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LOCALIZATION OF CALCIUM-ACCUMULATING STRUCTURES IN THE ANTERIOR BYSSAL RETRACTOR MUSCLE OF *MYTILUS EDULIS* AND THEIR ROLE IN THE REGULATION OF ACTIVE AND CATCH CONTRACTIONS

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SUMMARY

1. The localization of Ca-accumulating structures in the anterior byssal retractor muscle (ABRM) of *Mytilus edulis* and their role in the contraction–relaxation cycle were studied by fixing the ABRM at rest or during various phases of mechanical activity with a 1% osmium tetroxide solution containing 2% potassium pyroantimonate.

2. In the resting ABRM, electron-opaque pyroantimonate precipitate was observed at the inner surface of the plasma membrane, the vesicles and the mitochondria.

3. Electron probe X-ray microanalysis showed the presence of Ca in the precipitate, indicating that the precipitate provides a valid measure for Ca localization.

4. In the ABRM fixed at the peak of mechanical response to the Ca-removal or to acetylcholine, the precipitate was found to be diffusely distributed in the myoplasm in the form of a number of particles. At the completion of spontaneous relaxation, the precipitate was again seen at the inner surface of the plasma membrane.

5. During the catch state, the precipitate was found to be re-accumulated in the peripheral structures with a corresponding decrease of the precipitate in the myoplasm.

6. These results not only provide evidence for the involvement of the Ca-accumulating structures in the contraction–relaxation cycle in the ABRM, but also suggest that the transition from active to catch contractions is related to a decrease in myoplasmic free Ca ion concentration.

INTRODUCTION

Although it is generally accepted that, in vertebrate skeletal muscle, the contraction–relaxation cycle is regulated by the release of Ca from, and its uptake by, the sarcoplasmic reticulum (e.g. Ebashi & Endo, 1968), little information is at present available about the intracellular localization of Ca and its translocation during the mechanical activity in various kinds of smooth muscle. In the preceding paper (Sugi & Yamaguchi, 1976) it has been shown that the anterior byssal retractor muscle (ABRM) of *Mytilus edulis* contains intracellularly stored Ca in amounts sufficient to fully activate the contractile mechanism. The present experiments were undertaken to determine the Ca-accumulating structures within the ABRM fibres, and to study their role in the regulation of myoplasmic free Ca ion concentration, using K pyroantimonate which is known to penetrate intact cell membrane in the presence of Os to produce electron-opaque precipitates with intracellular cations (Komnick & Komnick, 1963; Legato & Langer, 1969). The opportunity was also taken to explore the possibility that the transition from active to catch contractions is related to a change in the myoplasmic free Ca ion concentration. Preliminary accounts of this work have been reported (Atsumi & Sugi, 1973; Sugi & Atsumi, 1973*a*, *b*; Atsumi, Sugi & Aikawa, 1974).

METHODS

The experimental methods used were identical with those previously described (Sugi & Yamaguchi, 1976). The fibre bundle of 0·5–1·5 mm diameter was mounted horizontally in the experimental chamber (3–5 ml.), and attached to the strain gauge to record isometric tension not only during the experiment but also during the time of fixation. At the end of each experiment, the fibres were fixed by replacing the experimental solution with a 1% OsO_4 solution (pH 6·0–6·2 by 0·01 N acetic acid) containing 2% K pyroantimonate $K[Sb(OH)_6]$. Then, the fibres were dehydrated with ethanol, and embedded in Epon 812. Sections with a gold colour were cut on a Porter–Blum ultramicrotome, and examined with a Hitachi HU-11DS or HU-12 electron microscope unstained or stained with uranyl acetate and lead citrate.

For chemical identification of the electron-opaque precipitate of pyroantimonate salts, the sections (unstained, 2000–3000 Å in thickness) were analysed with an energy dispersive X-ray microanalyser attached to a transmission scanning electron microscope (JEOL JEM 100B-ASID). The accelerating voltage was 50 kV, and the diameter of electron beam hitting the precipitate was about 100 Å.

The general ultrastructural organization of the ABRM was also examined by fixing the relaxed fibres in (*a*) 1% OsO_4 in 0·1 M cacodylate buffer (pH 7·4), (*b*) 5% glutaraldehyde in 0·1 M cacodylate buffer (pH 7·4) with or without 9% sucrose followed by (*a*).

The experiments were performed at room temperature (16–23° C) unless otherwise stated.

