CALCIUM MOVEMENTS DURING CONTRACTION IN MOLLUSCAN SMOOTH MUSCLE, AND THE LOCI OF CALCIUM BINDING AND RELEASE

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SUMMARY

1. Conventional $^{45}$Ca efflux studies of Buccinum and Neptunia pharangeal retractor and columella muscles revealed the presence of a fast and a slow calcium compartment. KCl-induced depolarization caused a stimulation of the $^{45}$Ca efflux from the slow compartment, suggesting that this compartment may be related to calcium diffusion from intracellular sources.

2. Fine structural studies showed a lack of organized sarcoplasmic reticulum in both muscles, and that submembrane vesicles and mitochondria were prominent.

3. Muscle sections examined by energy-dispersive X-ray microprobe analysis showed the presence of significant calcium accumulation in the submembrane vesicles and mitochondria.

4. Isolated submembrane vesicles and mitochondria of columella muscle showed strong powers of ATP-promoted calcium binding, while pre-loaded preparations could be induced to release $^{45}$Ca under in vitro conditions.

5. Synthetic detergent dispersal of columella muscle submembrane vesicles and mitochondria, followed by polyacrylamide gel electrophoresis revealed, in both fractions, a striking similarity of protein profile with those from skeletal muscle sarcoplasmic reticum. Two dominant protein bands were present, at 110,000-112,000 daltons and at 51,000-53,000 daltons, in a position similar to the Ca$^{2+}$/Mg$^{2+}$ ATPase and calsequestrin or high affinity proteins of skeletal muscle.

6. It is suggested that, in the absence of an organized sarcoplasmic reticum, the submembrane vesicles and mitochondria may act as the major calcium pools for contraction in these molluscan smooth muscles.

INTRODUCTION

The involvement of the calcium ion in the contraction/relaxation cycle of skeletal muscle fibres is now well documented (Sandow, 1965, 1970), and the sarcoplasmic reticulum has been firmly established as the major agency for subcellular release and binding of calcium during contraction and relaxation in skeletal muscle (Carvalho, 1966, 1968; Weber & Herz, 1968; Baskin, 1974), although some newer evidence points to a subsidiary role for mitochondria in muscle calcium regulation (Batra, 1974; Huddart, 1976; Huddart & Price, 1976).
Far less is known about calcium regulation in smooth muscle. In the longitudinal smooth muscle of mammalian ileum, depolarization induces a massive stimulation of $^{46}$Ca efflux, and in the absence of an organized sarcoplasmic reticulum, the membrane vesicles appear to possess the major power of calcium uptake and release (Huddart & Syson, 1975). In the smooth muscle of guinea-pig taenia caecum, the microsomal fraction also possesses powers of intracellular calcium regulation (Tomiyama, Takayanagi & Takagi, 1975). On the other hand, in vascular smooth muscle there is evidence that mitochondria may possess powers of calcium regulation in the contraction/relaxation cycle (Somlyo, 1972).

Nothing is known about the intracellular regulation of contraction and relaxation by the calcium ion in invertebrate smooth muscles, despite the interest shown in the contractile properties of molluscan muscles (Masai, 1951; Twarog, 1954; Millman, 1967; Burnstock, Greenberg, Kirby & Willis, 1967; Nystrom, 1967; Hill, Greenberg, Irisawa & Nomura, 1970; Sanger & Hill, 1973; Kobayashi, 1974). In this study we have attempted to detect the loci of calcium deposition in molluscan smooth muscle and to follow how this calcium is translocated during contraction. The loci of calcium binding and release have been correlated with the fine structure of the muscle cells in an attempt to deduce how the excitation-contraction coupling cycle operates.

**METHODS**

In this study, the columella and pharangeal retractor muscles of the marine whelks *Buccinum undatum* (L.) or *Neptuna antiqua* (L.) were used.

For efflux studies, small bundles of fibres were dissected from the muscles and loaded for 1 h in molluscan saline (Kobayashi, 1974) containing (mM): NaCl, 462; KCl, 9.4; CaCl$_2$, 9; MgCl$_2$, 54; Tris, 20. The pH of this saline was adjusted to 7.4 with 0.1 M-HCl and the loading salines contained 4 μCi/ml $^{46}$Ca. After loading, the preparations were quickly washed and placed in pipette tips attached to a constant flow pump which delivered non-isotopic molluscan saline at 1 ml/min. The effluent was collected on a conventional fraction collector. Depolarization and contraction of pre-loaded preparations was achieved using molluscan saline containing 200 mM-KCl (prepared by simple NaCl replacement). The $^{46}$Ca activity in the fractions (collected every 5 min) was estimated in a Packard tricarb liquid scintillation counter using Instagel as scintillant and emulsifier. The $^{46}$Ca activity leaving the preparations was expressed as a rate coefficient (Isaacson & Sandow, 1967). Further details of these techniques have been published elsewhere (Huddart & Syson, 1975).

