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CONTRACTIONS INDUCED BY A CALCIUM-TRIGGERED RELEASE OF CALCIUM FROM THE SARCOPLASMIC RETICULUM OF SINGLE SKINNED CARDIAC CELLS

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SUMMARY

1. Fragments of single cardiac cells were obtained by homogenization of ventricular tissue from adult rats. Remaining pieces of sacrolemma were removed by micro-dissection. Tension was recorded from the ends of the skinned (sarcolemma-free) cells with a photodiode force transducer.

2. In the presence of a strong buffering of the free [Ca^{2+}] with 4·0 mM total EGTA, a tonic tension was obtained that increased according to a sigmoid curve when the free [Ca^{2+}] was increased from $10^{-6.75}$ M to $10^{-5.0}$ M. This curve was not modified by the destruction of the sarcoplasmic reticulum (SR) by the detergent Brij 58. Therefore, the tonic tension corresponded to the direct effect of the free [Ca^{2+}] present in the buffer on the myofilaments.

3. In the presence of a slight buffering of the free [Ca^{2+}] with 0·050 mM total EGTA, cyclic contractions were observed that were attributed to cyclic releases and re-sequestrations of Ca^{2+} by the SR. The absence of effect of azide and ruthenium red on the cyclic contractions obtained at a free [Ca^{2+}] lower than $10^{-6.50}$ M demonstrated that the mitochondria played no role in the triggering of these contractions.

4. Cyclic contractions were induced by a slight variation of free [Ca^{2+}] in the buffer from $10^{-7.65}$ M to $10^{-7.40}$ M. Their amplitude at $10^{-7.40}$ M free Ca^{2+} was equal to the tonic tension developed by a free [Ca^{2+}] 20 times higher applied to the myofilaments when the SR was destroyed by detergent or functionally inhibited by high total [EGTA]. It was concluded that these cyclic contractions corresponded to a Ca^{2+}-triggered release of Ca^{2+} from the SR.

5. The cyclic contractions were induced by the filling of the SR with

Ca^{2+} to a critical level at which it released a fraction of the Ca^{2+} it contained. Each contraction was followed by a re-sequestration of Ca^{2+}, the kinetics of which conditioned the duration of the cycles.

6. The amplitude of the cyclic contractions increased when the free [Ca^{2+}] that triggered them was increased. This gradation was deemed incompatible with a simple regenerative process, which should produce an all-or-nothing response. Additional processes, such as a modulation of the Ca^{2+} release by free [Mg^{2+}] and [ADP] may help to explain the gradation of the contractions.

7. It was concluded that a Ca^{2+}-triggered release of Ca^{2+} from the SR of rat ventricular cells may amplify the Ca^{2+} flux crossing the sarcolemma during the plateau of the action potential, thereby permitting the activation of the myofilaments.

INTRODUCTION

In mammalian cardiac muscle, the trans-sarcolemmal inward current of Ca^{2+} that occurs during the plateau of the action potential seems insufficient for activating the contraction of the myofilaments (Bassingthwaighte & Reuter, 1972). It is assumed that additional Ca^{2+} is released from the internal stores (Bassingthwaighte & Reuter, 1972). A possible link between these two sources of Ca^{2+} for excitation–contraction coupling would be the triggering of the release of Ca^{2+} from the internal stores by the small trans-sarcolemmal flux of Ca^{2+}. To test this hypothesis, it is necessary to add known concentrations of exogenous free Ca^{2+} in the presence of the intracellular stores of Ca^{2+} and to measure the amount of Ca^{2+} released from these stores, which are presumably located in the sarcoplasmic reticulum (SR).

Single skeletal muscle cells from which the sarcolemma has been peeled (skinned fibres) permit the direct application of Ca^{2+} into the intracellular space. The concentration of free Ca^{2+} (free [Ca^{2+}]) can be buffered with ethyleneglycol-bis (β aminoethyl ether) *N*,*N*′-tetraacetic acid (EGTA). The release of Ca^{2+} from the internal stores can be inferred from the development of a contraction. Using these techniques Ford & Podolsky (1970) obtained quick phasic contractions by adding Ca^{2+} without EGTA buffering to the medium bathing skinned fibres of frog semi-tendinosus that had been previously loaded with Ca^{2+}. They attributed the quick contractions to a Ca^{2+}-triggered release of Ca^{2+} from the SR. A similar conclusion was obtained by Endo, Tanaka & Ogawa (1970), who studied the influence of free [Ca^{2+}] on the phasic contractions produced by caffeine in the presence of a slight buffering with a low total [EGTA]. The production of these phasic contractions by a release of Ca^{2+} from the internal stores has been