RESULTS

Structure of the ABRM

Pl. 1 shows electron micrographs of the resting ABRM fibres. In agreement with the previous reports (Twarog, 1967*a*; Heumann & Zebe, 1968; Twarog, Dewey & Hidaka, 1973), the ABRM consists of spindle-shaped individual fibres of 5–7 μm diameter containing the vesicles and the mitochondria in close apposition to the fibre surface (Pl. 1*A*). The vesicles are somewhat elongated along the longitudinal axis of the fibre, and appear to be arranged in rows in the longitudinal direction (Pl. 1*B*). The gap between the membrane of the vesicles and the plasma membrane was 100–170 Å, and electron-dense bridge-like structures analogous to those in the triadic junction of vertebrate skeletal muscle (Walker & Schrodt, 1966*a*, *b*) can be observed (Pl. 1*C*). The mitochondria are also markedly elongated (Pl. 1*B*), and in some regions the gap between the mitochondrial membrane and the plasma membrane appears to be even smaller than that between the vesicles and the plasma membrane.

Localization of Ca-accumulating structures in the resting ABRM

Localization of pyroantimonate precipitate

Pl. 2 shows the cross-section of the resting fibres fixed in the pyroantimonate-osmium solution. Before fixation, the fibres were made to relax by 5-hydroxytryptamine (10^{-6} M), and kept in the standard solution (artificial sea water, Sugi & Yamaguchi, 1976) for 10–30 min. The fibres showed a slight tension development (less than 5–10% of the maximum tension) during the course of fixation. In all the fibres examined, three distinct intracellular sites of pyroantimonate precipitate deposition, i.e. (1) the plasma membrane, (2) the vesicles and (3) the mitochondria could be observed.

Plasma membrane. A continuous line of electron-opaque precipitate was observed along the plasma membrane of the resting fibres (Pl. 2*A*). Examination of the plasma membrane with higher magnifications revealed that the precipitate was localized along the inner surface but not along the outer surface of the plasma membrane (Pl. 2*B*), indicating the localization of cation binding sites at the inner surface of the plasma membrane.

Vesicles. The precipitate was seen along both the inner and outer surfaces of the unit membrane of the vesicles (Pl. 2*C*). Furthermore, it was sometimes observed that the lumen of the vesicles were completely filled with the precipitate (Pl. 2*A*), suggesting that the Ca at the vesicles can be accumulated in the lumen as free ion as well as at both sides of the membrane in the bound form.

Mitochondria. In the mitochondria, the precipitate was present at both the inner and outer mitochondrial membrane but not at the mitochondrial matrix (Pl. 2*A*).

Other intracellular structures. Besides the above structures, the electron-opaque precipitate could be observed at the nuclear membrane, in the Golgi complexes and some other intracellular membranous structures. Minute particles of the precipitate were also seen in the myoplasm (Pl. 2*A*).

Effect of removal of external Ca or Na. The precipitate at the plasma membrane, the vesicles and the mitochondria was still present if the fibres were soaked in a Ca-free solution containing 3 mM-EGTA or in a Na-free solution in which NaCl was replaced by choline Cl or sucrose for 30 min before fixation, though its amount appeared to decrease to some extent.

Electron probe X-ray microanalysis of the precipitate

Since pyroantimonate was expected to produce an electron-opaque precipitate not only with Ca, but also with other cations such as Na, the energy dispersive electron probe X-ray microanalysis of the precipitate at the plasma membrane, the vesicles and the mitochondria was performed for chemical identification of its inorganic constituents. As shown in Pl. 2*D*, the energy dispersive X-ray spectrograph exhibited distinct peaks for Ca and Sb in the range of energy levels of 3·6–4·1 keV; the left peak is composed of Lα-radiation of Sb (3·6 keV) and Kα-radiation of Ca (3·7 keV), and the right peak is composed of Lβ-radiation of Sb (3·8 keV) and Kβ-radiation of Ca (4·1 keV). The peaks for Cu and Cl are due to the grid carrying the section and to Epon embedding the specimen respectively. Meanwhile, the spectrograph of the myoplasm where no precipitate was present showed the peaks for Cu and Cl but not the peaks for Ca and Sb. The above analysis clearly indicated that the electron-opaque precipitate actually contained calcium pyroantimonate and provides a valid measure for Ca localization, though the possibility that the precipitate also contains other cations is not excluded.

Translocation of the intracellularly stored Ca during mechanical activity

Further experiments were performed in which the fibres were fixed at various phases of mechanical activity to give information about the translocation of the intracellularly stored Ca. Attention was focused on the Ca accumulated at the peripheral structures, i.e. the plasma membrane, the vesicles and the mitochondria, since these three structures are expected to be readily influenced by the electrical activity of the fibre membrane or by the action of externally applied acetylcholine (ACh), both of which are regarded to be the normal trigger for contraction.

Mechanical response to Ca removal. In the preceding paper (Sugi & Yamaguchi, 1976) it has been shown that, when the ABRM is soaked in a Ca-free solution containing less than 10 mM-Mg, a marked tension development followed by spontaneous relaxation takes place. To examine the possible translocation of Ca during the mechanical response to the Ca removal, the fibres were fixed at the peak of the maximal mechanical response to a Ca-free solution containing 0·5 mM-EGTA or EDTA and zero Mg ions. A typical result is shown in Pl. 3*A*. It can be seen that the precipitate is diffusely distributed in the myoplasm in the form of a number of particles, while little or no precipitate is present at the plasma membrane, the vesicles and the mitochondria.

