Localisation of intracellular calcium stores in the striated muscles of the jellyfish *Polyorchis penicillatus*: possible involvement in excitation–contraction coupling

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Summary

When jellyfish striated muscles were stimulated directly, the amplitude of contractile tension increased as the stimulation frequency increased. Application of 10 mmol l⁻¹ caffeine reduced the amplitude of contractile tension and abolished this facilitatory relationship, indicating that calcium stores participate in excitation–contraction coupling. Calcium stores were identified ultrastructurally using enzymatic histochemistry to localize CaATPases, and potassium dichromate to precipitate calcium. Electron energy-loss spectroscopy was used to verify the presence of calcium in precipitates. Both CaATPase and calcium were localised in membrane-bound vesicles beneath the sarcolemma. We concluded that sub-sarcolemmal vesicles could act as calcium stores and participate in excitation–contraction coupling.

Key words: calcium stores, excitation–contraction coupling, jellyfish, *Polyorchis penicillatus*, caffeine, CaATPase.

Introduction

Muscle contraction is initiated by an increase in the intracellular free calcium concentration. This increase in intracellular calcium may occur as a result either of direct calcium influx through voltage-gated calcium channels in the plasma membrane, or of calcium release from intracellular stores such as the sarcoplasmic and endoplasmic reticulum. In striated muscles, the requirement for extracellular calcium influx for muscle contraction is normally inversely correlated with the development of the sarcoplasmic reticulum (Bers, 1991). For example, in the tunicate *Doliolum nationalis*, contraction of striated muscle is completely dependent on calcium influx through the sarcolemma since no sarcoplasmic reticulum has been observed (Bone et al., 1997). By contrast, in vertebrate skeletal muscles, which have extensive and well-organized sarcoplasmic reticulum, contraction depends almost exclusively on calcium release from the sarcoplasmic reticulum (Armstrong et al., 1972). Most invertebrate striated muscle and vertebrate cardiac muscle have a less pronounced sarcoplasmic reticulum. In many invertebrates, contraction depends both on calcium influx through the sarcolemma and on calcium release from the sarcoplasmic reticulum, in varying proportions (Bers, 1991; Ashley et al., 1993; Palade and Györke, 1993; Paniagua et al., 1996).

The sarcoplasmic reticulum of striated muscle is an intracellular tubular network with functionally discrete units that respond differently to various types of cellular stimulation (Golovina and Blanstein, 1997). The terminal cisternae of the sarcoplasmic reticulum have been identified as the likely calcium release and storage sites, and contain the calcium release channels, ryanodine receptors (Winegrad, 1965; Inui et al., 1987a; Inui et al., 1987b; Lai et al., 1988) and high-capacity and low-affinity calcium-binding proteins such as calsequestrin (Meissner et al., 1973; Jorgensen et al., 1983). Calcium uptake by the sarcoplasmic reticulum is mediated by Ca²⁺/Mg²⁺-ATPase pumps, which are a family of transmembrane proteins (Carafoli, 1991) with each pump molecule transporting two calcium ions from the cytoplasm into the lumen of the SR during each catalytic cycle at the expense of a single molecule of ATP (Tada et al., 1982; Inesi, 1987).

Myoepithelial cells in cnidarians represent some of the most 'primitive' types of muscle (Prosser, 1982), and are formed by an apical soma attached by a narrow neck to several contractile feet which are aligned in parallel arrays. Myoepithelial cells of polyps and most postural muscles in medusae are non-striated, while the subumbrellar myoepithelial cells of medusae have striated myonemes (Chapman et al., 1962; Fautin and Mariscal, 1991; Thomas and Edwards, 1991). The myofibrils of *Polyorchis penicillatus*, like other hydromedusae (Spencer and Satterlie, 1981) contain thick and thin myofilaments arranged in a hexagonal array of one thick filament surrounded by six thin filaments (Singla, 1978a; Singla, 1978b), and are joined end-on by desmosomes and laterally by gap junctions (Spencer, 1979). Despite their structural similarity to other striated muscles, little is known about the mechanism of excitation–contraction coupling in cnidarian striated muscle. In
this study, we present data on muscle contraction to show that calcium released from intracellular calcium stores plays a role in the contraction of jellyfish swimming muscle and we also localise putative calcium stores by cytochemical labelling of Ca$_{2+}$-ATPase and calcium.