confirmed by experiments using ^{45}Ca (Ford & Podolsky, 1972*a*, *b*) or the protein aequorin, which presents a bioluminescence sensitive to free $[Ca^{2+}]$ (Endo & Blinks, 1973). However, the role of this Ca^{2+}-triggered release of Ca^{2+} in physiological activation of skeletal muscle is uncertain since the free $[Ca^{2+}]$ necessary to trigger the transient contraction was more than sufficient to saturate the troponin. Furthermore, the contraction of individual myofibrils was found to be graded with the amplitude of localized depolarization applied to the surface membrane in intact single skeletal muscle fibres (Costantin & Taylor, 1973). This finding was deemed incompatible with a regenerative release of Ca^{2+} from the SR, which would imply an all-or-nothing response.

In isolated cardiac cells which had the sarcolemma disrupted by homogenization, transient contractions were induced by a free $[Ca^{2+}]$ much lower than that required to activate directly the myofilaments (Fabiato & Fabiato, 1972 and 1973). This result suggests that the Ca^{2+}-triggered release of Ca^{2+} may play a physiological role in excitation-contraction coupling of heart muscle. This process was found to be more developed in rat ventricle than in the ventricle of other mammalian species (Fabiato & Fabiato, 1972). Accordingly, the present study was done on single cells of rat ventricle. In this study the sarcolemma of the disrupted cardiac cells was removed by micro-dissection so that a preparation of skinned cardiac cells was obtained. This technical improvement eliminated the problems related to the restricted diffusion into the cells of externally applied media and precluded the possibility of the binding of Ca^{2+} to the disrupted sarcolemmas remaining on the broken cells that were obtained by homogenization. Furthermore, a direct measurement of the tension developed by the skinned cardiac cells permitted a quantitative study of the contractions produced by the Ca^{2+}-triggered release of Ca^{2+}. The relationship between the level of Ca^{2+} load of the internal stores and the contraction induced by release of Ca^{2+} has been explored. This permitted definition of the mechanism of the Ca^{2+}-triggered release of Ca^{2+} and the evaluation of its role in the physiological activation of cardiac cells. However, the amplitude of the contraction increased gradually when the free $[Ca^{2+}]$ used to trigger it was increased. According to Costantin & Taylor (1973) this result is not compatible with a simple regenerative release process. Additional mechanisms should be considered to account for the observed gradation. These may involve either additional processes modulating the amount of Ca^{2+} released from a single type of storage site, or the release of Ca^{2+} from different storage sites that have unequal affinities for Ca^{2+} (such as the SR and the mitochondria). These two possibilities have been explored.

METHODS

Solutions

In all cases the buffers contained dextrose 7 mM and tris maleate 18 mM; pH was 7·0 at 22° C. The concentrations of Na_2EGTA, $CaCl_2$, Na_2ATP, and $MgCl_2$ were varied. A computer programme developed by Reuben, Brandt, Berman & Grundfest (1971) was used to define the concentrations of those components necessary for obtaining the desired free $[Ca^{2+}]$, free $[Mg^{2+}]$, free $[ATP^{4-}]$, $[MgATP^{2-}]$, and total [EGTA]. The concentrations of free ions were expressed by their inverse $\log_{10}$: pCa, pMg, pMgATP, pATP. The following apparent stability constants were used at pH 7·0: CaEGTA, $4{\cdot}9 \times 10^6$ M^{-1} (Schwartzenbach, Senn & Anderegg, 1957; Ringbom, 1963); MgEGTA, 40 M^{-1} (Portzehl, Caldwell & Ruegg, 1964); CaATP, 5×10^3 M^{-1} (Nanninga, 1961); MgATP, $11{\cdot}4 \times 10^3$ M^{-1} (Nanninga, 1961). Since an increase of ionic strength depresses the tension developed by the myofilaments (Reuben *et al.* 1971; Gordon, Godt, Donaldson & Harris, 1973; Thames, Teichholz & Podolsky, 1974), it was kept constant at 0·16 M by the addition of an appropriate amount of KCl. Some preparations were treated for 30 min with the non-ionic detergent Brij 58 at a concentration of 0·5% (Orentlicher, Reuben, Grundfest & Brandt, 1974) obtained from Ruger Chemical Company (Irvington, N.Y., U.S.A.). Other preparations were treated for 20 min with the surfactant deoxycholate (1·0 mM) (Hellam & Podolsky, 1969). Deoxycholate, caffeine, dextrose, Tris maleate, azide, Na_2ATP (low CaATP), Na_2ITP (98–100% purity), EGTA, iodacetate, and ruthenium red (98% purity) were obtained from Sigma Chemical Company (St Louis, Mo., U.S.A.). All inorganic salts were reagent grade. Total Ca and Mg were measured in each solution by atomic absorption spectrophotometry (Perkin Elmer 303, Palo Alto, Calif., U.S.A.).