If, on the other hand, the fibres were fixed immediately after the completion of spontaneous relaxation, the precipitate was observed to be again located at the inner surface of the plasma membrane, whereas the precipitate in the myoplasm was markedly decreased (Pl. 3*B*, *C*). The total amount of the precipitate within the fibre was also diminished appreciably, indicating that a large fraction of the intracellularly stored Ca is removed during the mechanical response in the Ca-free medium. The above results may be taken to indicate that Ca ions are actually released from the plasma membrane, the vesicles and the mitochondria into the myoplasm to cause contraction, and that the plasma membrane is able to re-accumulate Ca during maintained depolarization in the Ca-free solution.

Mechanical response to ACh. The fibres were made to contract maximally by ACh (10^{-4}–10^{-3} M), and fixed at the peak of active contraction in the presence of ACh. As shown in Pl. 4*A*, the precipitate was also observed to be diffusely distributed in the myoplasm in the form of particles, while the precipitate at the peripheral sites was markedly diminished, indicating that Ca ions are also released from the peripheral structures into the myoplasm by the action of ACh. Similar results were obtained on the fibres fixed at the peak of active mechanical response to ACh at low temperatures (2–5° C), except that no precipitate remained around the periphery of the fibre.

If the fibres were fixed at the early phase of tension development by ACh, the precipitate was seen both in the myoplasm and at the peripheral sites except for the vesicles, suggesting that Ca ions may be released most readily from the vesicles. It was also noticed that, at the early phase of contraction, the precipitate at the plasma membrane was observed not only at the inner surface but also at the outer surface (Pl. 4*B*), suggesting some kind of interaction between the plasma membrane and the external cations.

Meanwhile, when the fibres were fixed during the phase of spontaneous

relaxation in the presence of ACh, the precipitate was observed to re-accumulate at the three peripheral structures with a corresponding decrease of the precipitate in the myoplasm.

Catch state. To explore the possible relation between the myoplasmic free Ca ion concentration and the catch mechanism, the fibres were fixed during the catch state attained within 3–4 min after the removal of ACh, the complete decay of the active state being ascertained by the quick release method (Abbott & Lowy, 1958). At room temperatures, the catch tension at the time of fixation was always smaller than the peak tension produced by ACh by more than 20%. The rate of gradual decline of tension after the removal of ACh could be markedly reduced by lowering the temperature to 2–5° C, and it was possible to maintain the catch tension close to the peak tension produced by ACh. Qualitatively similar results were obtained at both room and low temperatures. As can be seen in Pl. 4*C*, the precipitate is again observable at the plasma membrane, the vesicles and the mitochondria, while the precipitate is markedly diminished in the myoplasm. This result seems to indicate that the transition from active to catch contractions may be related to a decrease in myoplasmic free Ca ion concentration as a result of re-accumulation of Ca by these peripheral structures.

Mechanical response to K ions. Some experiments were also done in which the fibres were fixed during the mechanical response to a solution containing 200 mM-K. The difficulty which arose in the experiments with high-K solution was that the rate of spontaneous relaxation of the mechanical response (Sugi & Yamaguchi, 1976) was so rapid even at low temperatures that the tension at the time of completion of fixation was always reduced to less than 30% of the peak tension, while the fibres during the mechanical response to Ca-removal or to ACh could be fixed with little fall in tension. Therefore, the results obtained gave no definite information about the translocation of intracellular Ca at the peak of mechanical response, though the precipitate was always observed in the myoplasm as well as at the peripheral structures of the fibre.

DISCUSSION

Evidence for the role of Ca-accumulating structure in the contraction–relaxation cycle

It has been shown that, in vertebrate smooth muscles, cation binding sites are localized in some intracellular structures such as the sarcoplasmic reticulum, the mitochondria and the plasma membrane (Somlyo & Somlyo, 1971; Goodford & Wolowyk, 1972; Zelck, Jonas & Wiegershausen, 1972; Somlyo, Somlyo, Devine, Peters & Hall, 1974), and that the

isolated microsomal fraction can accumulate Ca in the presence of ATP (Carsten, 1969; Batra & Daniel, 1971; Hurwitz, Fitzpatrick, Debbas & Landon, 1973). These results, together with the close apposition of the sarcoplasmic reticulum and the mitochondria to the plasma membrane (Somlyo, Devine, Somlyo & North, 1971; Devine, Somlyo & Somlyo, 1972), suggest the possibility that Ca ions released from the sarcoplasmic reticulum and the mitochondria during membrane activity act as the trigger for contraction (Devine, Somlyo & Somlyo, 1973). In the case of *Mytilus* ABRM, Stössel & Zebe (1968) also isolated a fraction which accumulated Ca in the presence of ATP, and Heumann (1969) observed that Ca oxalate could be precipitated in the lumen of the vesicles, indicating that the vesicles can take up Ca as does the sarcoplasmic reticulum in vertebrate skeletal muscle. In spite of the above investigations, however, it is still uncertain how the intracellular structures in various kinds of smooth muscle are actually involved in the contraction–relaxation cycle.

