J. Physiol. (1971), **219**, *pp.* 233–251
With 2 plates and 7 text-figures
Printed in Great Britain

EXCITATION-CONTRACTION COUPLING IN AMPHIOXUS MUSCLE CELLS

BY S. HAGIWARA, MARYANNA P. HENKART AND Y. KIDOKORO
From the Department of Physiology, UCLA, Los Angeles, California 90024, U.S.A.

(*Received 5 July 1971*)

SUMMARY

1. Excitation-contraction coupling was studied in myotomal muscles of amphioxus, *Branchiostoma californiense*.

2. The action potential of a muscle cell produces a twitch with a rise time of 30–40 msec at 11° C and its Q_{10} is about 2·2.

3. The twitch increases in amplitude with increasing external Ca concentration and is abolished in Ca-free saline (1 mM-EGTA and 55·7 mM-$MgCl_2$); the twitch amplitude is suppressed by Co or La ions.

4. Caffeine at concentrations above 1 mM in the external saline causes a prolongation of the action potential and a contracture which lasts several minutes.

5. After exposure to caffeine the responsiveness of the muscle to subsequent applications of caffeine recovers in normal saline in 20–30 minutes but not in Ca-free saline.

6. The amplitude of the caffeine contracture is independent of the external Ca concentration and is unaltered after the twitch is eliminated in Ca-free saline.

7. After exposure to caffeine a full-sized twitch can be obtained before the responsiveness to caffeine shows any significant recovery.

8. It is concluded that the twitch is produced by the Ca influx resulting from the increased permeability of the muscle cell membrane to Ca during the action potential and that the Ca mobilized by caffeine is not necessary to the initiation of the twitch.

9. Electronmicroscopy shows the existence of sarcoplasmic reticulum.

INTRODUCTION

The myotome of amphioxus consists of lamella-like striated muscle cells about 1–2 μm thick. Electrophysiological studies of these muscle cells (Hagiwara & Kidokoro, 1971) show that the action potential results

primarily from an increase in Na permeability and includes an increase in membrane permeability to Ca. The Ca permeability increase should result in a significant influx of Ca and this could contribute to the initiation of a twitch. This is particularly interesting since Peachey (1961) and Flood (1968) have shown that the amphioxus muscle cell lacks transverse tubules and also probably sarcoplasmic reticulum.

The present paper is concerned with the relation between the Ca influx and contraction. We attempted to determine whether the twitch is produced exclusively by the Ca influx through the surface membrane or whether a release of Ca from intracellular stores contributes to the development of twitch tension. Tension development of single muscle cells as well as that of a muscle strip preparation was analysed under various experimental conditions. Electronmicroscopy was performed to determine the existence of possible Ca storage sites. A preliminary report has been published (Hagiwara, Henkart & Kidokoro, 1971).

METHODS

Specimens of amphioxus, *Branchiostoma californiense*, were obtained from the coast of Southern California. The preparation, method of electrical recording and salines used were similar to those described in the preceding paper (Hagiwara & Kidokoro, 1971). Caffeine was dissolved in salines at a concentration of 1–20 mM.

Mechanical recording. To record mechanical responses during the stimulation of a single muscle cell, one end of the chosen myotome was immobilized by a fine needle and the other end was impaled by a glass hook attached to a force-displacement transducer (see Text-fig. 1*C* 1). In most studies of mechanical properties, a muscle strip preparation was used. After the decapitation the animal was split into two symmetrical halves by a saggital section. Then a muscle strip was obtained by removing skin, notochord, etc. One end of the strip was immobilized by tying it with a thread to a small insect pin fixed to the floor of the chamber. The other end was connected to the stylus of the force-displacement transducer and the preparation was stretched slightly longer than its resting length (see Text-fig. 1*C* 2). Each muscle strip preparation included six to ten myotomal segments. A polyethylene tube with an inside diameter of about 0·5 mm was filled with the normal saline and a chlorided silver wire was inserted into the tube. The electrode tip was gently pressed onto the surface in the middle of the strip preparation. Negative voltage pulses were applied to the silver wire in the tube through a 2–12 kΩ resistor. A silver–silver chloride pellet electrode in the bath served as an indifferent electrode. The force-displacement transducer in conjunction with a carrier amplifier gave a sensitivity of 8 mV/mg. The range of tension observed was 1–100 mg. The rise time of the total recording system was about 5 msec which was sufficient for the present purpose. The recording condition represented approximately isometric tension. All the experiments were performed at 10–14° C.

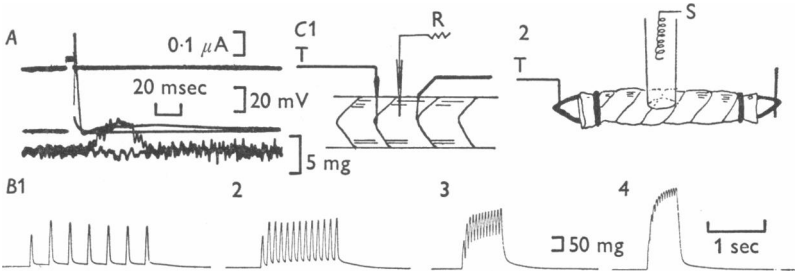
Electron microscopy. For electron microscopy the myotomal muscle of amphioxus was dissected as described above. Tissues were fixed in a solution having a final concentration of 3 g glutaraldehyde and 20 g sucrose/100 ml. of 100 mM cacodylate buffer at pH 7·4. The material was rinsed in 100 mM cacodylate buffer containing 20 g sucrose/100 ml., and post-fixed in 1 g OsO_4/100 ml. of 100 mM cacodylate buffer.

Some samples were dehydrated without treatment in OsO_4. Dehydration was in ethanol and the embedding medium was in Epon 812. Sections were cut on an LKB Ultratome, mounted on 300 mesh grids without supporting films and observed using a Siemens Elmiskop I or a Zeiss 9S-2 electronmicroscope.

RESULTS

Normal twitch and tetanus

Text-fig. 1*A* shows a simultaneous recording of an intracellular action potential produced by transmembrane polarization and the resulting twitch measured as shown in Text-fig. 1*C*1. The duration of the twitch measured between 10 % of the peak value on the rising phase and the 10 % point on the falling phase ranged from 50 to 80 msec at 10° C. For a



Text-fig. 1. *A*, simultaneous recording of the membrane potential change (middle trace) and tension development (bottom trace) of a single muscle cell when an outward current pulse (top trace) was applied through the intracellular recording pipette. The top trace also shows the potential level outside the cell. Temp. 12° C. *B*, tension development of a muscle strip preparation when the frequency of maximal stimuli (negative square-pulses of 3 msec in duration) was varied. The frequency was 3/sec in 1, 10/sec in 2, 18/sec in 3 and 25/sec in 4. Temp. 12° C. *C*, experimental arrangement of tension recording for single cell stimulation (1) and for a muscle strip preparation (2). T, stylus of transducer. S, stimulating pipette. R, recording and stimulating intracellular electrode.

stimulus frequency below 10/sec the mechanical response was a series of discrete twitches, while an almost complete tetanus was obtained at 25–35/sec (at 10° C). The peak amplitude of the twitch of a single myotome in response to intracellular stimulation of a single cell varied considerably and ranged from 4 to 10 mg. This variability could be due partly to the difficulty of recording from a single myotome and partly to variations in the number of cells to which the excitation spreads through electrotonic connections among muscle cells (Hagiwara & Kidokoro, 1971).