Materials and methods

Collection

Medusae of P. penicillatus (Eschscholtz) were collected from Bamfield Inlet or Pachena Bay, near Bamfield, British Columbia, Canada and held in aquaria with running sea water at 9–12°C. Medium-sized jellyfish (1.5–2 cm diameter in the bell opening) were used in this study.

Field stimulation of muscle strips

Preliminary studies indicated that muscle strips from the subumbrella (bell lining) and velum had similar pharmacologies. Due to the thickness and elastic property of mesoglea in the bell region, recordings from muscle strips obtained from this region were extremely variable and it was difficult to measure contractile amplitude. Therefore, only muscle strips from vela were used in this study. The velum consists of a thin lamina of mesoglea covered on the subumbrella side by a single layer of striated muscle cells, while the epithelium on the opposite surface has occasional strands of smooth muscle that are incapable of repetitive phasic contraction. The vela of medusae, anaesthetized in 1:1 isotonic MgCl$_2$ (0.33 mol l$^{-1}$) and artificial sea water (ASW; NaCl 376 mmol l$^{-1}$, $\text{Na}_2\text{(SO}_4\text{)}$ 26 mmol l$^{-1}$, MgCl$_2$ 41.4 mmol l$^{-1}$, CaCl$_2$ 10 mmol l$^{-1}$, KCl 8.5 mmol l$^{-1}$ and N-2-hydroxyethylpiperazine-N'2-ethanesulphonic acid (Hepes) hemisodium salt 10 mmol l$^{-1}$; pH 7.5), were excised so as to provide continuous strips of maximal width. Greater width ensured that tension was easily measured; however this did not compromise aerobic contraction as the tissue thickness was constant. To avoid any contamination by nervous tissue, each velar strip was bisected lengthwise into two strips and only strips free from nerve-ring tissue were used in the study. Muscle fibres ran parallel to the long axis of the velar strips. Vela with widths between 2–3 mm and lengths between 2 and 2.5 cm were used in this study. The free ends of each velar strip were pinned to the Sylgard base of a 35 mm Petri dish containing a pair of embedded Ag/AgCl$_2$ stimulating electrodes connected to a Grass S44 stimulator. The velar strip ran between the two stimulating electrodes and around a small hook attached to an isometric force transducer (Kent Scientific Corporation). The stimulation voltage was determined by increasing the voltage until there was no increase in the amplitude of contraction at the stimulation frequency of 0.1 Hz. The voltages used usually were between 30 and 40 V. The frequency of stimulation varied from 0.1 to 0.8 Hz, with each square pulse having a duration of 30 ms. The rate of perfusion was controlled by a peristaltic pump at 1.5 ml min$^{-1}$ and the perfusate was removed by a vacuum pump. All perfusion solutions were kept at 12–14°C during experiments by running the perifusion tubing through an ice bucket. The transduced tension was recorded on a digital Dash-IV pen-recorder (Astro-Med Inc.). The amplitude of contractile tension for each condition (control, drug effect and washed) was calculated by averaging ten contractions when the contraction was stabilised from each preparation. Caffeine was dissolved in the ASW to a final concentration of 10 mmol l$^{-1}$.