The present study has demonstrated not only the intracellular localization of the precipitate containing Ca in the resting ABRM but also the translocation of the precipitate during various phases of mechanical activity, providing the first experimental evidence for the role of intracellular Ca-accumulating structures in the contraction–relaxation cycle of smooth muscles. The localization of the precipitate at the three peripheral structures (Pl. 2) is completely in accord with the view of Sugi & Yamaguchi (1976) that the intracellularly stored Ca may be located at the inner surface of the fibre membrane or at the other structures electrically connected to the fibre membrane, and the marked translocation of the precipitate from these structures into the myoplasm (Pl. 3*A*) supports the suggestion that the mechanical response to Ca removal is due to the release of intracellularly stored Ca. The mode of translocation of the precipitate during the mechanical response to ACh (Pl. 4*A*) also seems to be consistent with the finding that ACh-contractures may be mainly due to the release of intracellularly stored Ca (Sugi & Yamaguchi, 1976). This implies that the Ca released from the peripheral structures, i.e. the plasma membrane, the vesicles and the mitochondria, may actually act as the trigger for normal contraction, since ACh is believed to be the mediator of the excitatory motor nerve in the ABRM (Twarog, 1954, 1960, 1967*b*; Cambridge, Holgate & Sharp, 1959). It seems possible that the role of the fibre membrane in the contraction–relaxation cycle observed in the ABRM is common to other kinds of muscles, since calcium oxalate can be precipitated along the surface membrane of vertebrate vascular smooth muscle (Zelck *et al.* 1972), and the sarcolemma of vertebrate skeletal muscle is shown to accumulate Ca in the presence of ATP (Sulakhe, Drummond & Ng, 1973).

Relation between myoplasmic free Ca ion concentration and the catch state

The re-accumulation of the precipitate in the peripheral structures during the catch state (Pl. 4*C*) suggests that the transition from active to catch contractions is in some way regulated by a decrease in myoplasmic free Ca ion concentration. Concerning the possible relation between the catch state and the myoplasmic Ca, it is of interest that the glycerol-extracted ABRM remains partly contracted if Ca-induced contraction in the presence of ATP is terminated with EGTA or EDTA, and the extracted preparation maintains passive tension with little energy expenditure (Rüegg, Straub & Twarog, 1963; Rüegg, 1971). More recently, Baguet (1973) showed that, in EDTA or Triton-X treated ABRM fibres, the catch state was spontaneously induced after the activation of the contractile mechanism with 10^{-6}–10^{-4} M-Ca, and the passive tension could be maintained with 10^{-9}–10^{-6} M-Ca, depending on both ionic strength and pH.

These results seem to be compatible with the idea that the catch tension may be maintained under a myoplasmic free Ca ion concentration which is much lower than that expected from the S-shaped relation between pCa and the active tension in the glycerol-extracted ABRM (Twarog & Muneoka, 1973; Baguet, 1973). It may be that, during the transition from active to catch contractions, the Ca ions in the myoplasm are gradually re-accumulated to their original sites of localization. The inhibitory effect of 5-HT on the catch tension (Twarog, 1954; Sugi & Yamaguchi, 1976) might be explained by assuming that the rate of decrease of myoplasmic free Ca ion concentration, which is increased by 5-HT (Hidaka, Osa & Twarog, 1967), should be below some critical value in order to produce the catch state.

Concerning the catch mechanism, Lowy & Millman (1963) and Nauss & Davies (1966) argue that both active and catch contractions are generated by the interaction between actin and myosin, while Johnson (1962), Rüegg (1971) and Heumann & Zebe (1968) are of the opinion that catch contraction is due to the interaction between paramyosin filaments and is independent of the actin–myosin system. Recent experiments of Tameyasu & Sugi (1976) have shown that the load–extension curve of the series elasticity in the ABRM is dependent on the tension at the moment of isotonic release as with vertebrate striated muscle fibres (Huxley & Simmons, 1972), and shows no significant difference between active and catch contractions, suggesting that both active and catch contractions are produced by the same actin–myosin system. On this basis, the present results imply that the linkages between actin and myosin filaments would

not break rapidly but would remain in a locked-on state to produce the catch state, when the myoplasmic free Ca ion concentration is slowly decreased after active contraction; the reduced rate of energy consumption during the catch state (Nauss & Davies, 1966; Baguet & Gillis, 1968) might reflect a reduced turn-over rate of the locked-on linkages.