Text-fig. 1*B* shows mechanical responses of a muscle strip preparation (illustrated in Text-fig. 1*C*2) when trains of stimuli of various frequencies

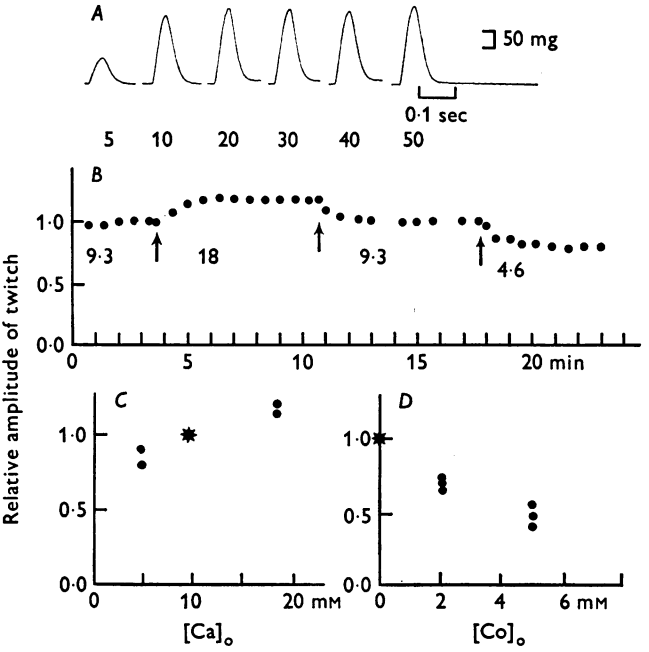
were applied through a large saline-filled external electrode. Each stimulus was a negative square-current pulse of 3 msec duration and produced a twitch. Within the range of stimulus intensities used, mechanical responses were always abolished when tetrodotoxin (TTX) was added to the saline at a concentration of 3 μM (see Text-fig. 3*A*). This indicates that the contractions are produced by means of the action potential but not by the applied current directly. The twitch duration, the maximum frequency of stimulation for a series of discrete twitches and the complete tetanus fusion frequency obtained with the strip preparation were nearly identical to those obtained with intracellular stimulation at the same temperature. This indicates that twitches recorded by the two different methods are produced by the same kind of muscle cells. Guthrie & Banks (1970) described two different types of twitches, slow and fast. The twitch studied in the present work corresponds to their fast type. The contraction time (time for the tension to reach its peak) for the twitch of the amphioxus muscle was 30–40 msec at 11° C. The contraction time of cat gastrocnemius muscle (Willis, 1942) is 20–25 msec at 37–39° C. The depressor muscle of the wing of a humming bird which is a very fast muscle has a contraction time of 8 msec at 40° C (Hagiwara, Chichibu & Simpson, 1968). With an increase in temperature from 11 to 21° C the contraction time of the amphioxus muscle decreased by a factor of about 2·2, i.e. its Q_{10} was about 2·2. Its contraction time of 30–40 msec at 11° C, therefore, indicates that the majority of the amphioxus body wall muscles are fast contracting muscles.

The amplitude of the twitch recorded from a muscle strip preparation increased with increasing stimulus intensity to a maximum. This is shown by Text-fig. 2*A*. Relative intensities are given for each trace. Maximum twitch amplitude was obtained at an intensity of 20 in this case. The amplitude of the maximal twitch of an individual preparation showed little variation during several hours of perfusion with the normal saline as long as the arrangement of the stimulating electrode was maintained. The amplitude of the maximal twitch in various muscle strips ranged from 20 to 100 mg. Effects of the ionic composition of the external saline upon the amplitude of the twitch were studied by observing changes in the maximal twitch. A decrease or increase of the amplitude of the maximal twitch was interpreted as reflecting a corresponding change in the twitch amplitude of each single muscle cell.

Effects of external Ca on the twitch

When the Ca concentration in the external normal saline was altered by replacing various amounts of $CaCl_2$ with $MgCl_2$ the amplitude of the maximal twitch increased or decreased with the Ca concentration. Text-fig. 2*B* shows the time course of the change in amplitude of the maximal

twitch during alteration of the external Ca concentration. The change in amplitude was complete within 2–3 min after application of the test solution. In some preparations the change was slightly slower but was always complete in no more than 5–6 min. Immersion of the preparation for 30 more minutes usually resulted in no significant additional change in the amplitude. Text-fig. 2*C* shows the relationship between the external



Text-fig. 2. *A*, the dependence of the amplitude of twitch on the intensity of stimulus in a muscle strip preparation. The stimulus was a negative square pulse of 3 msec duration. Relative amplitude of the pulse is listed under each trace. Temp. 12° C. *B*, the time course of change in amplitude of the maximal twitch for changes of the external Ca concentration. *C* and *D*, relationship between the amplitude of the maximal twitch and the external Ca concentration in *C* and that between the amplitude and the concentration of $CoCl_2$ in the normal saline in *D*. For *B*, *C* and *D*, the amplitude of the maximal twitch in normal saline (9·3 mM-Ca and no $CoCl_2$) is taken as unity.

Ca concentration and the amplitude of the maximal twitch in two preparations. The amplitude of the maximal twitch found in normal saline ($[Ca]_o$ = 9·3 mM) was taken as unity for each preparation. These results indicate that the amplitude of a twitch increases or decreases with the external Ca concentration.

When the Ca concentration was decreased to a much smaller value, however, the result was slightly different. When the preparation was

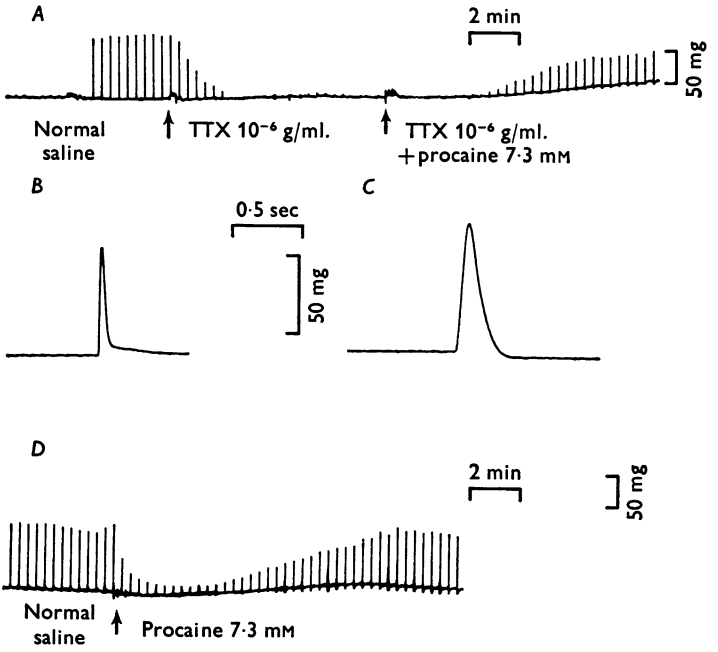
immersed in Ca-free saline (containing 57·7 mM-$MgCal_2$) the amplitude of the maximal twitch decreased within 2–3 min to about 60 % of the control obtained in the normal saline. This was followed by an additional decrease with a much slower time course. The amplitude reached 10–20 % of control in about 1 hr during which time the preparation was washed several times with fresh Ca-free saline. Immersion in Ca-free saline, for a few more hours usually resulted in no marked additional decrease, i.e. the twitch, as a rule, did not disappear in a simple Ca-free saline. When the Ca-free saline contained 1 mM-EGTA (ethyleneglycol-bis-[β-aminoethylether]-*N,N'*-tetra-acetic acid), however, complete elimination of the twitch occurred in 30–50 min (see Text-fig. 5*A* 2). At this stage the action potential of muscle cells remained unchanged (Hagiwara & Kidokoro, 1971). Upon return to the normal saline the twitch amplitude recovered to the original value within 2–3 min.