Localisation of Ca$_{2+}$-ATPase

Strips of the subumbrellar muscle sheet were removed from jellyfish by a pair of forceps and cut into pieces approximately 2 mm x 3 mm. The muscle pieces were washed and relaxed in Ca$_{2+}$-free ASW and then fixed with 2% paraformaldehyde and 0.25% glutaraldehyde in cacodylate-buffered saline at pH 7.8 (sodium cacodylate/HCl 100 mmol l$^{-1}$, NaCl 300 mmol l$^{-1}$, KCl 10 mmol l$^{-1}$) for 30 min on ice. Glutaraldehyde was added to provide better preservation of the ultrastructure (Ueno and Mizuhiro, 1984). After three washes (30 min each) in ice-cold cacodylate-buffered saline and two brief rinses in glyrine/NaOH buffer (250 mmol l$^{-1}$, pH 9.0), tissues were incubated at either 12°C or 37°C for 45 min in a solution containing glyrine/NaOH buffer 250 mmol l$^{-1}$, pH 9.0, ATP-Na 3 mmol l$^{-1}$, CaCl$_2$ 10 mmol l$^{-1}$, MgCl$_2$ 5 mmol l$^{-1}$, lead citrate 4 mmol l$^{-1}$ (Ando et al., 1981). Levamisole was added to a final concentration of 8 mmol l$^{-1}$ to exclude any contribution of non-specific alkaline phosphatase to ATP hydrolysis (Van-Noorden and Jonges, 1987). Ouabain (final concentration 10 mmol l$^{-1}$) was added to the incubation medium to inhibit Na$^+$-K$^+$-ATPase (Maggio et al., 1991). To examine ATP-, calcium- and substrate-dependence of the cytochemical reaction, the following controls were performed, with incubation in ATP-free medium, Ca$_{2+}$-free medium with 10 mmol l$^{-1}$ EGTA, or lead citrate-free medium. After incubation, samples were rinsed sequentially in glyicine buffer and cacodylate-buffered saline, post-fixed in 1% osmium tetroxide in cacodylate-buffered saline for 1 h, and then processed for conventional TEM examination. Sections of 70–90 nm thickness were collected on nickel grids and examined without staining.

Ultrastructural localisation of calcium

Calcium stores were localised using the method of Probst, 1986. Tissue samples were fixed with 4% paraformaldehyde and 2% glutaraldehyde in 0.1 mol l$^{-1}$ phosphate buffer (pH 7.4) for 2 h at 4°C and post-fixed in 1% osmium tetroxide and 2.5% potassium dichromate in 0.1 mol l$^{-1}$ phosphate buffer at 4°C for 24 h. After incubation, samples were rinsed in phosphate-buffered saline then processed for conventional TEM examination. Sections of 70–90 nm thickness were examined without staining.

Electron energy-loss spectroscopy

Unstained, ultrathin sections 30–50 nm thick from tissues prepared for calcium localisation (see above) were used for electron energy-loss spectroscopy (EELS) using an energy-filtering transmission electron microscope (EM 902, Zeiss,
Germany) and examined at an accelerating voltage of 80 kV. The inelastically scattered electrons with element-specific energy losses were used to obtain high-resolution imaging of calcium distribution in the sections. For calcium element mapping, energy-filtered images (energy width 20 eV) were recorded above and below the edge of electron absorption specific for calcium (Ca L₂,3 edge 346 eV) at 355 eV and 330 eV, respectively. The image taken at 330 eV served as a reference image for background levels. Net calcium distribution images were obtained by computer-assisted image processing of the difference between images taken at 355 and 330 eV.