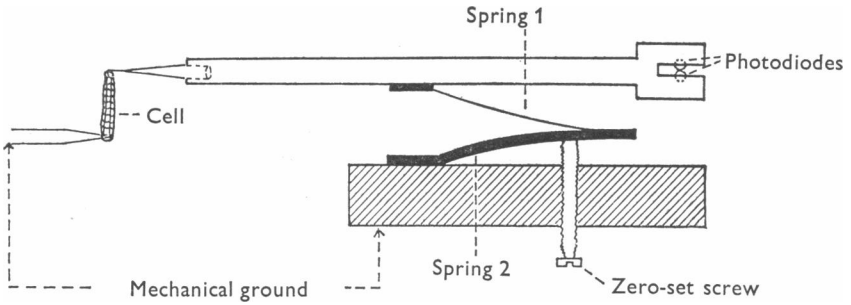
Preparation of skinned cardiac cells

Adult Sprague–Dawley rats of 200 g ± 20 g were decapitated and the heart was rapidly removed. In some experiments the heart was perfused for 1 hr by the Langendorff technique with a medium containing 0·20 mM total EGTA, pCa 8·40, pMg 3·50, pMgATP 2·50, and pATP 3·0 (total Ca was 0·004 mM, $MgCl_2$ 3·48 mM, Na_2ATP 4·04 mM). In all cases the tissue was minced into cubic fragments of approximately 2 mm in each dimension, and the fragments were washed several times in the same medium at 2° C. The minced tissue (500–700 mg) was homogenized in 25 ml. of the same buffer at 2° C, using a Virtis 45 blender, into fragments of about 50 μm in length and 12 μm in width, i.e. slightly smaller than a single cell. A fraction of the homogenate was diluted to 1/200 in the same buffer, and placed in a 0·05 ml. perfusion chamber on the stage of a Biovert inverted microscope (Reichert, Vienna, Austria) with anoptral phase contrast or Nomarski differential interference. This microscope had a thermoelectrically controlled heating and cooling stage (Cambion, Cambridge, Mass., U.S.A.). A temperature of 22° C ± 0·1° C was used in this study.

Fragments made of a single broken cell were used for skinning, and those made of two broken cells separated by an intercalated disk were discarded. During homogenization the two ends of the single cell were generally broken transversally (approximately perpendicular to the axis of the myofibrils) so that the disrupted cell had the same width as the intact cell (less than 15 μm in a 200 g rat) but had a slightly shorter length (less than 60 μm). Disruption of the sarcolemma was visible at the ends of the cell where the myofibrils were partly dissociated. The lateral limits of the cell were generally regular suggesting the presence of sarcolemma, which was confirmed in some cases under electron-microscopy. One end of the broken cell was

immobilized with a glass micro-tool (heat occluded micro-electrode). A 0·5 μm tip glass micro-needle (prepared as a micro-electrode) was used to pull the sarcolemma and superficial myofibrils longitudinally along the entire length of the cell, thereby stripping the sarcolemma from the cell (Pl. 1). Some superficial myofibrils and mitochondria were removed with the sarcolemma. During the skinning care was taken not to stretch the broken cell beyond 30% of its initial length. Complete skinning without overstretch was obtained in about one cell out of six. The skinning was done in the medium used for homogenization, which contained 0·20 mM total EGTA and pCa 8·40. In this medium the cell was relaxed. Larger total [EGTA] would have resulted in adherence of the myofibrils to the glass and to the sarcolemma, thereby preventing the skinning.

In the presence of a low free [Ca^{2+}] myofibrils and broken cells developed strong adherence to the glass. The non-skinned broken cells and the large bundles of myofibrils present in the homogenate were attached to a glass micro-tool and were removed from the perfusion chamber. Consequently, the skinned cell was the only major piece of tissue present in the perfusion chamber. Perfusion was done continuously with several inlets and one suction outlet. Each medium was brought by a different inlet under pressure with a DeFonbrune screw syringe (Baudouin, Paris, France). The inlets and outlet were placed at about 50 μm from the lateral sides of the cell. All the inlets were placed on one side and the outlet on the other. The perfusion medium was not re-circulated. Complete change of the medium bathing the cell was achieved in 1 sec.