The amplitude of the maximal twitch was suppressed when Co or La was added to the external saline. Suppression reached a steady state in several minutes after the application of Co. Text-fig. 2*D* shows the result obtained when 2·5 and 5·0 mM-$CoCl_2$ were added to the normal saline. The steady-state amplitude of the maximal twitch is plotted against the Co concentration. The amplitude of the twitch in the absence of Co was taken as unity. This effect of Co or La was reversible. As mentioned above, the maximal twitch decreases very slowly if the preparation is immersed in Ca-free saline containing no EGTA and complete suppression of the twitch was not normally obtained. If 2–5 mM-$CoCl_2$ was added to the Ca-free saline the twitch disappeared completely in 30–50 min. A much smaller concentration of La was usually sufficient to obtain the same suppression as with a given concentration of Co. The above result suggests that the important Ca for the twitch is that at sites somewhere near or in the cell membrane rather than that in bulk solution. Co or La ions may occupy the same sites and suppress the twitch by reducing the Ca concentration at those sites. Small but significant twitches remain in nominally Ca-free solution. A tentative explanation is that Ca ions extruded from the interior of the cell may occupy the sites even in nominally Ca-free solution.

Contraction in the absence of the normal action potential

In the preceding paper (Hagiwara & Kidokoro, 1971) it has been shown that procaine restores a regenerative potential change after elimination of the normal action potential either by removal of the external Na or by the addition of tetrodotoxin (TTX). The potential is produced as a result of a Ca permeability increase of the membrane. The procaine-induced Ca action potential was associated with contraction. In the experiment shown in Text-fig. 3*A* maximal twitches were produced in normal saline at a rate

of 1/20 sec and then TTX was added to the saline at a concentration of 3 μM. This abolished the twitch in 2–3 min. After complete abolition of the twitch, procaine (7·3 mM) was added to the TTX-containing saline. Twitch-like contractions re-appeared and the amplitude of the maximal contraction increased gradually, reaching a final steady amplitude 8–10 min after addition of procaine. These represent contractions produced by procaine-induced Ca action potentials. The amplitude of the maximal



Text-fig. 3. Effects of TTX and procaine on the amplitude of the maximal twitch elicited by a negative voltage pulse of 3 msec duration at 1/20 sec in a muscle strip preparation. *A*, TTX was first applied and then procaine was added. Temp. 12° C. *B* and *C*, the maximal twitch in the normal saline (*B*) and the maximal contraction obtained after TTX and procaine (*C*) obtained with the same muscle strip preparation. Temp. 14° C. *D*, procaine was applied without previous application of TTX. Temp. 11° C.

contraction produced by the procaine-induced Ca action potential was usually comparable to that of the maximal twitch obtained with the same preparation in normal saline. The time course of the former, however, was much slower than that of the latter. This is shown by Text-fig. 3*B* and *C*. This probably corresponds to the longer duration of the procaine-induced Ca action potential. In the experiment shown in Text-fig. 3*D*, procaine (7·3 mM) was applied in the absence of TTX. The amplitude of twitch was

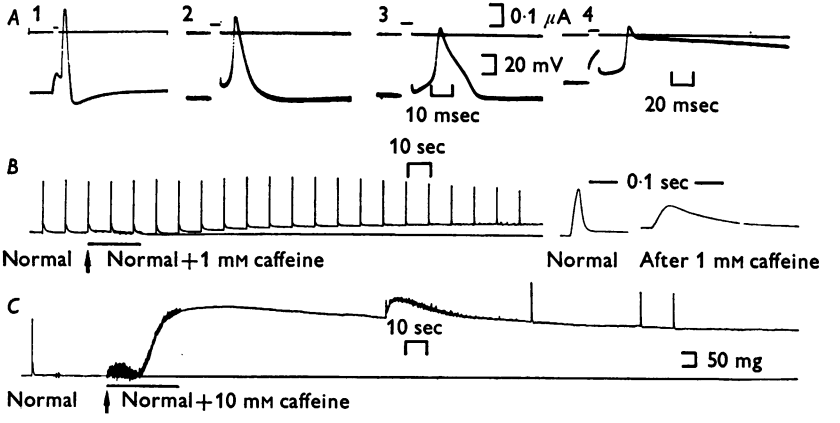
first reduced, and this effect was complete in 2–3 min. This is presumably due to elimination of the Na action potential by procaine. This initial decrease in the amplitude of the maximal twitch was followed by a slow recovery corresponding to the development of a procaine-induced Ca action potential. These results show that the amplitude of the maximal contraction associated with the procaine-induced Ca action potential is comparable to that of the maximal twitch when the Ca concentration is the same. This suggests that a similar number of Ca ions moves into the muscle cells in these two cases.

Effects of caffeine

Action potential. The addition of caffeine to the normal saline produced a marked prolongation of the falling phase of the action potential. The effect was observed at 0·5 mM and became more marked as the concentration was increased. Text-fig. 4*A* shows the action potential in normal saline. *A* 2 and *A* 3 were obtained from two other cells of the same preparation after caffeine was added at concentrations of 2·5 and 5·0 mM respectively. In some cases, the initial peak was followed by a plateau as shown by Text-fig. 4*A* 4 (obtained at 2·5 mM caffeine). The effect tended to become more marked with time in caffeine solution. Prolongation of the action potential was usually associated with a reduction in peak amplitude of the action potential. For caffeine concentrations lower than 5 mM, no marked changes were seen in the resting potential. The prolongation of the action potential by caffeine found in amphioxus muscle is remarkable since in other muscles caffeine produces a contracture with little effect on the electrical properties of the muscle fibre membranes (Axelsson & Thesleff, 1958). Etzensperger (1957) showed a slight prolongation of action potential in frog skeletal muscle fibre in 1·9–3·7 mM caffeine. This is, however, much less marked than that found in amphioxus muscle cells. The ionic mechanism of the prolongation by caffeine was not analysed in the present study. The prolonging effect of caffeine on the action potential was completely suppressed by 5 mM-$CoCl_2$ in the external saline.