Results

Field stimulation of muscle strips

Velar strips rarely contracted spontaneously after being mounted in the perfusion chamber and maintained consistent contractions to field stimulation without significant changes in amplitudes for 3–4 h. The mean contractile tension measured at a stimulation frequency of 0.1 Hz was 0.275±0.079 mN (N=30). The time course of contraction was very similar to that measured previously (Spencer and Satterlie, 1981) for subumbrellar muscle, with a time-to-peak tension of approximately 130 ms for jellyfish of the size used in these experiments. The contractions were symmetrical with relaxation following a similar time course. Fig. 1A,B shows that the maximum contractile tension increased stepwise as stimulation frequency was increased from 0.1 to 0.8 Hz (P<0.01, N=9, ANOVA test, followed by Student–Neuman–Keuls multiple comparison test). This force–frequency relationship suggested the presence of calcium stores and calcium release that was induced by an influx of extracellular calcium (Bers, 1991). Caffeine was used to examine if calcium stores were involved in muscle contraction. At the onset of bath-application of caffeine at 10 mmol l⁻¹, when the stimulation rate was 0.1 Hz, caffeine immediately increased the amplitudes of several contractions higher than the ASW control (Fig. 1C). The inhibitory effect of caffeine on contractile force appeared within the first minute after the application of caffeine. The force–frequency relationship appeared to be mediated by caffeine-sensitive calcium stores, as the presence of 10 mmol l⁻¹ caffeine abolished the frequency-dependent increase in tension (Fig. 1C,D); there were no differences among the mean amplitudes of contraction at 0.1 Hz, 0.4 Hz and 0.8 Hz. Nevertheless, the mean amplitude of contraction in the presence of caffeine was reduced to 75.9±3.6 % of control at 0.1 Hz, 61.7±2.3 % at 0.4 Hz and 49.5±0.8 % at 0.8 Hz (Fig. 1D, N=3). However, application of caffeine alone without field stimulation did not evoke any contraction (data not shown). Ryanodine at 100 μmol l⁻¹ had no effect on field stimulation of jellyfish striated muscles (data not shown).

We conclude that although calcium influx through voltage-gated calcium channels is sufficient to evoke contraction,
contractions normally involve the addition of calcium released from caffeine-sensitive intracellular stores during repetitive contraction.

**Ultrastructural localisation of Ca^{2+}-ATPase**

Fig. 2 shows the ultrastructure of the swimming muscle where each cell is separated into a somal region and a myofibrillar region attached to the mesoglea. As previously reported (Singla, 1978b; Spencer, 1979), no T-tubular structures have been observed in *P. penicillatus* myofibrils; however, several membrane-bound vesicles in the subsarcolemmal cytoplasm were found close to contractile filaments. The distribution of these vesicles did not show any specific pattern. To establish if these sub-sarcolemmal vesicles are indeed sarcoplasmic reticulum and possibly involved in excitation–contraction coupling in swimming muscle cells, we used a cytochemical method (Ando et al., 1981) to test the presence of Ca^{2+}-ATPase activity in these vesicles. When tissue was incubated in medium containing calcium, magnesium ions and ATP, an electron-dense precipitate of lead phosphate indicated the presence of Ca^{2+}-ATPase activity. We found labelling in somal regions, such as the nucleus (Fig. 3A), the Golgi apparatus (Fig. 3B), vesicles of the endoplasmic reticulum and the lateral and apical plasma membranes (Fig. 3C). Precipitates on the outer side of these latter membranes presumably arose from non-specific ecto-ATPase activity (Ogawa et al., 1986; Nasu and Inomata, 1990).

The densest precipitates, however, were found on the inner side of the sarcolemma surrounding the myofibrils and in subsarcolemmal vesicles, that were distributed sparsely along the sarcolemma, that were distributed sparsely along the side of the sarcolemma surrounding the myofibrils and in sub-

**Ultrastructural localisation of calcium using dichromate precipitation and electron energy loss spectroscopy**

We also used potassium dichromate precipitation to indicate the presence of calcium stores (Probst, 1986). This histochemical method localizes loosely bound calcium and hence indicates calcium-binding sites. In somata, electron dense precipitates (EDPs) were found in the nucleus, mitochondria, the inner surface of the plasma membrane and sarcoplasmic reticulum-like vesicles (Fig. 4A,B). EDPs were also found in sub-sarcolemmal vesicles (Fig. 4C,D), and lining the inner side of the sarcolemma (Fig. 4D). Fine EDPs were also found in the mesoglea adjacent to myofibrils.