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EXPLANATION OF PLATES

PLATE 1

Electron micrographs of the resting ABRM fibres. Prefixed in 5% glutaraldehyde (cacodylate buffer) with 9% sucrose, and post-fixed in 1% OsO_4 (cacodylate buffer). Stained with uranyl acetate and lead citrate.

A, cross-section showing the vesicles (V) and the mitochondria (M) in close apposition to the plasma membrane. Calibration 1 μm. × 22,000.

B, longitudinal section showing longitudinally elongated shape of the vesicles (V) and the mitochondria (M). Calibration 1 μm. × 15,000.

C, high magnification micrograph illustrating bridge-like structures (arrows) at the gap between the vesicle and the plasma membrane. Calibration 0·1 μm. × 58,000.

PLATE 2

Localization and identification of electron-opaque precipitate in the resting ABRM fibres fixed in a 1% OsO_4 solution containing 2% K pyroantimonate.

A, cross-section showing the localization of the precipitate along the plasma membrane (P), at the vesicles (V) and at the mitochondria (M). Unstained. Calibration 1 μm. × 12,000.

B, high-magnification view of the plasma membrane illustrating the localization of the precipitate at the inner surface. Arrows indicate the outer surface of the plasma membrane. Stained. Calibration 0·1 μm. × 101,000.

C, high magnification view of the vesicle. The precipitate can be seen at both the inner and outer surfaces of the membrane of the vesicle. Unstained. Calibration 0·1 μm. × 153,000.

D, X-ray spectrum of the precipitate showing the presence of Ca and Sb.

PLATE 3

Translocation of the precipitate during and after the mechanical response to a Ca-free solution containing 0·5 mM-EDTA and zero Mg.

A, cross-section of the ABRM fibres fixed at the peak of the mechanical response. The precipitate is distributed in the myoplasm in the form of particles, while it is hardly observable at the peripheral structures of the fibre. Unstained. Calibration 1 μm. × 12,000.

B, cross-section of the ABRM fibres fixed at the completion of spontaneous relaxation of the mechanical response. The precipitate is again seen along the plasma membrane. Unstained. Calibration 1 μm. × 12,000.

C, high-magnification view of the plasma membrane showing the re-accumulation of the precipitate along the inner surface. Stained with lead citrate and uranyl acetate. Calibration 0·1 μm. × 101,000.