Mechanical properties. The application of caffeine resulted in a contracture. This was seen at caffeine concentrations as low as 1 mM (Text-fig. 4*B*). At this concentration no marked change was seen in the amplitude of the maximal twitch in the early period of the contracture. As the contracture developed, the amplitude of the twitch tended to decrease. This result differs from that in frog twitch muscle where a small amount of caffeine augments the twitch amplitude (Lüttgau & Oetliker, 1968). The decrease in the twitch amplitude during the caffeine contracture in amphioxus muscle was associated with a marked increase of the twitch duration as shown by Text-fig. 4*B* (right). This is probably due at least in part to corresponding changes in the action potential in caffeine. With

higher caffeine concentrations such as 10 mM, the contracture developed more rapidly and reached a much higher amplitude (Text-fig. 4*C*). A single shock applied to the preparation relatively early in the contracture sometimes resulted in a prolonged contraction superimposed on the contracture as shown by Text-fig. 4*C*. Irregular oscillations were often seen in the tension during the prolonged contraction. The amplitude of a twitch

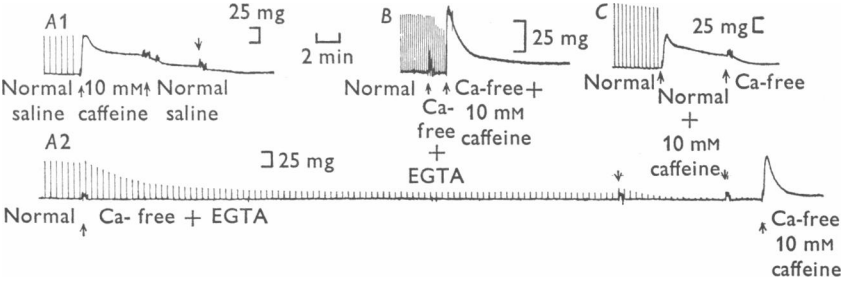


Text-fig. 4. *A*, action potentials of amphioxus muscle cells in normal saline containing caffeine. Caffeine concentration, 0 mM in *A* 1, 2·4 mM in *A* 2 and *A* 4, and 5 mM in *A* 3. Records *A* 1–3 were obtained from different cells in the same preparation. The upper trace of each record shows the potential level outside the cell and the applied current. *B* and *C*, effects of caffeine in normal saline on the tension development of a muscle strip preparation. The caffeine concentration was 1 mM in *B* and 10 mM in *C*. Maximal twitches are also shown. Irregular oscillation on the tension recording just after the time indicated by an arrow is an artifact due to the exchange of the external solution. Temp. 12° C. Two traces on the right-hand side of *B* show maximal twitches of the same preparation before and after the application of 1 mM caffeine in normal saline.

superimposed on the contracture became smaller with time. Once the contracture reached maximum, it decayed even in the presence of caffeine. When the preparation was brought back to the normal saline containing no caffeine, this decay was accelerated. Upon returning to the normal saline, the amplitude of the twitch was restored. When the preparation was kept in normal saline more than 30 min after exposure to 10 mM caffeine, a subsequent exposure to 10 mM caffeine resulted in a similar contracture. No recovery, however, was seen when the preparation was kept in Ca-free saline (no EGTA). The time course of the recovery will be described later in detail.

Caffeine contracture and external Ca

The caffeine contracture in amphioxus muscle can be interpreted in either of the following ways. (1) Caffeine increases the Ca influx through the surface membrane without significantly changing the membrane potential, or (2), caffeine releases Ca from some storage site inside the muscle cells. To distinguish these possibilities, caffeine contracture was observed in Ca-free saline containing 1 mM-EGTA. Text-fig. 5*A* 1 shows a contracture produced by 10 mM caffeine in normal saline. After incubation of the preparation in normal saline for 30 min the same preparation was immersed in Ca-free saline containing 1 mM-EGTA (Text-fig. 5*A* 2). The



Text-fig. 5. Effects of the external Ca concentration on caffeine contracture. *A* 1 and *A* 2 were obtained from the same preparation. *A* 2 was taken after *A* 1 and the preparation was kept in normal saline for 30 min between the two recordings. In all recordings maximal twitches were obtained with negative square pulses of 3 msec in duration. 15° C for *A*, 11° C for *B* and *C*. Fast irregular oscillation seen just after the time indicated by an arrow is an artifact due to the exchange of the external solution.

amplitude of the maximal twitch decreased progressively in this solution. Occasional washing with fresh solution accelerated this decrease, as shown by the two downward directed arrows. After the twitch was abolished, 10 mM caffeine in Ca-free saline (no EGTA) was applied. It was found that the peak amplitude of the contracture was essentially unchanged by this treatment. The result, therefore, suggests that the caffeine contracture is produced by the release of Ca from some intracellular storage.

Although the peak amplitude was not altered significantly, the rate of decay of contracture became much faster in Ca-free saline. Text-fig. 5*A* 1 shows that the contracture produced by 10 mM caffeine in normal saline declined in the presence of caffeine with a half-decay time of about 300 sec (14° C). After the elimination of twitches in Ca-free saline the half-decay time became about 40 sec (14° C) as shown by Text-fig. 5*A* 2. The shortening of contracture after treatment with Ca-free saline was a constant phenomenon. Half-decay times of contracture produced by 10 mM

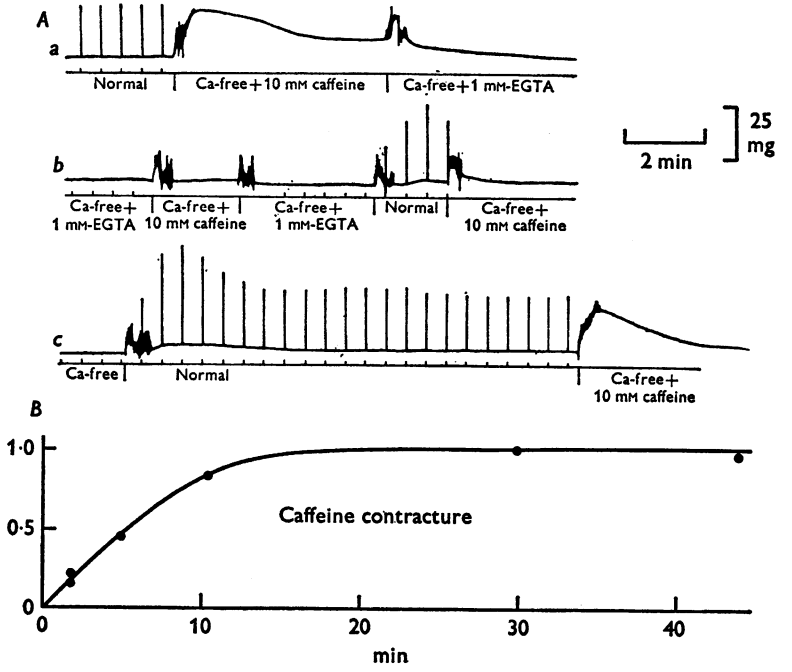
caffeine observed in five other preparations in normal saline were 300, 400, 240, 240 and 350 sec whereas those obtained with three other preparations after the elimination of twitches in Ca-free saline containing 1 mM-EGTA were 65, 35 and 20 sec. All of these were obtained at 13–15° C. In the case shown in Text-fig. 5*B*, the preparation was immersed only 1 min in Ca-free saline containing EGTA and the amplitude of the maximal twitch was reduced by only 25%. This was followed by the application of 10 mM caffeine in Ca-free saline (no EGTA). A substantially faster decay was observed (100 sec half-decay time). The above result indicates that the rate of decay of contracture depends on the external Ca concentration during contracture. In the experiment shown in Text-fig. 5*C*, 10 mM caffeine was first applied in normal saline and this resulted in a contracture of a slow decay. During the decaying phase of contracture, Ca-free saline (no EGTA) containing 10 mM caffeine was applied and this accelerated the rate of decay significantly. This suggests that low external Ca concentration enhances Ca extrusion from inside the muscle cell during caffeine contracture. These results also show that the amplitude and time course of the caffeine contracture have no correlation with the diminution of the amplitude of the maximal twitch. The finding suggests that they depend on different sources of Ca, i.e. the twitch depends on the Ca at sites in or near the surface membrane whereas the caffeine contracture depends on Ca in some intracellular storage site.