We used electron energy loss spectroscopy to establish whether the EDPs contained calcium. The net calcium distribution image (Fig. 5A) was generated by subtracting the image taken below the absorption edge specific for calcium (served as a background reference image) from the image taken above the calcium edge. The bright spots on the image represent the calcium signals, which were almost identical to the position of the EDPs on the conventional TEM image taken at zero energy loss (Fig. 5B), indicating that EDPs contained calcium.

**Discussion**

While it has been shown that generation of action potentials and contraction of the swimming muscles of *P. penicillatus* depend on the influx of extracellular calcium through voltage-gated calcium channels (Spencer and Satterlie, 1981; Lin and Spencer, 2001), the findings in this report provide further evidence that intracellular sources of calcium are required to give full tension development and the facilitatory force–frequency relationship seen during repetitive contraction, which is the normal swimming mode of this jellyfish (Spencer and Satterlie, 1981). As shown in Fig. 1A,B, an increase in the frequency of stimulation results in a stepwise increase in the amplitude of contraction, indicating the possible involvement of a calcium-induced calcium release mechanism in
Calcium stores in jellyfish striated muscles

Fig. 3. Ca\(^{2+}\)-ATPase activity in the swimming muscle of Polyorchis penicillatus. The reaction product (lead phosphate precipitate) of Ca\(^{2+}\)-ATPase activity is located in (A) the nucleus; (B) Golgi stacks; (C) the outer leaflet of the lateral plasma membrane (arrowhead) and vesicles of the sarcoplasmic reticulum (arrow). The apical region, at the top of panel, shows little precipitation and there is an absence of reaction products in the mitochondria. (D) Precipitates were found along both sides of the sarcolemma surrounding myofibres and at their basal attachment to the mesoglea, but none were seen in the mitochondria. Arrows point to subsarcolemmal vesicles showing Ca\(^{2+}\)-ATPase activity. The arrowhead indicates a tubular structure that does not have any precipitate. (E) Negative control for the Ca\(^{2+}\)-ATPase activity in swimming muscle. A section through the nuclear region showed no lead phosphate precipitate when tissues were incubated in a medium without ATP. (F) Negative control for the Ca\(^{2+}\)-ATPase activity in swimming muscle. Tangential section through myofibrils showing that there was no lead phosphate precipitation when tissues were incubated in a calcium-free medium with 10 mmol l\(^{-1}\) EGTA. The arrow indicates one of the vesicles and the arrowhead the sarcolemmal membrane, both of which are free of precipitate. Mi, mitochondria; Me, mesoglea. Scale bars, 1 \(\mu\)m (A,E); 50 nm (B); 100 nm (C); 0.5 \(\mu\)m (D,F).
excitation–contraction coupling. The sequence of excitation–contraction coupling in vertebrate muscle cells starts with a propagated depolarisation invading the plasma membrane. This change in potential across the membrane activates voltage-gated, calcium channels (dihydropyridine-sensitive receptors, DHPRs), which either leads to a direct increase in intracellular Ca\(^{2+}\) levels or to the release of Ca\(^{2+}\) from internal calcium stores in the sarcoplasmic reticulum (SR) through Ca\(^{2+}\) release channels (Winegrad, 1965; Inui et al., 1987a; Inui et al., 1987b). The mechanism of calcium release via ryanodine-sensitive channels (RyRs) in the sarcoplasmic reticulum differs for skeletal and cardiac muscles. In skeletal muscle, Ca\(^{2+}\) influx through DHPRs is not required to initiate Ca\(^{2+}\) release from the SR (Nabauer et al., 1989) since DHPRs interact directly with RyRs at a molecular level (Flucher and Franzini-Armstrong, 1996). In contrast, cardiac muscle requires Ca\(^{2+}\) influx through DHPRs in cardiac muscle to activate Ca\(^{2+}\) release from the SR through a process known as calcium-induced calcium release (Fabiato, 1983). Calcium-induced calcium release is generally accepted as the major source of calcium for regulation of contraction in cardiac muscle, although there may be some contribution from Ca\(^{2+}\) influxing through DHPRs (Bers et al., 1993).

