982). Skinned muscle fibres from the barnacle (Fig. 34Aa) were treated with 0.5% Brij 58 to disrupt the SR, and were then exposed to different free [Ca^{2+}] in the presence or absence of 10^{-3}M AR-L. Data was fitted to the Hill equation by a least-squares method. pCa values corresponding to 50% activation of force were 5.74 (zero AR-L) and 5.68 (10^{-3}M AR-L) in barnacle (n = 4), 5.89 and 5.82 respectively in frog (n = 6), at 5°C. Thus 10^{-3}M AR-L has very little direct effect upon the Ca^{2+} sensitivity of either barnacle or frog myofibrils, in contrast to heart muscle.

However, 10^{-3}M AR-L caused a marked yet reversible potentiation of force and aequorin light in barnacle muscle fibres (Fig. 34Ab) and a 1.23-fold increase in half-time of relaxation, but the relation between peak force and light was unchanged. These results suggest that the effect of AR-L in these preparations is primarily on the free Ca^{2+} transient, and not on the Ca^{2+} sensitivity of the contractile apparatus.

9.6.8.2 Deuterated Water (D_2O). It is known that D_2O can inhibit the contraction of skeletal muscle. As a rule, a pD (pD = $-\log_{10}$[D]) of 0.4 pH

Fig. 34 (*p. 240–242*). **A** *Effect of potentiating agents and D_2O* (**a**) Normalised tension: pCa relation in barnacle myofibrillar bundle (•), control; (○), 10^{-3} M AR-L (pre-equilibration 30 min). *Bars* show standard error (SE) of mean. Solutions contained: KCl 100 mM, EGTA 25 mM, TES 60 mM, MgATP 5 mM, free Mg^{2+} 1 mM, creatine phosphate 20 mM, creatine kinase 20 units ml^{-1}. pH 7.00; Ca^{2+} added to vary pCa; sarcomere length 8 μm (laser diffraction); temp., 4°C. (**b**) Single fibre and aequorin light responses in the presence and absence of 10^{-3} M AR-L, following a single voltage clamp pulse ΔV + 32 mV, voltage clamp holding potential, e_m, minus 40 mV; pulse 200 ms; temp., 7°C. Larger light and force after 10 min in 10^{-3} M AR-L saline. Light trace time constant 10 ms; resting light 40 nA. (From Ashley and Griffiths 1984). **B** The effects of D_2O on contraction: isometric tension responses of a barnacle myofibrillar bundle (d, 200 μm) to 2.5 mM caffeine. Bathing solutions contained: 150 mM K^+, 20 mM Na^+, 140 mM Cl^-, 1.0 mM free Mg^{2+}, 5 mM ATP, 0.3 mM EGTA, 60 mM TES, 900 mM sucrose, 10 mM creatine phosphate, 20 μml^{-1} creatine kinase. pH = 7.1; pH meter reading in D_2O, 7.1. Temp., 16°C. (Griffiths and Lea 1984). **C** Effect of applying a single electrical stimulation of 75 ms duration to a single barnacle muscle fibre injected with aequorin, before (**a**) and after (**b**) 20 min exposure to dantrolene Na (35 μM) in the external medium. *Trace 1*, membrane depolarisation; *trace 2*, Ca^{2+} transient; *trace 3*, isometric peak force. c-f, time course of the effect of dantrolene (35 μM) on several processes involved in stimulus contraction coupling of the same muscle fibre. *Abscissa*, time in min.; c, isometric peak force; d, Ca^{2+} transient (relative to the initial resting level of light output); e, membrane depolarisation in mV; f, time from peak to half-decay of the Ca^{2+} transient in ms. Fibre diameter 1.6 mm; mean intracellular resting potential −56 mV; temp 22°C. (From Desmedt and Hainaut 1977)