Text-fig. 1. Transducer for recording of tension.

Recording of contraction

Each end of the skinned cell was attached to a glass micro-needle (Pl. 2). The cell was impaled by the micro-needles, and in a few seconds the myofibrils developed such a strong adherence to the glass that excessive stretching caused the cell to break transversely rather than to detach itself from the micro-needles. One micro-needle was immobilized, and the other was directly connected to the light 10 cm brass lever of the transducer (Text-fig. 1). The mobile micro-needle impaled the entire cross-section of the cell, and its direction was kept strictly perpendicular to the axis of the myofibrils (Pl. 2) in order to record the full tension developed by the myofilaments. The micro-needles were made of 2 mm external diameter Corning glass tubing and were pulled with a David Kopf (Tujunga, Calif., U.S.A.) micro-pipette puller. The tips of the micro-needles were heat-occluded with a DeFonbrune micro-forge (Baudouin, Paris, France). The micro-needle attached to the transducer had 1 μm tip. The micro-needle attaching the fixed end of the cell had a 3 μm tip.

The free end of the transducer lever (Text-fig. 1) was enlarged in an opaque vane

that had a 1 mm horizontal slit in its centre, and the light given by an infra-red light-emitting diode (General Electric, SSL-5C) passed through the slit and illuminated two photodiodes (Texas Instruments, H-38). The diameter of each photodiode was 1 mm. Therefore, each of them was half-illuminated when the slit was in the central position. Any force applied to the lever resulted in a slight movement of the vane. Consequently, the illumination of one of the photodiodes was increased while that of the other was decreased. A Wheatstone bridge permitted the differential change in illumination to be reflected as change in electrical resistance. To increase sensitivity the centre of the lever was attached to a brass spring that served as a pivot and a restoring force. The sensitivity was 500 mV/mg, the linearity was 99% for a 10 mg range, the drift was 0·002 mg/min, the natural frequency was 50 Hz. The compliance of the transducer was 2 μ/mg. This highly sensitive isometric transducer was made in accordance with plans given by R. A. Meiss (1974). The electrical output of the transducer was filtered above 50 Hz and displayed on a DC linear pen-writer (Hewlett Packard, Cupertino, Calif., U.S.A.).

Micro-manipulations for the skinning and attachment of the glass micro-needles for tension recording were done with three Leitz (Rockleigh, N.J., U.S.A.) and several specially made micro-manipulators. The transducer was attached to one of the Leitz micro-manipulators. The microscope and all the micro-manipulators were placed on a vibration-free table (Vibrostat, Milo, Freeport, N.Y., U.S.A.) in a copper Faraday cage to avoid electrical interference and in a Plexiglass cage to avoid mechanical interference due to air movement. The force transducer was protected by an additional shielding against air currents and temperature drift.

In each experiment the length and the width of the preparation were measured, and in some experiments the thickness was measured. Sarcomere length was adjusted by pulling the skinned cell under observation with a Unitron Filar eyepiece micrometer (Unitron, Newton, Mass., U.S.A.). The distance between eleven striations was measured and divided by ten. Photographs of the skinned cell were taken to document sarcomere length with a Zeiss (New York, N.Y., U.S.A.) photomicrographic camera. A sarcomere length of $2{\cdot}3 \pm 0{\cdot}1$ μm was always used in the present study and corresponded to the maximum tension developed. The length and width of the skinned cells (as indicated in the figures) were always measured when the cell was stretched to this sarcomere length.

Measurement of the total calcium content of the tissue

The total Ca content of the tissue was measured in fifteen homogenates of disrupted cardiac cells. These cells were bathed in a medium of the same composition as that in which a Ca^{2+}-triggered release of Ca^{2+} was produced. The tissue was weighed and homogenized at 2° C in 0·20 mM total EGTA with pCa 8·40, as previously described. The homogenate was spun for 3 min at 1000 *g* at 2° C. The supernatant fluid was removed and the pellet was re-suspended in a medium containing 0·050 mM total EGTA at pCa 7·65 with pMg 3·50 and pMgATP 2·50. This new homogenate was kept at 22° C for 20 min. It was then re-centrifuged for 20 min at 200,000 *g*. The pellet was precipitated by trichloracetic acid and total calcium was measured by atomic absorption spectrophotometry. The same measurement was done in fifteen homogenates of cells that were submitted to the same procedures with the addition of a re-suspension in a medium containing 0·5% Brij 58 with pCa 9·0 and 4·0 mM total EGTA for 30 min. This re-suspension was done between the initial homogenization and the re-suspension at pCa 7·65 and 0·050 mM total EGTA.