The sarcoplasmic reticulum in striated muscle can be elaborate, as in vertebrate skeletal muscle, where SR and T-tubules form triads that are precisely positioned above the Z line in each sarcomere (Fawcett, 1986). In cases like this, muscle contraction is completely dependent on calcium released from sarcoplasmic reticulum. At the other extreme, where a sarcoplasmic reticulum is absent, such as the striated muscles of the tunicate Doliolum nationalis, muscle contraction depends on calcium influx through the sarcolemma (Bone, 1997). In other striated muscles showing varying degrees of development of the sarcoplasmic reticulum, including vertebrate cardiac and invertebrate muscles, calcium influx is essential for muscle contraction and triggering the release of calcium from sarcoplasmic reticulum. Thus, it appears that the requirement for extracellular calcium influx for muscle contraction is inversely correlated with the development of the sarcoplasmic reticulum (Bers, 1991; Ashley et al., 1993; Palade and Györke, 1993; Paniagua et al., 1996).

Caffeine was applied to determine whether calcium stores were directly involved in mediating contraction and in producing the force–frequency relationship. In most tissues, caffeine empties intracellular stores by making the store leaky to calcium or increasing the open probability of calcium

![Fig. 4. Localisation of calcium in the swimming muscle of Polyorchis penicillatus by potassium dichromate precipitation. (A) Within the nucleus, grainy electron-dense precipitates (EDPs) can be seen. (B) Vesicles in the somal region show a high calcium content. The arrowhead indicates the plasma membrane, showing EDPs located on the inner side of the membrane. (C) Vesicles (arrow) in the subsarcolemma (arrowhead) contain EDPs. (D) Large vesicles between mitochondria and myofilaments in the subsarcolemma contain EDPs (arrow), which are located on the inner side of the membrane. The arrowhead indicates the sarcolemma membrane. Mi, mitochondria; Me, mesoglea. All scale bars are 1 \(\mu\)m.]
releasing channels (Weber and Herz, 1968; Rousseau et al., 1988). In our experiments, caffeine increased the amplitude of the first few contractions (Fig. 1C). This could be due to caffeine sensitising myofilaments to calcium (Wendt and Stephenson, 1983), or to the sensitisation of calcium releasing channels treated with caffeine (Herrmann-Frank et al., 1999). After the initial facilitated contractions, caffeine produced a reversible inhibitory effect; it reduced the steady-state peak tension by 75% at 0.1 Hz and 50% at 0.8 Hz, and abolished the progressive tension increase that normally accompanies increased stimulation frequency (Fig. 1) (Lin and Spencer, 2001). These results indicate the presence of caffeine-sensitive calcium stores. However, caffeine did not evoke any contraction or contractures when applied alone without field stimulation. One possible explanation could be that the amount of calcium released from the calcium stores alone was not enough to induce contraction or the tension developed was below threshold for the transducer. Ryanodine is known to be an effective channel blocker for caffeine-sensitive calcium stores (Winegrad, 1965; Inui et al., 1987a; Inui et al., 1987b; Lai et al., 1988); however, no inhibitory effects of ryanodine at 100 μmol l⁻¹ were observed during field stimulation of jellyfish striated muscles (data not shown). This raised the possibility that the inhibitory effects of caffeine we observed were non-specific ‘blocking’ of voltage-gated calcium channels due to the high concentration of caffeine (10 mmol l⁻¹). Alternatively, the calcium releasing channels on caffeine-sensitive stores in jellyfish muscles could not be blocked by ryanodine.