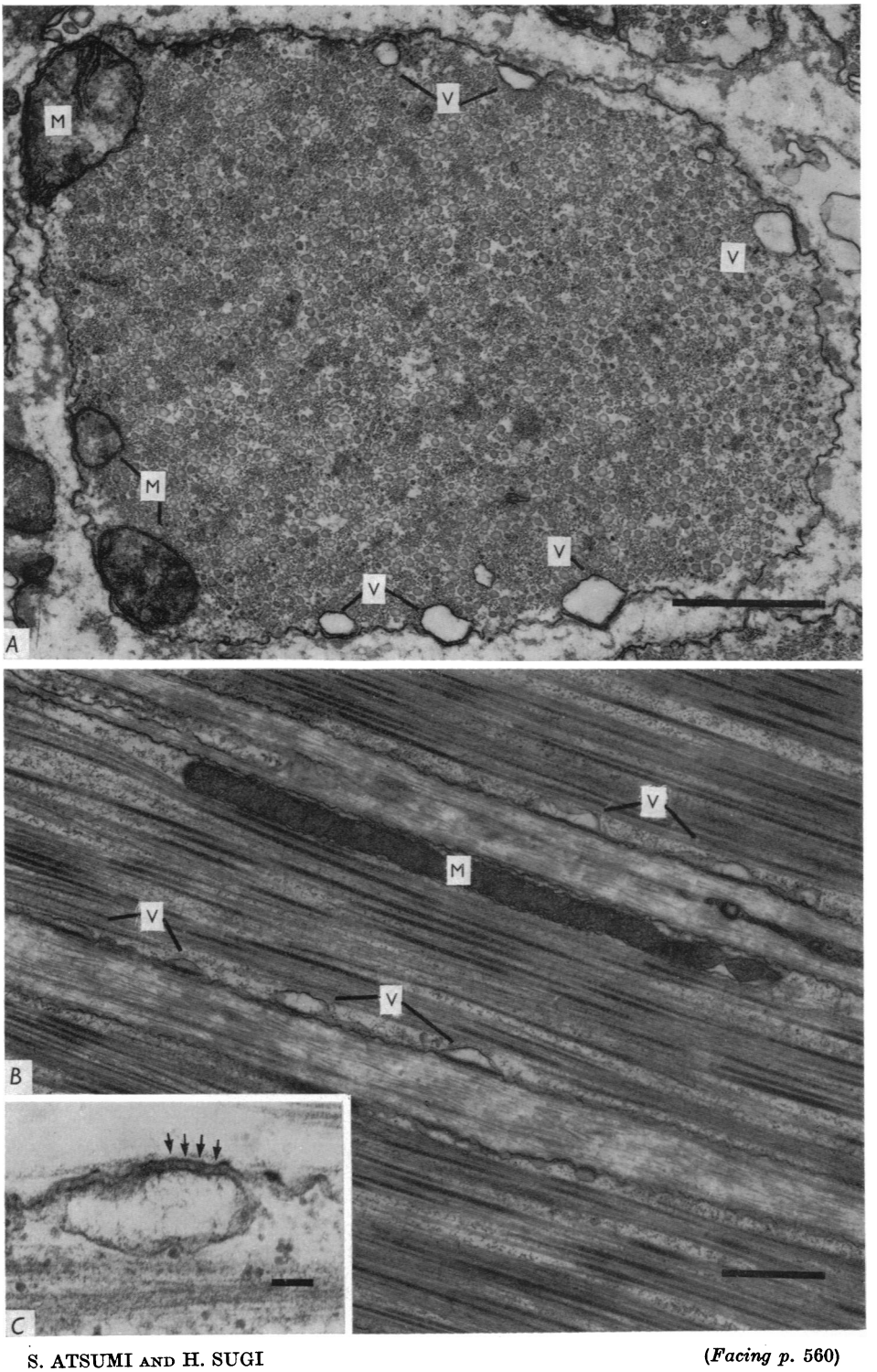
PLATE 4

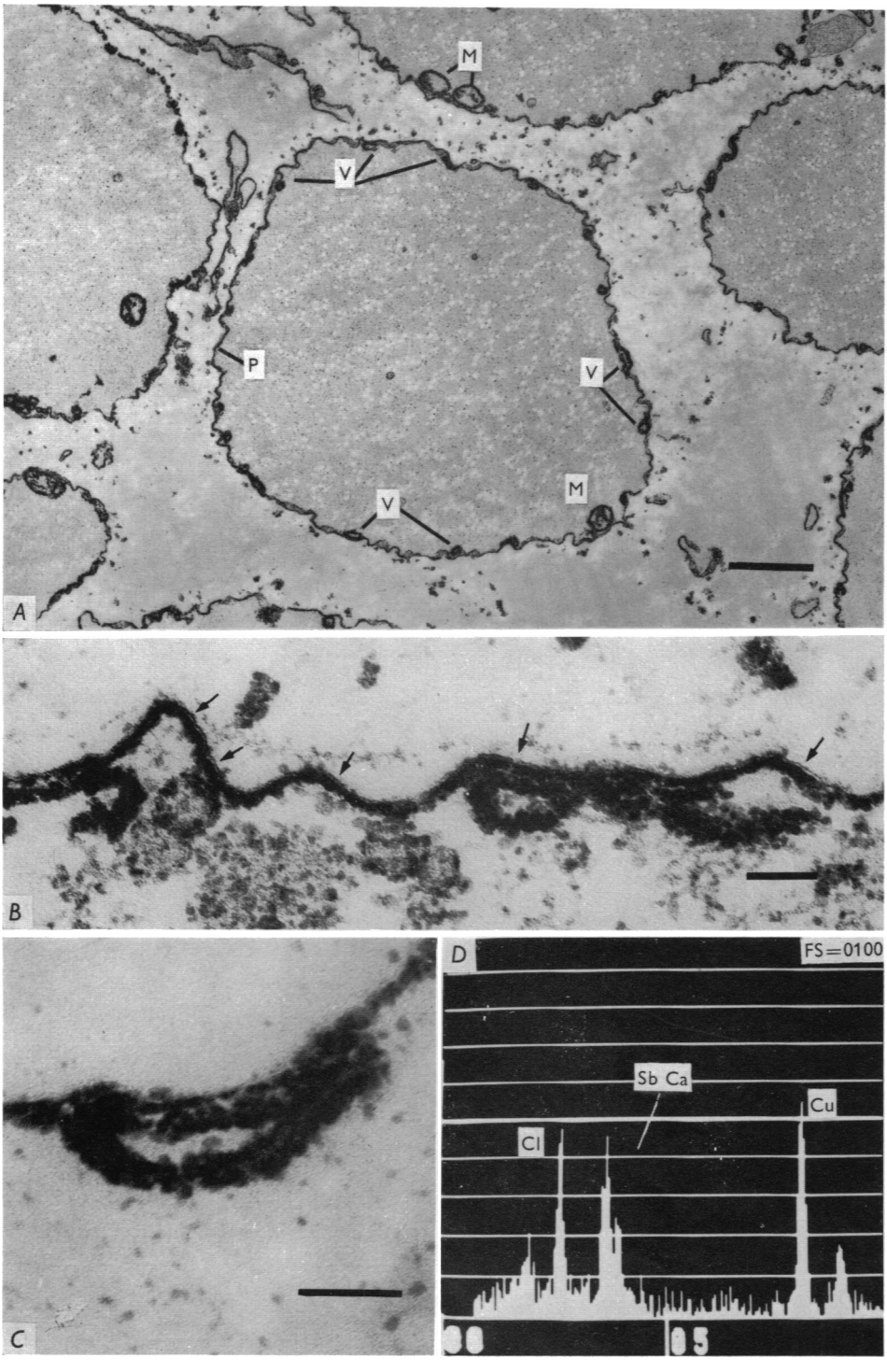
Translocation of the precipitate during active and catch contractions induced by ACh.