RESULTS

Tension developed by a single skinned cardiac cell

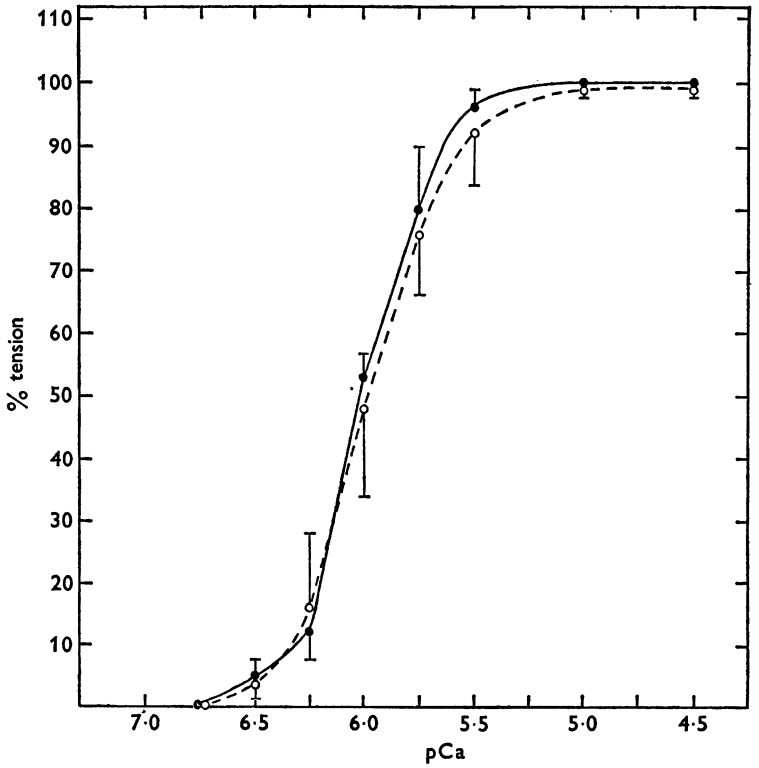
Fifteen skinned cells of $10{\cdot}5 \pm 0{\cdot}1$ μm (S.D.) width had a thickness of $7{\cdot}8 \pm 1{\cdot}1$ μm (S.D.) when they were stretched at a sarcomere length of 2·3 μm. These cells developed a force of 0·57 mg $\pm$ 0·04 mg (S.D.) when fully activated (at sarcomere length 2·3 μm) in the presence of 4·0 mM total EGTA at pCa 5·0 with pMg 3·50, pMgATP 2·50, and pATP 3·0 (total Ca 3·97 mM, $MgCl_2$ 3·48 mM, Na_2ATP 4·08 mM). This corresponded to a mean tension of 0·89 kg/cm^2, which is about 64 % of the tension developed by skinned fibres of skeletal muscle (1·40 kg/cm^2 according to Hellam & Podolsky, 1969).

In the presence of 4·0 mM total EGTA a curve of tension as a function of pCa was obtained while pMg was maintained at 3·50, pMgATP at 2·50, and pATP at 3·0 (Text-fig. 2). These values of pMg and pMgATP are within the range assumed to be physiological in intact cardiac cells (Polimeni & Page, 1973). A curve of the tension *vs.* the pCa was also obtained in skinned cells that had been treated for 0·5–1 hr in a relaxing solution (pCa 9·0, 4·0 mM total EGTA) containing a concentration of 0·5 % of the detergent Brij 58. This curve was not significantly different from that obtained without pre-treatment with Brij 58 (Text-fig. 2). According to studies done on skeletal muscle one half hour of treatment with 0·5 % Brij 58 should be sufficient to destroy the SR almost completely (Orentlicher *et al.* 1974). This result demonstrates that internal stores of Ca^{2+} play no role in the presence of 4·0 mM total EGTA even when the SR is not structurally destroyed. The capacity of Ca^{2+} binding of the SR is small as compared to that of 4·0 mM total EGTA (Solaro & Briggs, 1974). Movements of Ca^{2+} into or out of the internal stores would not modify the pCa bathing the myofilaments. Therefore, the tension developed at a given pCa set in the buffer represented the direct effect of this pCa on the myofilaments when the total [EGTA] was 4·0 mM.