Dependence of the twitch on the caffeine-sensitive Ca

The preceding results suggest that the twitch amplitude depends primarily on Ca at sites near or in the muscle cell membrane. However, they do not exclude the possibility that the twitch amplitude depends on Ca in intracellular storage sites as well. Based upon a series of experiments with skinned muscle fibres of a frog, Ford & Podolsky (1970) and Endo, Tanaka & Ogawa (1970) have proposed that the release of intracellularly stored Ca is triggered by an increase of the Ca concentration in the vicinity of the storage site. The Ca influx through the surface membrane in amphioxus muscle cells increases the internal Ca concentration and this may trigger release of Ca from the storage sites. Since the release should be eliminated when the Ca influx vanishes no twitches should be elicited in Ca-free saline containing EGTA even though the Ca in the storage site is intact. The following experiment was performed to find out if there is any dependence of the twitch upon stored Ca which is sensitive to caffeine.

The responsiveness of the preparation to caffeine was examined by observing contractures produced by 10 mM caffeine in Ca-free saline containing no EGTA. Text-fig. 6*Aa* shows a control response to the test solution obtained in normal saline. Twitches elicited before the application

of caffeine gave the control amplitude of the maximal twitch. After the contracture had decayed the preparation was kept in Ca-free saline with 1 mM-EGTA for 30–50 min. The preparation became incapable of producing twitches (Text-fig. 6*Ab*). A subsequent application of the 10 mM caffeine test solution produced no contracture (Text-fig. 6*Ab*). If a slight response was seen the preparation was kept in the caffeine test solution for



Text-fig. 6. Effect of depletion of caffeine-sensitive Ca upon the amplitude of maximal twitch. *Aa–c* were obtained from the same preparation. The lower trace of each record indicates the timing of stimulation for the maximal twitch as well as that of exchange of the external solution. Fast irregular oscillations are artifacts due to the exchange of the external solution. Stimulus pulses did not produce twitches in Ca-free solution containing 1 mM-EGTA. 12° C. *B*, the time course of recovery of caffeine contracture. The amplitude of contracture produced by 10 mM caffeine in Ca-free saline (no EGTA) is plotted against the incubation time in normal saline. The amplitude obtained in normal saline before applying various test solutions was taken as unity.

10 min and then in Ca-free saline with 1 mM-EGTA containing no caffeine for 10–20 min. The responsiveness of the preparation to 10 mM caffeine was then totally eliminated. At this stage no contracture was seen even when the caffeine concentration was raised to 30 mM. When the preparation was brought back to normal saline the responsiveness to caffeine and the twitch both recovered. The purpose of the present experiment was to

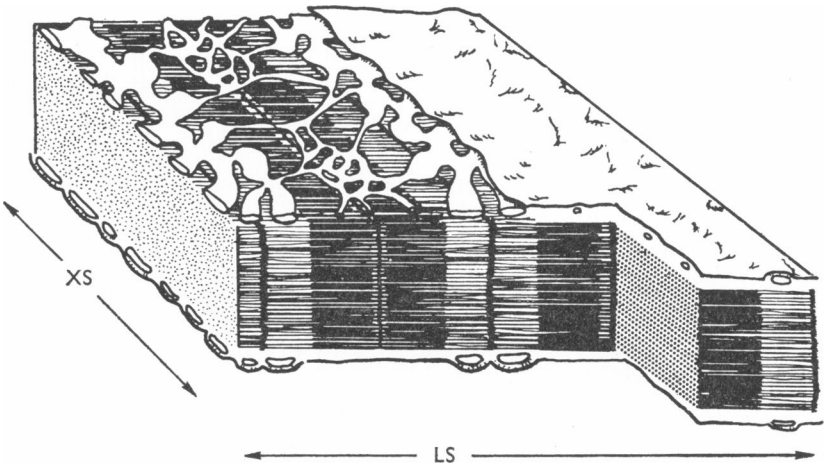
compare the time courses of their recoveries. Text-fig. 6*A b* and *c* show contractures produced by the 10 mM caffeine test solution after the preparation was kept in normal saline for 2 and 14 min respectively. The amplitude of the contracture showed only a slight recovery in *b* but the recovery was almost complete in *c*. In Text-fig. 6*B* the amplitude of the caffeine contracture to the test solution was plotted against the time during which the preparation was allowed to recover in normal saline before the application of the test solution. The control amplitude was taken as unity, and the data were obtained with two preparations.

The time course of recovery in the amplitude of the maximal twitch in the normal saline is seen in Text-fig. 6*A b* and *c*. A supernormal phase was found before the amplitude returned to the control value. A prolonged perfusion of the preparation with Ca-free saline containing no caffeine did not eliminate the supernormal phase. When application of caffeine was omitted the amplitude recovered without supernormal phase and the recovery was complete in 2–3 min. This indicates that the supernormal phase is due to the preceding application of caffeine. Although the recovery was complicated by the supernormal phase in the present case, the result definitely shows that the amplitude of the twitch recovers almost completely before the responsiveness of the preparation to caffeine shows any significant recovery. The finding leads to a conclusion that the amplitude of the twitch does not depend on the Ca which is mobilized by caffeine.

Sarcoplasmic reticulum in amphioxus muscle

Since the mechanism by which caffeine produces a contracture is thought to be by releasing Ca from the sarcoplasmic reticulum (Weber & Herz, 1968), and since a caffeine contracture was easily evoked in amphioxus muscle even in Ca-free solutions, it seemed worthwhile to re-examine the fine structure of this muscle. Electronmicroscope studies of the body wall muscle of amphioxus have been done by Peachey (1961) and by Flood (1968), and we will, therefore, not discuss the morphology in detail except for the point on which our results differ from those of the previous studies. In amphioxus muscle a transverse tubular system is lacking, but our results indicate the presence of a sarcoplasmic reticulum. Text-fig. 7 is a drawing indicating the distribution of SR over the lamellar amphioxus muscle cell. Pl. 1 is a somewhat oblique cross-section and Pl. 1*b* is a longitudinal section of the narrow dimension of several lamellae. In such sections cisternae are apparent immediately beneath and in specialized contact with the surface membrane, principally at the levels of the I-bands. *En face* views (Pl. 1*c* and *d* and 2*a*) indicate that the cisternae are actually sections through a reticulum that envelops each lamella. The lumen of this reticulum which surrounds each I-band and forms areas of association with the

surface membrane is continuous in many places with the lumen of a smaller diameter reticulum that overlies the A-bands at intervals. The SR at the level of the A-band rarely makes contact with the surface membrane. Ribosomes are often seen in association with portions of the SR membrane, particularly in the A-band region, but also occasionally on membranes of the cisternae over the I-bands (Pl. 2*a*). It was not determined to what extent the lumina of the elements of the SR are continuous over a whole muscle lamella.



Text-fig. 7. A reconstruction of a portion of one lamella of amphioxus muscle designed to indicate the approximate distribution of the sarcoplasmic reticulum. The cell membrane has been removed from the surface of one sarcomere showing a small diameter reticulum overlying the A-band and a system of interconnected cisternae overlying the I-bands and Z-lines. The number of inter-connexions between the two portions of the sarcoplasmic reticulum are not known. LS and XS indicate longitudinal and cross-sections.

The areas of association between the SR and the surface membrane (Pl. 2*b*) are characterized by the approach of the membranes to a distance of 120–160 Å and somewhat increased electron opacity in the space between the two membranes. In some cases periodic densities appear to extend between the two membranes. The content of the SR cisternae is a granular or finely fibrous material which is generally similar in appearance to the content of the terminal cisternae of the SR of frog twitch muscle (Peachey, 1965). There are often small amounts of an extracellular amorphous material in the vicinity of the areas of association between the SR and the surface membrane.