In vertebrate striated muscles a substantial portion of stored calcium resides in the sarcoplasmic reticulum (Bers, 1991). In large diameter muscle cells, however, these stores may be distant from the external plasma membrane, which provides the initial influx of external Ca²⁺ to trigger calcium release. To ensure efficient triggering of calcium-induced calcium release, larger muscle cells have T-tubules that are invaginations of the plasma membrane, to bring this triggering signal rapidly to Ca²⁺ stores deep within the muscle. T-tubules are specialized to provide the external Ca²⁺ signal since their membranes have a high density of voltage-gated calcium channels (Carl et al., 1995). Small muscle cells, such as those in frog and lizard hearts, however, do not possess T-tubules and all compartments of the sarcoplasmic reticulum are relatively close to the external plasma membrane (Fabriato, 1982; Anderson et al., 1989; McLeod et al., 1991). T-tubules have never been observed in electron micrographs of P. penicillatus swimming muscle, presumably because there is only one layer of myofibrils, and each is about 2 μm in diameter, thereby positioning the contractile apparatus close to the triggering calcium signal from the plasma membrane (Singla, 1978b; Spencer, 1979) (this study).

At least three characteristics are necessary for an intracellular compartment to act as a ‘calcium store’ involved in muscle contraction: (1) the presence of membrane pumps to replenish Ca²⁺ in the store; (2) calcium-release channels, which release calcium into the cytoplasmic space; and (3) calcium-binding proteins, which bind calcium in the store. As the nature of calcium-releasing channels in jellyfish is not known, we chose to map Ca²⁺-ATPase activity and demonstrate the presence of calcium by using histocytochemical methods to identify possible calcium stores. We used an enzyme cytochemical method to map the activity of Ca²⁺-ATPase ultrastructurally, since this method has been used to demonstrate Ca²⁺-ATPase activity in a wide variety of animal tissues, including marine invertebrates (Maggio et al., 1991; Cario et al., 1996). Ca²⁺-ATPase is known to participate in...
sequestering calcium into stores as well as pumping calcium into extracellular space (Carafoli, 1991). At least two types of \textsuperscript{Ca}\textsuperscript{2+}-ATPase have been identified; one is located on the sarcolemma or plasmalemma and the other type is located on sarcoplasmic reticulum (Carafoli, 1991). The method used in this study does not distinguish between these two types of \textsuperscript{Ca}\textsuperscript{2+}-ATPases, nor any non-specific ecto-ATPase activity (Ogawa et al., 1986; Nasu and Inomata, 1990). This latter activity is characterised by its location, which is at the outer surface of the plasma membrane (Ando et al., 1981). The plasmalemmal \textsuperscript{Ca}\textsuperscript{2+}-ATPase functions to remove calcium from intracellular spaces during the relaxation phase of the muscle contraction cycle (Carafoli, 1991). The sarcolemmal region of \textit{P. penicillatus} swimming muscle is more heavily labelled by \textsuperscript{Ca}\textsuperscript{2+}-ATPase specific precipitates than the apical region. This polarized distribution of \textsuperscript{Ca}\textsuperscript{2+}-ATPase activity is to be expected and parallels the functional polarization of the cells into somal and contractile compartments. A polarised distribution of \textsuperscript{Ca}\textsuperscript{2+}-ATPase has been reported for vertebrate epithelium, pancreatic and salivary gland cells, photoreceptors (Seguchi et al., 1982; Lee et al., 1997; Krizaj and Copenhagen, 1998) and crustacean posterior caecal epithelium (Meyran and Graf, 1986). Plasmalemmal \textsuperscript{Ca}\textsuperscript{2+}-ATPase has been localised to the caveolae or uncoated plasma membrane invaginations in mouse endothelial cells, smooth muscle cells, cardiac muscle cells, epidermal keratocytes and mesothelial cells by using a specific antibody generated against erythocyte membrane \textsuperscript{Ca}\textsuperscript{2+}-ATPase (Fujimoto, 1993). Although we did not see caveolae-like structures in the sarcolemma, the plasma membrane surrounding myofibrils is heavily invaginated and ruffled, especially at the junctions between adjacent myofibrils where \textsuperscript{Ca}\textsuperscript{2+}-ATPase activity is very high.