Presence of a Ca sink within the cell

In the presence of a slight buffering with 0·050 mM total EGTA at pCa 7·65 a skinned cell was in relaxation. Decreasing pCa to 7·0 induced cycles of contraction and relaxation (Text-fig. 3*A*). When the same experiment was done with increasing total [EGTA], the amplitude of the cyclic contractions decreased (Text-fig. 3*B* and *C*), and they were absent with more than 0·150 mM total EGTA (Text-fig. 3*D*). No tonic tension was observed in the presence of 4·0 mM total EGTA because pCa 7·0 corresponded to a free [Ca^{2+}] below the contraction threshold of the myofilaments (Text-fig. 2). This experiment suggests that the cyclic

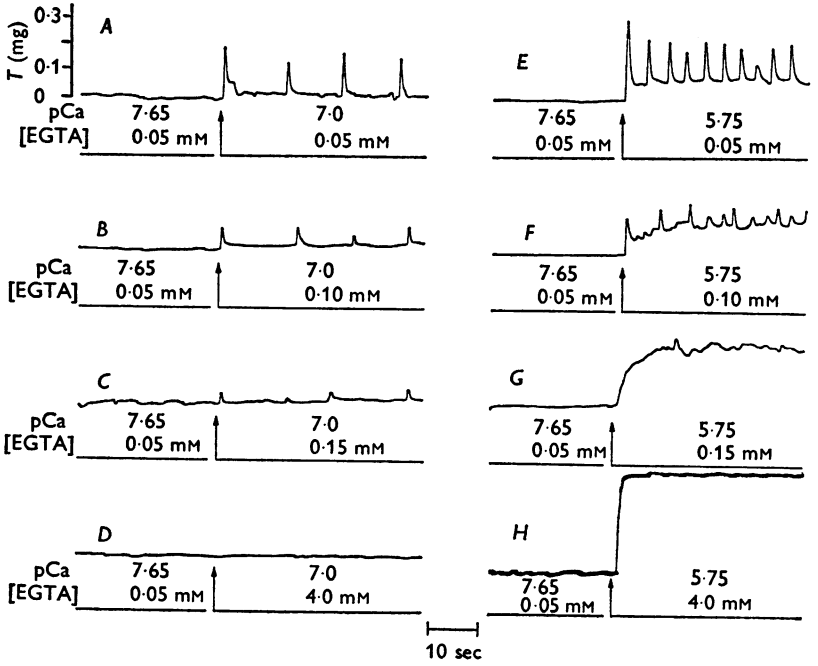
contractions were related to the presence of a sink of Ca^{2+} within the cell that was in competition for Ca^{2+} with the EGTA of the buffer. Hence the cyclic contractions were only observed in the presence of a weak EGTA-Ca buffer with which the intracellular sink of Ca^{2+} could compete. Their amplitude decreased when the capacity of the buffer for Ca^{2+} was enhanced by increasing the total [EGTA]. Furthermore the cyclic contractions



Text-fig. 2. Tension developed by skinned cardiac cells as a function of the pCa in the bathing medium, in the presence of 4·0 mM total EGTA with pMg 3·50 and pMgATP 2·50. The continuous curve with filled circles (●—●) was obtained from cells that had not been submitted to detergent. the interrupted curve with open circles (○- - -○) corresponds to cells which had been treated for one half hour with the non-ionic detergent Brij 58 at a concentration of 0·5%. Tension developed by a given cell at a given pCa was expressed by the percentage of the tension developed by the same cell at pCa 5·0 without pre-treatment with Brij 58. The tension 0% corresponds to the tension developed at pCa 9·0. Each point is the mean, and each vertical bar is the standard deviation for twenty observations, except for the point at pCa 6·0 without Brij 58 which corresponds to fifty observations. For clarity, the standard deviation is shown in one direction only. The segments of skinned cells had a width of 8–13 μm and a length of 35–60 μm.

corresponded to transient releases of Ca^{2+} from this intracellular sink since they were observed at a pCa too high to permit the direct activation of the myofilaments.