When 5 mM-Ca is added to the fixative or the muscle is exposed to a solution containing isotonic Ca before fixation, the contents of the sarcoplasmic reticulum appear in many places to be condensed into very dense

granules (Pl. 2*c*). The density of these granules is not due to staining with lead or uranyl salts since dense granules also appear in unstained sections. Although the mechanism of the production of the granules is unknown, it appears likely that it is in some way related to Ca accumulation by the SR.

Given the effects of lanthanum on the muscle contraction and on the procaine-induced Ca action potential (Hagiwara & Kidokoro, 1971) it was of interest to study its distribution using the electronmicroscope. The binding of La under physiological conditions can produce a specific distribution of La particles visible in the electronmicroscope. This has been studied in detail in the giant muscle fibres of the barnacle, *Balanus nubilus*, and the experiments and arguments in favour of the specificity and usefulness of the method will be published elsewhere (M. P. Henkart, in preparation). Amphioxus muscle in which the contraction and, hence, the membrane conductance increase to Ca ions had been suppressed by 1 mM-La was transferred to glutaraldehyde fixative without the addition of further La. Osmium post-fixation was omitted. Material prepared under these conditions showed an accumulation of La in the regions of association between the SR and the surface membrane (Pl. 2*d*).

DISCUSSION

The present experimental results obtained for the twitch of amphioxus muscle cells show that (1) the peak amplitude decreases with decreasing external Ca concentration, (2) the removal of the external Ca by 1 mM-EGTA in Ca-free saline eliminates twitches, and (3) the twitch amplitude is suppressed by Co or La ions in the external saline. The experimental results described in the preceding paper (Hagiwara & Kidokoro, 1971) show that during the normal action potential, which is primarily produced by an increase of the membrane Na permeability, there is an increase of the membrane permeability to Ca ions. The dependence on the external Ca concentration, EGTA, Co or La is nearly identical to that found for the twitch, i.e. the Ca influx decreases with decreasing Ca concentration, is suppressed by Co or La and eliminated in Ca-free saline with 1 mM-EGTA. Neither the change in Ca concentration nor the application of Co, La or EGTA, however, alters the amplitude or the shape of the normal action potential significantly, at least over the ranges of concentration used for these experiments. These findings lead to a conclusion that the twitch is produced primarily by the influx of Ca ions through the cell membrane as a result of an increase of the membrane permeability to Ca ions. The experimental analyses suggest that the Ca influx depends on the Ca concentration at sites somewhere near or at the cell membrane rather than the concentration in bulk solution (Hagiwara & Kidokoro, 1971).

The experimental results show that there is a source of Ca which is mobilized by caffeine. The amount of this Ca is independent of changes of the external Ca concentration lasting at least 1–2 hr. Caffeine contractures are found in frog twitch muscle fibres and this is thought to be due to a release of Ca from intracellular storage sites. The same Ca is presumably released during an action potential and this provides the major portion of the Ca for twitch initiation (Ebashi & Lipmann, 1962; Jöbsis & O'Connor, 1966; Winegrad, 1970; Ashley & Ridgway, 1970). In amphioxus muscle, however, the caffeine-sensitive Ca does not seem to be necessary for the initiation of a twitch since a full-sized twitch can be obtained even after the caffeine-sensitive Ca has been depleted. It is concluded, therefore, that the twitch of amphioxus muscle is produced exclusively by the influx of Ca ions through the cell membrane. This coupling mechanism is considered inadequate to explain the short delay between the action potential and the twitch in muscle fibres of relatively large diameters such as frog twitch or crustacean muscle fibres (Hill, 1948). In these preparations the delays found are always much shorter than those expected from diffusion of ions from the surface membrane to the interior of such fibres. This need not be considered a difficulty in amphioxus muscle, however. Because of their small thickness, time spent by diffusion should be negligible compared with actual delays found between an action potential and a twitch. The preceding work (Hagiwara & Kidokoro, 1971) has shown that the increase of the Ca permeability occurring during the action potential probably increases the internal Ca ion concentration at least up to $0{\cdot}7–1{\cdot}5 \times 10^{-5}$ M per action potential. It has been shown that a contraction occurs in frog skeletal muscle fibres when the internal Ca ion concentration exceeds about 10^{-6} M. This effect of Ca saturates at about $1–3 \times 10^{-5}$ M (Ebashi, Endo & Ohtsuki, 1969). Somewhat lower values have been obtained by other authors in frog skeletal and crustacean muscle fibres (Hellam & Podolsky, 1966; Portzehl, Caldwell & Rüegg, 1964; Hagiwara & Nakajima, 1966). The above difference may be due partly to the difference in estimation of binding constant of EGTA used for Ca ion buffers. The figure of $0{\cdot}7–1{\cdot}5 \times 10^{-5}$ M per action potential in amphioxus may be slightly low but it does not seem to reject the above conclusion.

Peachey (1961) and Flood (1968) have studied the fine structure of the muscle of amphioxus myotomes and described them as lacking sarcoplasmic reticulum. The present results show that there is a sarcoplasmic reticulum in amphioxus. A possible reason for the failure of the previous authors to recognize this is that they used osmium fixation which apparently collapsed the SR cisternae. At the present stage we have no direct experimental evidence to correlate our electrophysiological result to the sarcoplasmic reticulum. As mentioned above, the experimental result shows that

there are two sources of Ca in amphioxus muscle cells. The first Ca is directly related to the initiation of a twitch and this Ca can be replaced by lanthanum. The fact that no La particles are found inside the SR after lanthanum staining suggests that the above Ca is not located inside the SR. La particles were found near the surface membrane. This may indicate that the sites for the above Ca are located near the surface membrane. A marked accumulation of lanthanum particles is found in the region of coupling between the SR and the surface membrane. The fact may indicate that this region of the membrane plays an important role for the Ca entry. Our present data is, however, insufficient for any further interpretation of this fact.

It is known that the caffeine sensitive Ca is located in the SR in frog skeletal muscle fibres (Weber & Herz 1968). If the Ca in the amphioxus SR exclusively represents caffeine–sensitive Ca the present result indicates that the Ca in SR is not necessary for the initiation of twitch in amphioxus muscle. This may further indicate that the primary role of the SR in amphioxus muscle is the sequestration and extrusion of Ca ions. In muscle treated with high Ca before fixation or fixed in a solution containing Ca the SR appears swollen and dense granules frequently appear in the vesicles. Although the mechanism of the production of the granules is not clear, it appears in some way related to calcium accumulation by the vesicles. This suggests that the vesicles in association with the surface membrane in amphioxus muscle are capable of accumulating calcium, as are elements of the SR in other muscles.

Significant contribution of Ca ions entering through the surface membrane to the contraction has been suggested in a few other striated muscles, such as in crustacean muscles (Orkand, 1962; Hagiwara, Takahashi & Junge, 1968; Chiarandini, Reuben, Brandt & Grundfest, 1970). Chiarandini *et al.* (1970) have concluded that there are two sources of Ca for contraction. The one is mobilized by caffeine and is likely to be located in the SR, and the other is mobilized by depolarization and strongly dependent on the entry of Ca from the external medium. These two sources are very similar to those found in amphioxus muscle. However, in crustacean muscle fibres there seems to be a definite contribution to the twitch of Ca ions released from intracellular storage. In contrast, practically no contribution to the twitch of Ca from storage sites is necessary in amphioxus muscle cells.