For the isolation of subcellular fractions to be used in calcium uptake and release experiments, pieces of columella muscle were passed through a steel muscle press with 0.5 mm holes and homogenized in KCl-imidazole buffer (Nakamaru & Schwartz, 1972) using a conventional coaxial homogenizer. Mitochondria were sedimented at 8000 g while the membrane vesicular fraction (microsomal fraction) was sedimented at 28000 g following the procedure of Van Der Kloot (1965). The final suspensions were adjusted to a concentration of 1 mg protein/ml after biuret analysis. As a check for purity of the preparations, sample pellets were fixed in osmic acid and prepared for electric microscopic examination as described below. For calcium uptake studies, the technique of Nakamaru & Schwartz (1972) was employed. Suspensions
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of mitochondria and membrane vesicles were mixed with tris-maleate buffer containing 0.1 μCi/ml 45Ca, the ATP and calcium concentration being varied as described in the various experiments. After incubation, the 45Ca-loaded fractions were collected by passage through membrane filters with a pore size of 0.45 μm, and after washing and drying the activity on the filters was estimated by liquid scintillation counting. These techniques have been described in detail elsewhere (Huddart, Greenwood & Williams, 1974). For calcium release studies, fractions which had been loaded with 45Ca as described above were collected on membrane filters and the filters were placed in a constant drip apparatus. The effluent was collected on a fraction collector and the 45Ca activity in the fractions was estimated. Further details of this technique have been published elsewhere (Huddart & Price, 1976).

For conventional electron microscopy, small pieces of muscle were fixed in 5% glutaraldehyde, postfixed in 2% osmic acid, dehydrated through an ethanol series and embedded in Epon. Sections of about 50 nm thickness (estimated by diffraction colour) were stained with uranyl acetate and lead citrate before examination on an AEI EM 801 operated at 80 kV accelerating voltage.

Material for energy dispersive X-ray microprobe analysis had to be treated to minimize calcium loss from the cells. This was achieved using the recently developed direct aqueous polymerization method of Yarom, Peters & Hall (1974). After 30 min fixation in 2% glutaraldehyde containing 1% sodium oxalate and 0.1 M sodium cacodylate buffer with 0.25 M sucrose, muscle fibres were washed in the buffer and placed in a urea/glutaraldehyde mixture at pH 4.2 and allowed to polymerize overnight. Sections for analysis were cut at a number of thicknesses between 130 nm and 0.5 μm and were mounted on Formvar- and carbon-coated copper grids. The sections were examined on an AEI CORA analytical transmission microscope fitted with a KEVEX 152 eV energy dispersive X-ray detector and a LINK/AEP 290 series elemental analysis system. Each analysis lasted 200 s.

For examination of the protein composition of muscle fractions, samples were incubated for protein dispersal in 0.01 M sodium phosphate buffer (pH 7.4) for 24 h, the buffer containing 1% sodium dodecyl sulphate (SDS) and 8 M urea. After incubation, samples were placed on 5% polyacrylamide gel columns and electrophoresis was performed at 8 mA per gel. Gels were calibrated using proteins with molecular weights ranging from 10^6 to 2 x 10^6 daltons. After electrophoresis, the gels were stained with 1% bromophenol blue. Further details of these techniques have been described by Williams & Price (1974).