A, cross-section of the ABRM fibres fixed at the peak of active contraction in response to ACh. Diffuse distribution of the particles of the precipitate in the myoplasm can also be seen. Unstained. Calibration 1 μm. $\times$ 12,000.

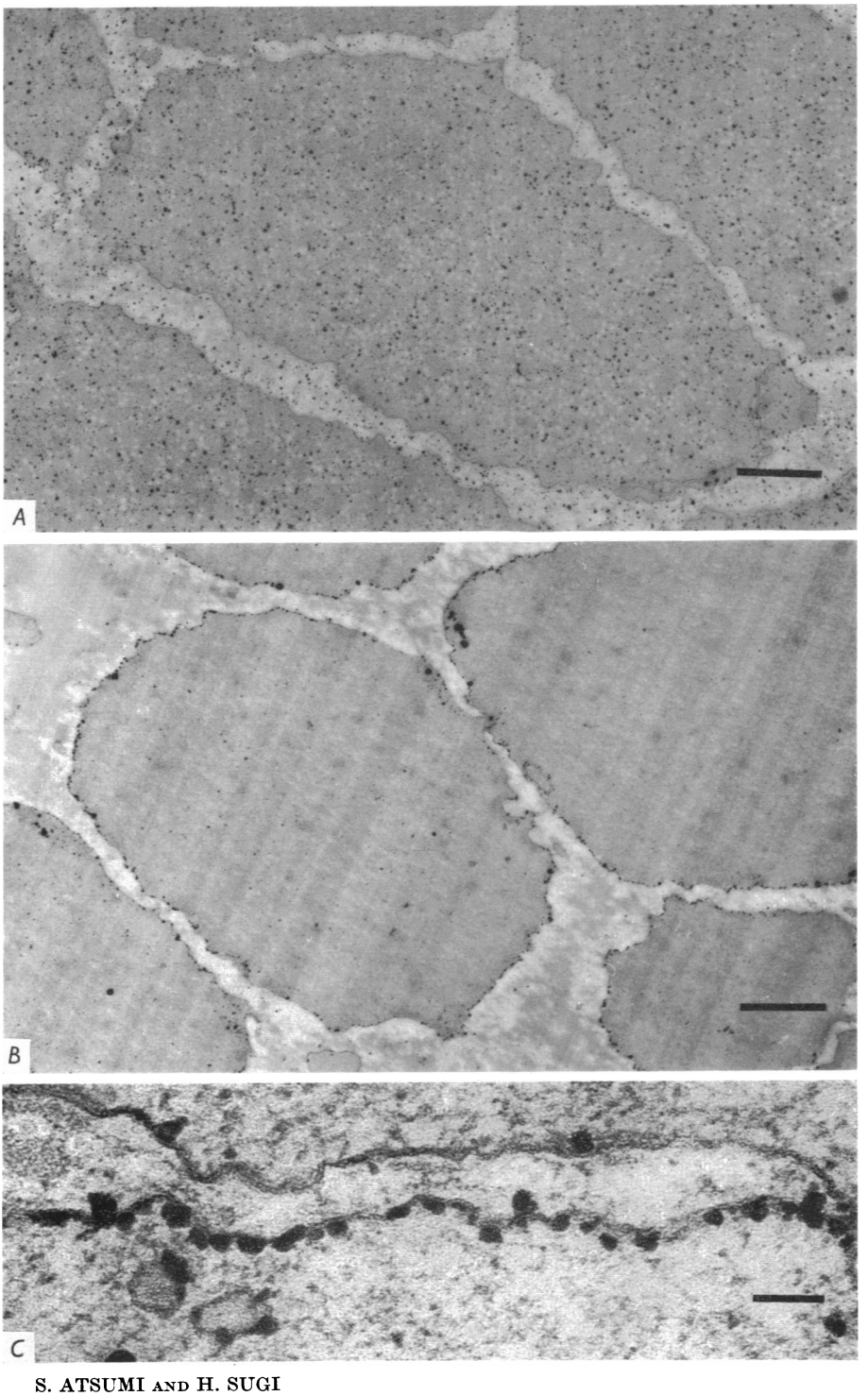
B, high magnification view of the plasma membrane of the ABRM fibre fixed at the early phase of active contraction. The precipitate can be observed at both the inner and outer surfaces of the plasma membrane (arrows). Unstained. Calibration 0·1 μm. $\times$ 101,000.