The authors wish to express their indebtedness to Drs A. D. Grinnell and R. K. Orkand for their criticism while the manuscript was in preparation. This research was aided by U.S. Public Health Service Grant NS 09012 to Dr Hagiwara and by post-doctoral fellowships 1F2 NB 41,652 to Dr Henkart.

REFERENCES

ASHLEY, C. C. & RIDGWAY, E. B. (1970). On the relationships between membrane potential, calcium transient and tension in single barnacle muscle fibres. *J. Physiol.* **209**, 105–130.

AXELSSON, J. & THESLEFF, S. (1958). Activation of the contractile mechanism in striated muscle. *Acta physiol. scand.* **44**, 55–66.

CHIARANDINI, D. J., REUBEN, J. P., BRANDT, P. W. & GRUNDFEST, H. (1970). Effects of caffeine on crayfish muscle fibres. I. Activation of contraction and induction of Ca spike electrogenesis. *J. gen. Physiol.* **55**, 640–664.

EBASHI, S., ENDO, M. & OHTSUKI, I. (1969). Control of muscle contraction. *Q. Rev. Biophys.* **2**, 351–384.

EBASHI, S. & LIPMANN, F. (1962). Adenosine triphosphate-linked concentration of calcium ions in a particulate fraction of rabbit muscle. *J. cell Biol.* **14**, 389–400.

ENDO, M., TANAKA, M. & OGAWA, Y. (1970). Calcium induced release of calcium from the sacroplasmic reticulum of skinned skeletal muscle fibres. *Nature, Lond.* **228**, 34–36.

ETZENSPERGER, J. (1957). Modifications du potentiel d'action de la fibre musculaire striée provoquées par la caféine et la quinine. *C. r. Séanc. Soc. Biol.* **151**, 587–590.

FLOOD, P. R. (1968). Structure of the segmental trunk muscle in Amphioxus. *Z. Zellforsch. mikrosk. Anat.* **84**, 389–416.

FORD, L. E. & PODOLSKY, R. J. (1970). Regenerative calcium release within muscle cells. *Science, N.Y.* **167**, 58–59.

GUTHRIE, D. M. & BANKS, J. R. (1970). Observations on the electrical and mechanical properties of the myotomes of the lancelet (*Branchiostoma lanceolatum*). *J. exp. Biol.* **52**, 401–417.

HAGIWARA, S., CHICHIBU, S. & SIMPSON, N. (1968). Neuromuscular mechanisms of wing beat in hummingbirds. *Z. vergl. Physiol.* **60**, 209–218.

HAGIWARA, S., HENKART, M. P. & KIDOKORO, Y. (1971). Action potential and excitation contraction coupling of muscle cells in amphioxus. *Biophys. J.* **11**, 203*a*.

HAGIWARA, S. & KIDOKORO, Y. (1971). Na and Ca components of action potential in amphioxus muscle cells. *J. Physiol.* **219**, 217–232.

HAGIWARA, S. & NAKAJIMA, S. (1966). Effects of the intracellular Ca ion concentration upon the excitability of the muscle fiber membrane of a barnacle. *J. gen. Physiol.* **49**, 807–818.

HAGIWARA, S., TAKAHASHI, K. & JUNGE, D. (1968). Excitation-contraction coupling in a barnacle muscle fiber as examined with voltage clamp technique. *J. gen. Physiol.* **51**, 157–175.

HELLAM, D. C. & PODOLSKY, R. J. (1966). The relation between calcium concentration and isometric force in skinned frog muscle fibres. *Fedn Proc.* **25**, 466.

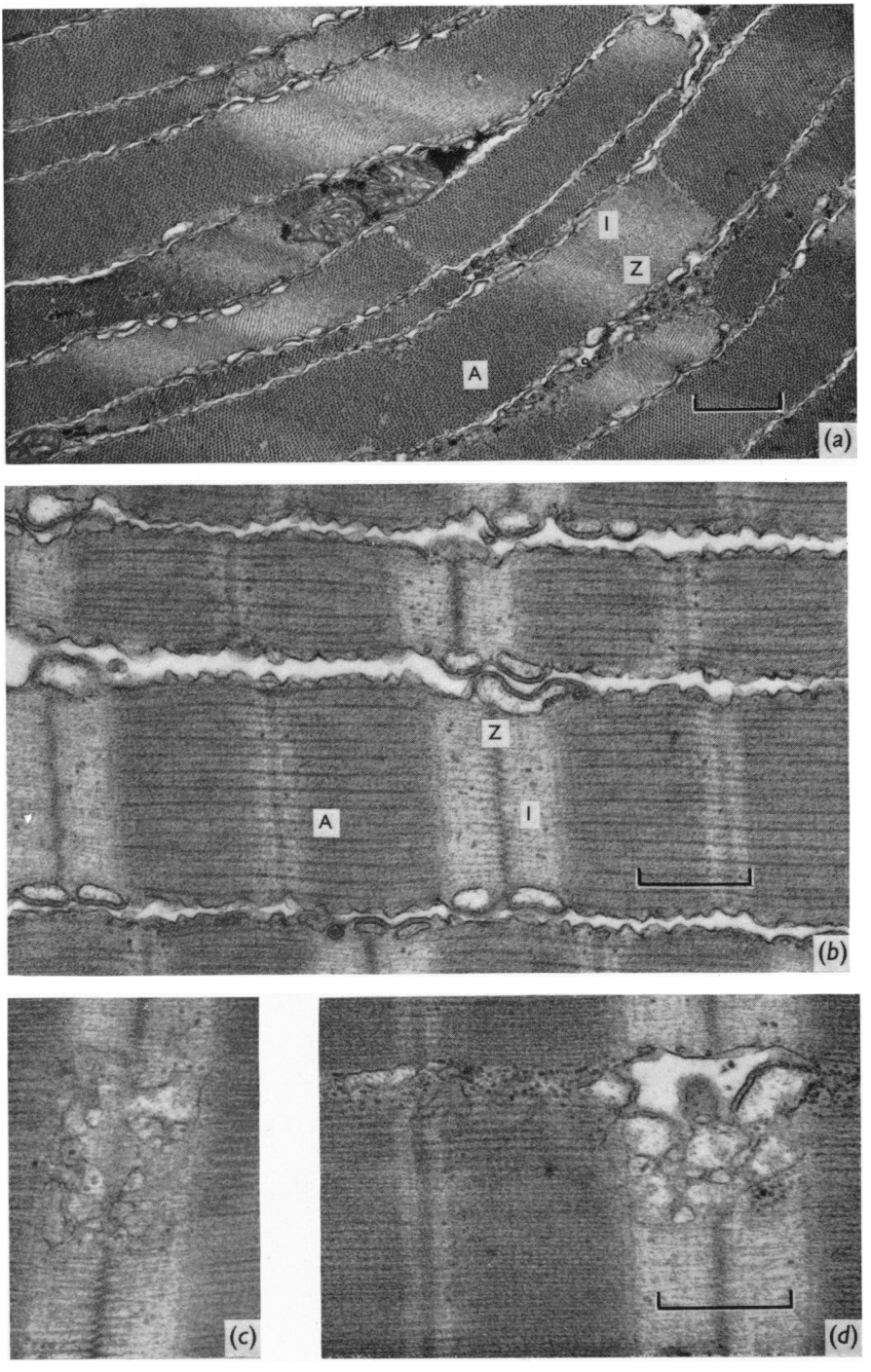
HILL, A. V. (1948). On the time required for diffusion and its relation to processes in muscle. *Proc. R. Soc.* B **135**, 446–453.