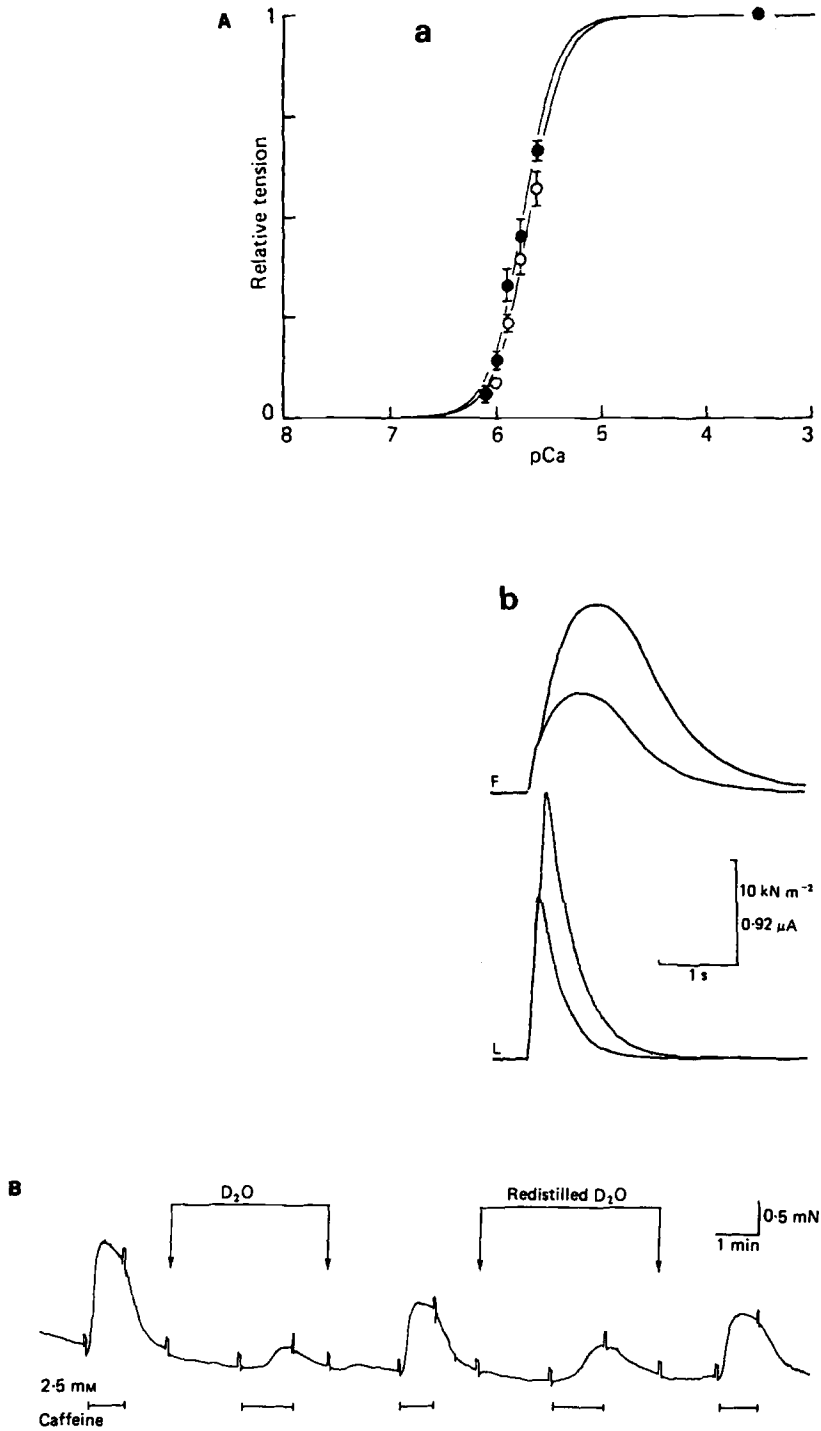
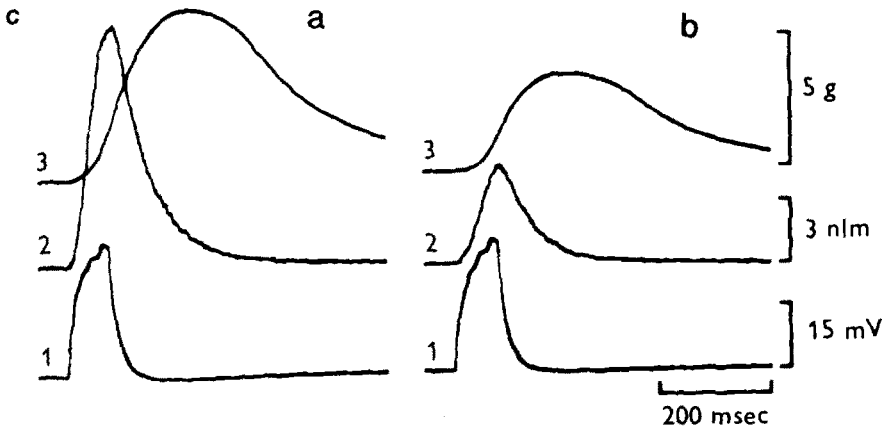

A
a
1
0
Relative tension
8
7
6
5
4
3
pCa
b
F
L
10 kN m⁻²
0·92 μA
1 s
B
D₂O
Redistilled D₂O
0·5 mN
1 min
2·5 mM
Caffeine