C, cross-section of the ABRM fibres fixed during catch contraction attained after the removal of ACh. The precipitate is again observable along the plasma membrane and at the vesicles (V), while the precipitate in the myoplasm is markedly diminished. Unstained. Calibration 1 μm. $\times$ 12,000.

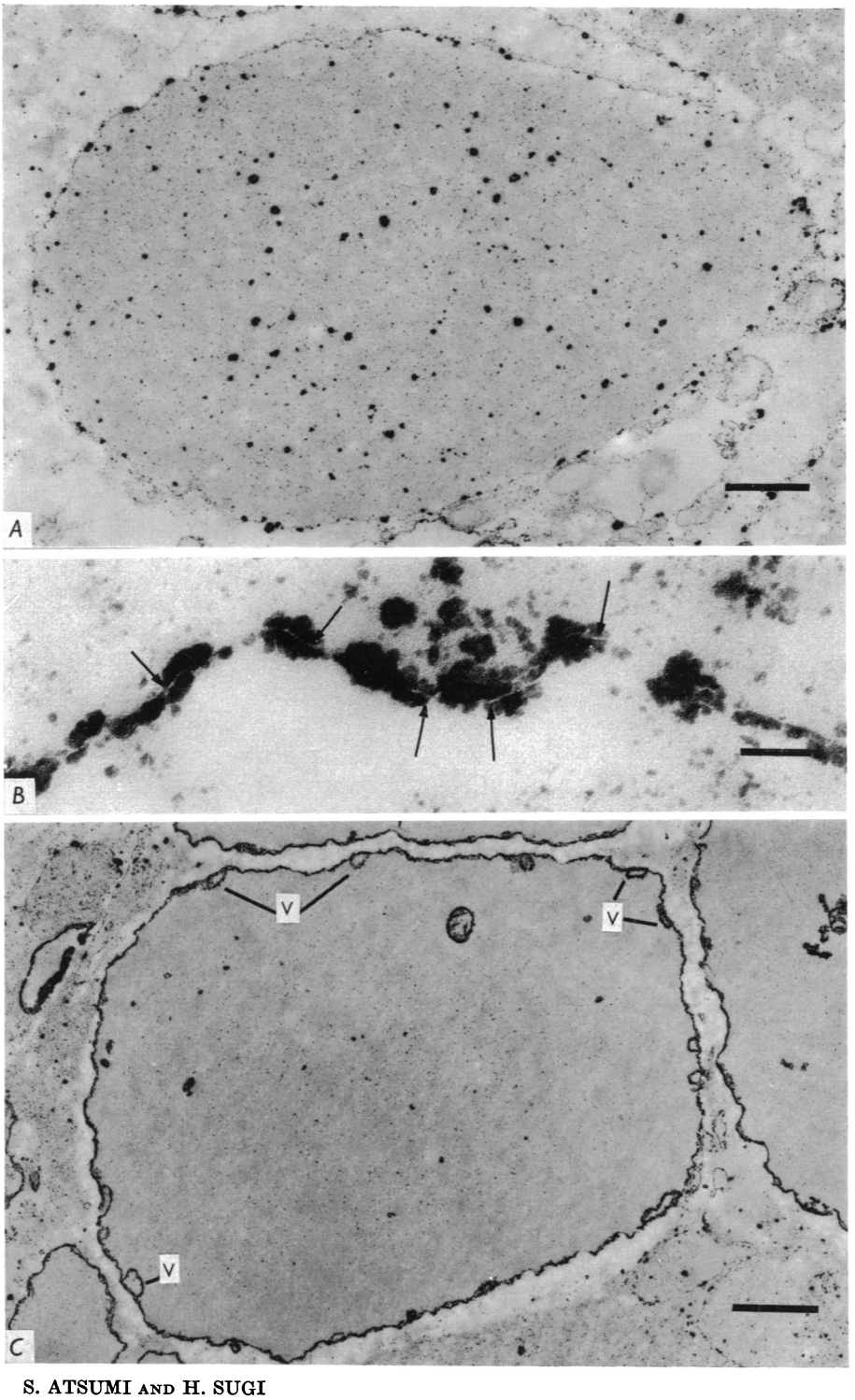

M
V
V
M
V
V
A
V
M
V
V
B
C



S. ATSUMI AND H. SUGI



S. ATSUMI AND H. SUGI


A
B
V
V
V
C