RESULTS AND DISCUSSION

Calcium efflux studies of molluscan muscle fibres

Determination of the gross fibre calcium exchange of molluscan smooth muscle has not previously been attempted. The initial experiments of this study were directed towards establishing the nature of the intracellular calcium compartment by conventional 45Ca efflux methods. The control experiments for columella muscle and pharangeal retractor muscle are shown in Fig. 1, where the 45Ca efflux has been plotted as a rate coefficient. The efflux curves of both muscles consist of an initial fast component, presumably related to calcium of extracellular origin, and a delayed sustained
slow component, presumably related to calcium of intracellular origin, as is regarded to be the case in skeletal muscle at least (Isaacson & Sandow, 1967). In these respects, these molluscan smooth muscles are little different from invertebrate skeletal muscle (Huddart & Syson, 1975). Because the slow component is of the greatest interest in terms of calcium mobilization during contraction, attempts were made to study how it was affected by activation of the muscle fibres, by depolarizing them with 200 mM KCl saline during the slow efflux phase. Fig. 1 shows that there was indeed a stimulation of the efflux, although this stimulation is somewhat less than that found in mammalian smooth muscle under identical conditions (Huddart & Syson, 1975). Unlike with skeletal muscle, the experimental efflux remained somewhat above control levels well after washout of the high KCl saline; a phenomenon also noticed in mammalian smooth muscle. This could mean that the slow phase is not a simple reflection of intracellular calcium changes; a loss from some very slowly equilibrating extracellular site could also be involved. However, if the effluxing medium is changed
Fig. 2. (a) Low-power photomicrograph of pharangeal retractor muscle. Note the dense packing of the cells in this muscle, the prominent central mitochondria and peripheral subsurface cisternae. Scale bar = 2 μm. (b) High-power view of the surface organization showing the prominent vesicles and short finger-like plasma membrane incursions. Scale bar = 0.5 μm.

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Fig. 3. (a) Low-power photomicrograph of edge of columella muscle. As in retractor muscle (Fig. 2), the central mitochondria and peripheral subsurface vesicles are prominent. Scale bar = 1 μm. (b) High-power view of part of the subsurface organization of columella muscle. Scale bar 0.5 μm.

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for one without calcium, no alteration of efflux pattern is seen, making this latter suggestion rather unlikely.

Fine structure of the muscle cells

To gain clues as to the physical agencies within the cell which could be responsible for calcium binding and release, the fine structure of both muscles was examined. Figs. 2 and 3 show the essential ultrastructural details of the cells of columella and pharangeal retractor muscle. The cells contain a central core of mitochondria and occasional peripheral mitochondria but the great bulk of the cells is filled by the contractile apparatus. Both muscles lack an organized sarcoplasmic reticulum and a transverse tubular system, although at the periphery of the cells there is an array of subsurface cisternae—finger-like, short invaginations of the plasma membrane and rounded vesicles, which are quite extensive at many places. In mammalian smooth muscle, there is a similar but more extensive structure that has been loosely termed 'sarcoplasmic reticulum' (Somlyo, Devine, Somlyo & North, 1971). However, in these molluscan muscles, the structure bears little resemblance to the sarcoplasmic reticulum of skeletal muscle, so the term 'membrane vesicles' has been used here to describe this cellular fraction.

The detection of calcium-binding loci by microprobe analysis

In recent years, direct detection of cellular calcium deposits in tissue sections has become possible by wavelength-dispersive and energy-dispersive X-ray microprobe analysis (Yarom et al. 1974; Huddart & Price, 1976). In this study, energy-dispersive microprobe analysis was employed on thick unstained sections of columella muscle to detect the most obvious cellular loci of calcium deposition. By experience we have found that sections of 130 nm thick gave adequate display outputs with a probe size of approximately 100 nm diameter, and a 200 s analysis, and at the same time permitted reasonable resolution of cellular detail in the unstrained sections. The fine structural appearance of a typical section is shown in Fig. 4. Probes were made of the four most obvious areas of the cell and its surroundings: the mitochondria; the sarcoplasm; the membrane area including the subsurface organization; and the extracellular space. Typical X-ray spectra in the 1–5 keV range from these areas are shown in Fig. 5, and it can be readily seen that appreciable calcium deposits (revealed at 3.69 keV) are present in the membrane vesicle area and in the mitochondria, with somewhat less calcium in the extracellular space, and very little calcium indeed in the sarcoplasm. This evidence is admittedly of a comparative and qualitative nature but it clearly indicated that the mitochondria and membrane vesicles should be examined for calcium binding in an in vitro situation, so attempts were made to prepare relatively pure suspensions of these cellular fractions by sequential centrifugation. Because of the large bulk of muscle required, these studies were confined to columella muscles. Electronmicrographs of sections through these fractions are shown in Fig. 6, and it can be seen that it was possible to prepare membrane vesicle suspensions relatively free from mitochondrial contamination although some vesicular contamination of mitochondria was always present. Fractions such as these were used routinely in studies of calcium uptake and release under in vitro conditions.
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Calcium uptake and release by muscle fractions