Fig. 34A,B


c
a
b
3
2
1
5 g
3 nlm
15 mV
200 msec

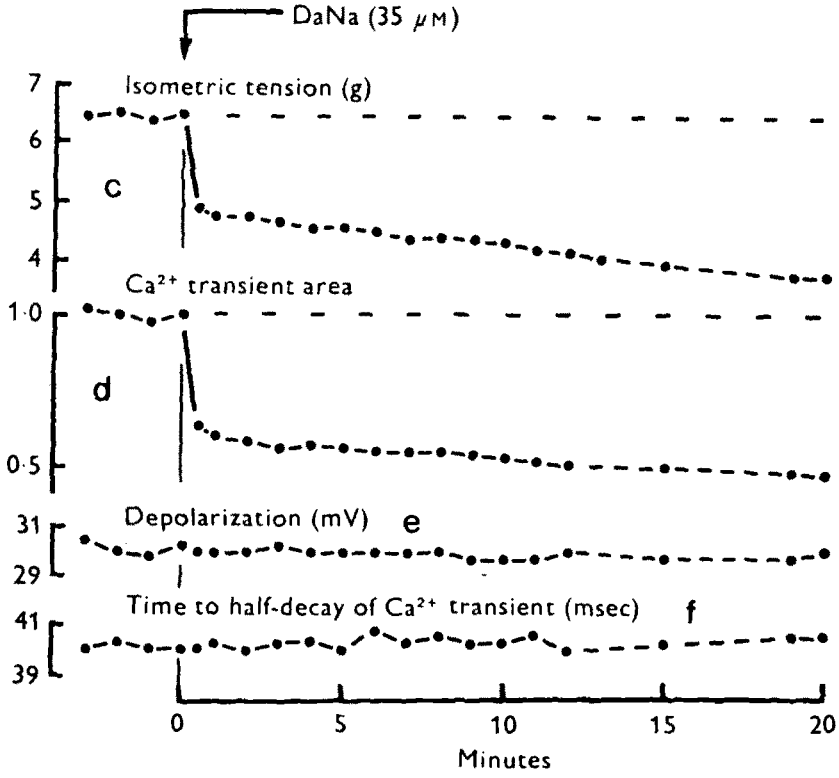

DaNa (35 μM)
Isometric tension (g)
7
6
5
4
c
Ca2+ transient area
1·0
0·5
d
Depolarization (mV)
31
29
e
Time to half-decay of Ca2+ transient (msec)
41
39
f
0
5
10
15
20
Minutes

Fig. 34C

meter units below the pH of H_2O saline has been used in physiological experiments. The free Ca^{2+} has been measured in D_2O and H_2O salines buffered with EGTA (20 mM) either by aequorin light or by Ca^{2+} electrode measurements. Both methods indicated that the free Ca^{2+} obtained from a given ratio of Ca^{2+} and EGTA was similar in D_2O and H_2O when adjusted to a pH meter reading of 7.0 and (free Ca^{2+} was about 0.15 pCa units higher in D_2O), while the application of the 0.4 units pD correction caused an increase in free Ca^{2+} of 0.8 pCa units. Myofibrillar bundles were exposed to 2.5 mM caffeine and 100% CO_2 (agents known to cause Ca^{2+} release from the SR) in H_2O and D_2O adjusted to the same pH meter reading. It was found that brief exposure to D_2O caused a reversible inhibition of force development by either of these (Fig. 34B). Redistillation of the stock D_2O did not prevent this effect. Intact barnacle muscle fibres showed a rapid and reversible loss of force and aequorin light responses to voltage clamp pulses in the presence of D_2O salines adjusted to the same pH meter reading as used for H_2O saline. Inhibition of force responses could be partially reversed in D_2O by larger voltage clamp depolarisations. We suggest that some reports of D_2O action in the literature may have been influenced by the elevated Ca^{2+} level resulting from the 0.4 pH unit correction in EGTA-buffered salines used to test myofibrillar bundle contractility: that is to a shift in the $K_{appCa-EGTA}$ by H^+.

9.6.8.3 Dantrolene-Sodium and A23187. The kinetics of the rapid increase in cytosolic free Ca^{2+} and its subsequent re-uptake by the SR can be analysed quantitatively from the phasic increase in light output in aequorin-injected fibres (Ashley and Ridgway 1970). 1-[(p-nitrophenyl)-furfurilidene-aminol] hydantoin sodium hydrate (dantrolene sodium) has muscle relaxant properties and depresses E-C coupling without interfering with either neuromuscular transmission or conduction of muscle action potentials. Experiments with the internal Ca^{2+} indicator aequorin were undertaken to investigate dentrolene's effect on free Ca^{2+} events associated with E-C coupling.