JÖBSIS, F. F. & O'CONNOR, M. J. (1966). Calcium release and reabsorption in the sartorius muscle of the toad. *Biochem. biophys. Res. Commun.* **25**, 246–252.

LÜTTGAU, H. C. & OETLIKER, H. (1968). The action of caffeine on the activation of the contractile mechanism in striated muscle fibres. *J. Physiol.* **194**, 51–74.

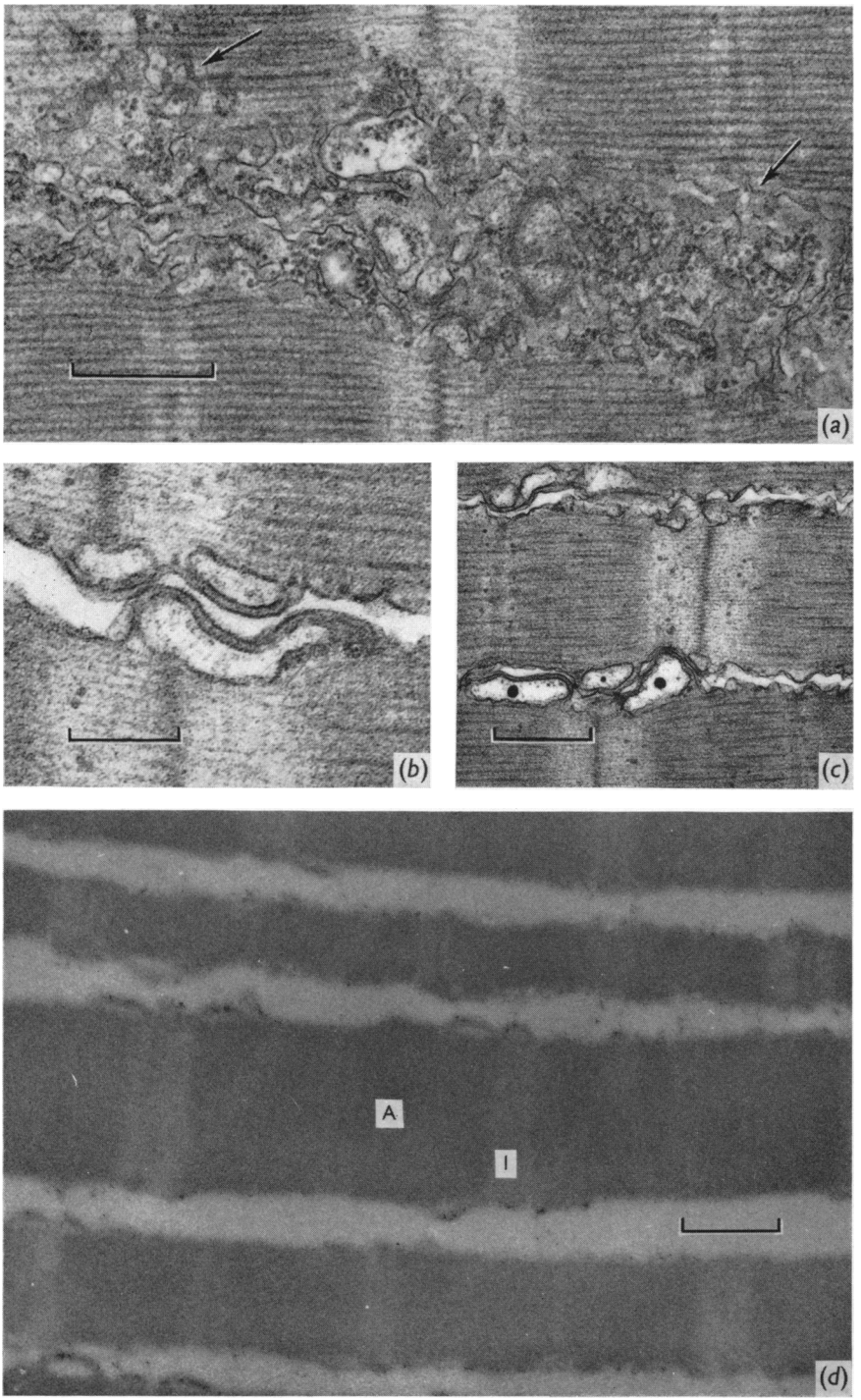
ORKAND, R. K. (1962). The relation between membrane potential and contraction in single crayfish muscle fibres. *J. Physiol.* **161**, 143–158.

PEACHEY, L. D. (1961). Structure of the longitudinal body muscles of Amphioxus. *J. biophys. biochem. Cytol.* **10** (suppl.), 159–179.



S. HAGIWARA, M. P. HENKART AND Y. KIDOKORO

(*Facing p.* 250)


(a)
(b)
(c)
A
I
(d)

PEACHEY, L. D. (1965). The sarcoplasmic reticulum and transverse tubules of the frog's sartorius. *J. cell Biol.* **25**, 209–231.

PORTZEHL, H., CALDWELL, P. C. & RÜEGG, J. C. (1964). The dependence of contraction and relaxation of muscle fibres from the crab *Maia squinado* on the internal concentration of free calcium ions. *Biochim. Biophys. Acta* **79**, 581–591.

WEBER, A. & HERZ, R. (1968). The relationship between the caffeine contracture of intact muscle and the effect of caffeine on reticulum. *J. gen. Physiol.* **52**, 750–759.

WILLIS, J. H. (1942). Speed of responses of various muscles of cats. *Am. J. Physiol.* **136**, 623–628.

WINEGRAD, S. (1970). The intracellular site of calcium activation of contraction in frog skeletal muscle. *J. gen. Physiol.* **55**, 77–88.

EXPLANATION OF PLATES

All plates are electron micrographs. Plates 1*a–d* and 2*a–c* are micrographs of material fixed in glutaraldehyde and osmium and stained with uranyl acetate and lead citrate. Symbols: A = A-band, I = I-band, Z = Z-line.

PLATE 1

a is a cross-section through portions of several muscle lamellae. Cisternae of the sarcoplasmic reticulum appear as vesicles beneath the surface membrane at the levels of the I-bands and Z-lines. The bar indicates 1 μm.

b is a longitudinal section through the narrow dimension of parts of two lamellae. The sarcoplasmic reticulum again appears principally at the level of the I-bands and Z-lines. The bar indicates 0·5 μm.

c and *d* are *en face* views of the sarcoplasmic reticulum overlying the I-bands and Z-lines. The cisternae are interconnected in a true reticulum. The bar represents 0·5 μm.

PLATE 2

a shows the smaller diameter reticulum overlying the A-bands as indicated by the arrows. Over the Z-line in the center of the picture are sections through several of the larger diameter cisternae. The bar indicates 0·5 μm.

b shows the area of association between cisternae of the sarcoplasmic reticulum and the surface membrane. In this section periodic densities appear to connect the two membranes. The SR contains a somewhat granular, amorphous material. The bar represents 0·25 μm.

c is a longitudinal section of muscle soaked for 30 min in isotonic $CaCl_2$ before fixation. The cisternae of the sarcoplasmic reticulum contain dense granules. The bar represents 0·5 μm.

d is an electron micrograph of material exposed to 1 mM-$LaCl_3$ for 30 min before fixation in glutaraldehyde. No osmium fixation or further stains were used. There is some shrinkage of the fibres which produces light gaps between them. The A- and I-bands are clearly distinguishable. Lanthanum is deposited as particles on the surface of the lamellae, particularly in the areas of association between the surface membrane and the cisternae of the sarcoplasmic reticulum. The bar represents 0·5 μm.