To determine any possible calcium-binding capacity of mitochondria and membrane vesicles, suspensions of these fractions were exposed to $^{46}$Ca for 1 h in the presence of ATP and excess calcium (6 mM). The dependency of calcium uptake on the ATP concentration in the incubation medium is shown in Fig. 7. In the case of both mitochondria and membrane vesicles, calcium uptake increased sharply up to 2 mM ATP, a situation also found with isolated microsomes from mammalian taenia caecum (Tomiyama et al. 1975), and this ATP concentration was employed in all subsequent studies.

The relationship between calcium uptake by mitochondrial and membrane vesicular fractions and the calcium concentration of the incubation medium is shown in Fig. 8. In the case of both fractions, uptake was maximal at about 2 mM calcium. These uptake figures were obtained without the use of oxalates or phosphates in the media to enhance binding, and the figures compare well with those of Tomiyama et al. (1975), obtained from taenia caecum microsomes, where uptake was enhanced to about 40 nmol/mg protein by the use of 5 mM sodium oxalate in their incubation media. In all our experiments, the membrane vesicular fraction showed a consistently higher uptake capacity for calcium than the mitochondrial fraction.

The estimation of calcium efflux from pre-loaded vesicular and mitochondrial fractions was considerably more difficult to achieve than the influx data above, for technical reasons. Suspensions were incubated with $^{46}$Ca-containing media for 1 h,
Fig. 4. Appearance of a typical thick (130 nm) unstained section used for energy-dispersive X-ray microprobe analysis. Even at this thickness, the central mitochondria and subsurface organization can be recognized. Scale bar = 2 μm.
Fig. 6. (a) Section of a pellet of membrane vesicles isolated from columella muscle. Note the freedom from contamination by mitochondrial fragments. Scale bar = 0.5 μm. (b) Section of a suspension of mitochondria prepared from columella muscle, showing some slight vesicular contamination. Scale bar = 0.5 μm.

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Fig. 7. Uptake of $^{40}$Ca by (●) membrane vesicles and (○) mitochondria as a function of ATP concentration in the incubation media. Each point is the mean of five experiments ± S.E.

then collected on membrane filters and quickly washed in isotope-free media. The filters were placed in a constant drip apparatus and the effluent was collected at short time intervals and counted. Typical efflux figures are summarized in Fig. 9. To see if efflux could be stimulated in an in vitro situation, the preparations were briefly exposed to a MgCl$_2$ or a CaCl$_2$ drip. In all cases an efflux stimulation was seen, but whether this simple test in any way mimics an in vivo situation is debatable. A possible interpretation of these results is that they may simply reflect the level of Ca/$^{40}$Ca and Mg/$^{40}$Ca passive exchanges at the vesicular or mitochondrial membranes. However, if ATP is omitted from the media, virtually no stimulation of efflux is seen, suggesting that the above results involve an ATP-promoted release of calcium.

The protein constituents of muscle fractions

In recent years considerable attention has been paid to the protein composition of skeletal muscle sarcoplasmic reticulum in relation to calcium transport and binding. In vertebrate skeletal muscle, polyacrylamide gel electrophoresis and gel permeation chromatography have revealed the presence of two proteins of high calcium-binding affinity, the 100 000 dalton Ca$^{2+}$/Mg$^{2+}$ ATPase and the 42 000–46 000 Dalton calsequestrin (MacLennan, Ostwald & Stewart, 1974; Huddart & Price, 1976). These proteins represent about 60% of the total protein present. Since the membrane
vesicles and mitochondria of molluscan smooth muscle possess calcium-binding activity, it was decided to examine the protein composition of these fractions to see if there was any similarity with the protein profiles of skeletal muscle. Typical polyacrylamide gel protein profiles obtained after SDS dispersal treatment of mitochondria and membrane vesicles are shown in Fig. 10, along with the molecular weight calibrations of the individual bands. As in the case of skeletal muscle, two bands are dominant in both fractions, constituting about 60–70% of the total protein present.

The gross fibre efflux experiments in this study indicate the presence of two major compartments to the calcium diffusion system in these molluscan smooth muscles. That the slow component, which is related to calcium leaving intracellular structures (Isaacson & Sandow, 1967; Isaacson, 1969), is associated with the calcium utilized during contraction is shown by the experiments in which the preparations were depolarized by KCl salines. Under these conditions, a sustained stimulation of efflux was seen.