In this section we discuss whether the divalent cation ionophore A23187- and dantrolene-induced changes of free Ca^{2+} transients affect one or more of the following steps during E-C coupling: (1) the electromechanical threshold; (2) the kinetics of Ca^{2+} release from SR; (3) the process of sequestration of cytosolic free Ca^{2+} back into the SR. Rectangular depolarising current pulses of 75–150 ms duration were used. These pulses did not exceed 40 mV in intensity and elicited no regenerative action potentials (Hagiwara and Naka 1964) and consistent results were obtained. The use of an extensive range of subthreshold depolarisations allowed the relations between the passive membrane depolarisations and the corresponding Ca^{2+} transient and mechanical force output to be examined.

The evidence that dantrolene reduces the steady leakage of Ca^{2+} from intracellular stores (Desmedt and Hainaut 1977) is in agreement with the present finding that this drug markedly inhibits the free Ca^{2+} transient and decreases the mechanical force of the contraction (Fig. 34C). The actual depolarisation produced by a given electrical current delivered through the intracellular electrode across the outer membrane was not changed by dantrolene. A rapid inhibitory effect of dantrolene on the Ca^{2+} transient and force appeared after a few seconds and further increased at a slow rate over the next few minutes. This was comparable to the pattern recorded for the reduction of resting aequorin light emission. The reduction of the Ca^{2+} transient by dantrolene no doubt accounts for the simultaneously recorded depression of the mechanical force output of the muscle fibre.

In contrast to dantrolene, A23187 which enhances Ca^{2+} release from the SR and across the plasma membrane when applied to an aequorin-injected fibre, enhances both the transient free Ca^{2+} and the force event (Desmedt and Hainaut 1977) when the fibres are stimulated electrically.

10 Summary

In this review, aspects of the ways in which Ca^{2+} is transported and regulated within muscle cells have been considered, with particular reference to crustacean muscle fibres. The large size of these fibres permits easy access to the internal environment of the cell, allowing it to be altered by microinjection or microperfusion. At rest, Ca^{2+} is not in equilibrium across the cell membrane, it enters the cell down a steep electrochemical gradient. The free $[Ca^{2+}]$ at rest is maintained at a value close to 200 nM by a combination of internal buffering systems, mainly the SR, mitochondria, and the fixed and diffusible Ca^{2+}-binding proteins, as well as by an energy-dependent extrusion system operating across the external cell membrane. This system relies upon the inward movement of Na^{+} down its own electrochemical gradient to provide the energy for the extrusion of Ca^{2+} ions. As a result of electrical excitation, voltage-sensitive channels for Ca^{2+} are activated and permit Ca^{2+} to enter the cell more rapidly than at rest. It has been possible to determine both the amount of Ca^{2+} entering by this step, and what part this externally derived Ca^{2+} plays in the development of force as well as in the free Ca^{2+} change. The latter can be determined directly by Ca^{2+}-sensitive indicators introduced into the cell sarcoplasm. A combination of techniques, allowing both the total and free Ca^{2+} changes to be assessed during electrical excitation, has provided valuable information as to how muscle cells buffer their Ca^{2+} in order to regulate the extent of the change in the free Ca^{2+} concentration. The data indicate that the entering Ca^{2+} can

only make a small direct contribution to the force developed by the cell. The implication here is that the major source of Ca^{2+} for contraction must be derived from the internal Ca^{2+} storage sites within the SR system, a view reinforced by caged Ca^{2+} methods.

The ability to measure the free Ca^{2+} concentration changes within a single cell during activation has also provided the opportunity to analyse, in detail, the likely relations between free Ca^{2+} and the process of force development in muscle. The fact that the free Ca^{2+} change precedes the development of force implies that there are delays in the mechanism, either at the site of Ca^{2+} attachment on the myofibril, or at some later stage in the process of force development that were not previously anticipated. The experimental evidence also supports the notion that Ca^{2+} is still attached to the contractile system during the relaxation phase of the mechanical event, and that it returns to the SR when the cytosolic concentration has already declined to a relatively low level.

This article emphasises the versatility of these large, single muscle fibres for physiological investigations. It also stresses the importance of the ideas derived from these experiments, and their applicability to other muscle cells where similar effects and, in particular, time relations between free Ca^{2+} and force development have recently been shown to occur.

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