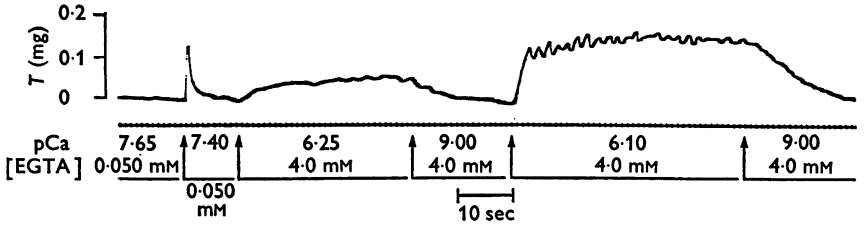
When the same experiment was done with a pCa of 5·75 (Text-fig. 3*E* to *H*), the amplitude of the cyclic contractions also decreased when the total [EGTA] was increased. In addition, a resting tension was observed, the amplitude of which increased when the total [EGTA] was increased.



Text-fig. 3. Effect of total [EGTA] on the amplitude of cyclic contractions. Tracings *A*, *B*, *C*, and *D* were obtained from a single skinned cell of 10 μm width and 40 μm length. Tracings *E*, *F*, *G* and *H* are from another skinned cell of 10 μm width and 52 μm length. Arrows indicate perfusion change; pCa and total [EGTA] are also indicated. In all cases pMg was 3·50, pMgATP 2·50, and pATP 3·06.

This experiment suggests a competition for Ca^{2+} among three pools: the myofilaments, the EGTA-Ca buffer, and a sink of Ca^{2+} within the cell. With low total [EGTA], the binding of Ca^{2+} by the intracellular sink rendered the pCa in the myofilament space much higher than the pCa set in the buffer, as shown by a lower plateau of tension than with high total [EGTA] (Text-fig. 3*G* and *H*). When cyclic contractions were observed with low total [EGTA], the tonic tension slowly reached its plateau, but even after several minutes the plateau remained lower than with high total [EGTA]. In cells treated for one half hour with Brij 58 (0·5 %) no

cyclic contractions were observed at any level of total [EGTA] and the plateau of tension was not significantly different for a given pCa obtained either with 4·0 mM or with 0·20 mM total EGTA (data not shown).



Text-fig. 4. Phasic contraction produced by a Ca^{2+}-triggered release of Ca^{2+} in a skinned cell of 11 μm width and 45 μm length. Arrows indicate the changes of perfusion medium. The pCa and total [EGTA] are indicated. Total Ca content of the solutions with 0·050 mM total EGTA was 0·005 mM for pCa 7·65 and 0·008 mM for pCa 7·40. The pMg was 3·50, the pMgATP 2·50, and the pATP 3·06 in all media (with either 0·050 mM or 4·0 mM total EGTA).

Ca-triggered release of Ca from the intracellular sink

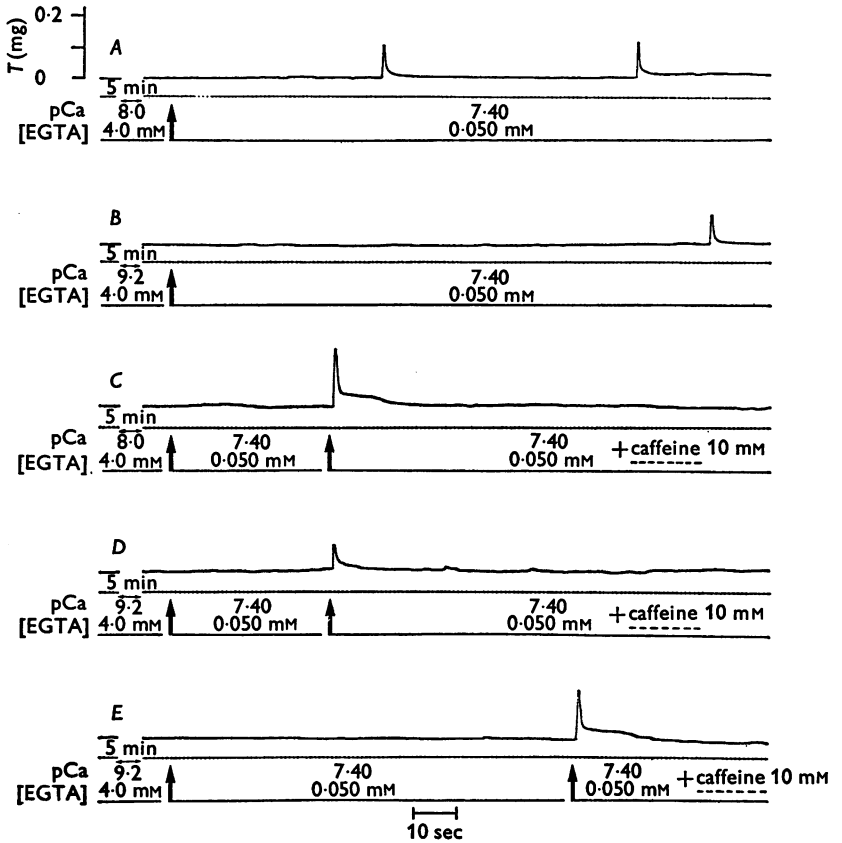
In the presence of 0·050 mM total EGTA a skinned cell was quiescent at pCa 7·65. Slightly decreasing the pCa to 7·40 was sufficient to induce a contraction of 0·130 mg (Text-fig. 4). Thereafter the total [EGTA] was increased to 4·0 mM to study the direct effect of the free [Ca^{2+}] set in the buffer on the myofilaments in the same cell. The tonic tension observed at pCa 6·25 with 4·0 mM total EGTA was less than half of the amplitude of the phasic contraction. A pCa 6·10 with 4·0 mM total [EGTA] was necessary to obtain a tonic contraction of the same amplitude as the phasic contraction obtained at pCa 7·40 (a twenty times lower free [Ca^{2+}]). This large phasic contraction, which was induced by a slight variation of the free [Ca^{2+}] in the perfusing solution, suggests a Ca^{2+}-triggered release of Ca^{2+} from the intracellular sink.