Another type of \textsuperscript{Ca}\textsuperscript{2+}-ATPase is located on the sarcoplasmic reticulum membrane and pumps calcium into the sarcoplasmic reticulum during the relaxation phase of muscle contraction (Lytton and Nigam, 1992), and is considered to be an indicator protein for calcium stores. In \textit{P. penicillatus} muscle, the presence of \textsuperscript{Ca}\textsuperscript{2+}-ATPase activity in sub-sarcolemmal vesicles close to myofilaments indicates these these vesicles perform the function of sarcoplasmic reticulum.

The calcium precipitation method revealed a similar distribution pattern to that for \textsuperscript{Ca}\textsuperscript{2+}-ATPase localisation; however, it should be noted that although calcium precipitates were present in mitochondria there was no \textsuperscript{Ca}\textsuperscript{2+}-ATPase activity. This is to be expected as mitochondria can act as calcium sinks, with calcium ion influx \textit{via} a calcium uniporter rather than a \textsuperscript{Ca}\textsuperscript{2+}-ATPase pump (Crompton et al., 1983; Kessar and Crompton, 1983). Calcium precipitates also outlined the inner leaflet of the sarcolemma of \textit{P. penicillatus} swimming muscle, which has been reported for a variety of cell types where calcium ions are probably bound by negatively charged glycoproteins (Carafoli, 1987). Lullman and Peters (Lullman and Peters, 1977; Lullman and Peters, 1979) proposed that these membrane-bound calcium ions could be released during depolarisation of the sarcolemma and participate in cardiac myocyte contraction. However, further studies showed that neither the intracellular calcium concentration, nor muscle contractile force increases during depolarisation without calcium influx (Rich et al., 1988; Nabauer et al., 1989). Thus the inner leaflet of the sarcolemma might simply serve as a calcium-buffering site.

Calcium precipitates were also found in similar vesicular structures in the soma and beneath the sarcolemma of myofibrils. As is implied by the \textsuperscript{Ca}\textsuperscript{2+}-ATPase activity, these vesicles underneath the sarcolemma may function as calcium stores during excitation–contraction coupling, since they are adjacent to the myofibrils. Calcium channels are more abundant on the sarcolemma of myofibrils than on the somal plasmalemma (Lin and Spencer, 2001) to ensure rapid excitation–contraction coupling. Thus cytochemical localisation of \textsuperscript{Ca}\textsuperscript{2+}-ATPase and calcium indicates the presence of a relatively poorly organized sarcoplasmic reticulum and the physiological actions of caffeine on muscle contraction indicate that calcium influx through voltage-gated calcium channels is sufficient to cause muscle contraction. Thus the sarcoplasmic reticulum may play a regulatory role in muscle contraction. For example, the normal swimming pattern for \textit{P. penicillatus} is a series of bouts. Spencer and Satterlie (Spencer and Satterlie, 1981) noted that there is a stepwise increase in the tension of the first four or so contractions in each bout as well as a decrease in duration. The facilitation in the amplitude of contractile tension is likely to be the result of refilling calcium stores after a period of rest and may represent a case of post-rest potentiation. Together these frequency-dependent changes in contraction dynamics are presumably an adaptation for overcoming the inertia of an animal at rest.

The presence of \textsuperscript{Ca}\textsuperscript{2+}-ATPase activity and calcium precipitates in the mesoglea close to myofibrils is intriguing. One possible function for calcium and \textsuperscript{Ca}\textsuperscript{2+}-ATPase in mesoglea could be similar to opacification (blanching) of the mesoglea in the siphonophore \textit{Hippopodius hippopus}, which is a calcium dependent process (Bassot et al., 1979). Blanching is due to temporary formation of light-scattering granules in response to the propagation of action potentials in overlying epithelia. Thus in both cases the mesoglea may act as a long-term store for calcium, with dynamic exchange between the epithelium and mesoglea.

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