To determine the nature of the intracellular calcium compartment, a number of
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Fig. 9. \(^{46}\text{Ca}\) efflux from mitochondria (a, b) and membrane vesicles (c, d) isolated from molluscan columella muscle. In both cases, efflux could be stimulated by increase in external calcium or magnesium.

approaches were taken. Although these muscles were without an organized sarcoplasmic reticulum, as is the case in other molluscan smooth muscles (Philpott, Kahlbrook & Szent-Gyorgi, 1960; Santander & Garcia-Blanco, 1972; Campbell & Burnstock, 1968; Hunt, 1972; Sobieszek, 1973; Wabnitz, 1975) and in some mammalian smooth muscles (Syson, 1974), consistent ultrastructural features were the centrally placed mitochondria and the peripherally placed subsurface vesicular organization.

The X-ray spectra obtained from our studies clearly indicate significant calcium deposition in the mitochondria and membrane vesicle fractions. That these two compartments do possess large calcium deposits is no evidence that they will bind and
release such calcium under conditions related to the in vivo situation; such an indication was obtained by in vitro studies. The relative purity of our isolated mitochondria and vesicular fractions gives some confidence that the calcium influxes observed here under in vitro conditions could be ascribed to the separate activities of these fractions. The experiments where ATP concentration and calcium concentration in the media was varied indicate that both cell fractions possess strong ATP-dependent calcium uptake properties. The calcium efflux data from these isolated fractions are more difficult to interpret since it is impossible under in vitro conditions to stimulate efflux in a way related to cellular conditions during the contraction/relaxation cycle. However, what these influx and efflux experiments do show is that both mitochondrial and membrane vesicular fractions possess strong powers of calcium binding and release.

In mammalian skeletal muscle, studies of calcium transport and binding to sarcoplasmic reticulum and mitochondria have been carried one stage further by examination of the protein components of these membranes by polyacrylamide gel electrophoresis (MacLennan et al. 1974; Huddart, 1976; Huddart & Price, 1976). The application of this technique to our isolated fractions has revealed a striking overall similarity of gel protein profiles with both mammalian and arthropod skeletal muscle profiles. In both fractions, the bulk of the protein resolves into two major bands at between 110000–112000 mol wt and 51000–53000 mol wt. These values are about 10% higher than the corresponding figures for the Ca^{2+}/Mg^{2+} ATPase and calsequestrin or high affinity proteins of mammalian muscle sarcoplasmic reticulum (MacLennan, Yip, Isles & Seeman, 1972; MacLennan et al. 1974), these being the bands occupying similar positions in the gel profiles of mammalian muscle. The technique of polyacrylamide gel electrophoresis is subject to an error of about 10% in molecular weight determination, so the variation observed in our molluscan material could be due to the electrophoretic techniques used in this laboratory. However, our electrophoretic separations of vertebrate and arthropod skeletal muscle sarcoplasmic reticular and mitochondrial proteins give gel profiles which are in very close agreement with the figures of MacLennan and his co-workers (Huddart, 1976; Huddart & Price, 1975), and this suggests that there may be slight compositional differences between these proteins and those of our molluscan material. Only amino acid analyses of purified isolated proteins can confirm this suggestion.

So far, due to the difficulty in obtaining enough material, it has not proved possible to isolate individual proteins from molluscan muscle solubilized fractions by gel permeation chromatography in sufficient quantity to study the kinetics of their calcium binding and their composition. This is now actively under study in this laboratory.

CONCLUSIONS

Our experiments indicate that in molluscan smooth muscle, the calcium pools for 'utilizable' calcium in contraction may reside in the membrane vesicles and mitochondria. Both fractions are able to bind calcium in a capacity and manner similar to that of the sarcoplasmic reticulum of skeletal muscle. In addition, their protein profiles are strikingly similar to that of skeletal muscle sarcoplasmic reticulum, with a similarity too great to be accounted for by simple chance. How the two proposed
Fig. 10. Polyacrylamide gel protein profiles of columella muscle membrane vesicles and mitochondria after SDS electrophoresis. Note in both cases the prominence of bands at 110000-112000 daltons and 51000-53000 daltons.
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Calcium pools in these muscles interact during the contraction/relaxation cycle awaits investigation.

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