This skinned cell had not been submitted to any pre-load with Ca^{2+}. The heart was perfused for 1 hr in a relaxing solution with 0·20 mM total EGTA and pCa 8·40. Fragments of minced tissue were rinsed for several minutes and were homogenized and skinned in this relaxing solution. The skinned cell was then perfused for 3 min at pCa 7·65 with 0·050 mM total EGTA, and no cyclic contractions were observed. Decreasing pCa to 7·40 induced a phasic contraction after a delay of less than 1 sec.

An approximation of the importance of the Ca^{2+} load of the skinned cardiac cell under these conditions was obtained by measuring the total calcium content of the tissue in homogenates of cardiac cells with disrupted sarcolemmas maintained for 20 min at 22° C with pCa 7·65 and 0·050 mM total EGTA (see Methods), The tissue was found to contain 0·31 m-mole/kg wet weight ± 0·03 m-mole (S.D.) of total Ca. The same measurement done in homogenates of cells which had been treated with Brij 58 showed the tissue to contain 0·20 m-mole/kg wet weight ± 0·04 m-mole (S.D.) of total Ca.

Relationship between Ca load and induction of the phasic contractions

The Ca^{2+} contained in the internal stores of segments of skinned cells was controlled by a 6 min perfusion in a solution containing 4·0 mM total EGTA at various pCa. These segments of cells were then perfused in a solution containing 0·050 mM total EGTA, where cyclic contractions due to a release of Ca^{2+} could be observed (Text-fig. 5). When the initial

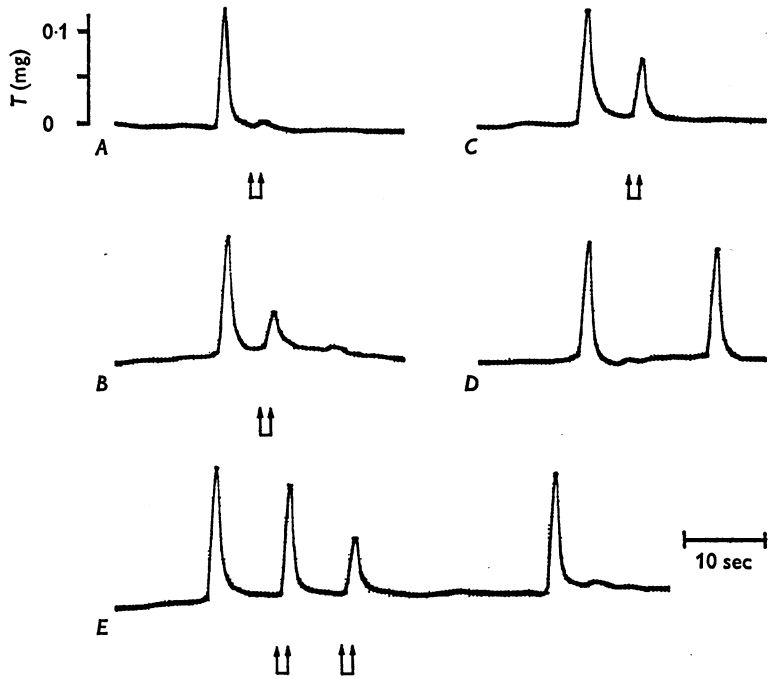


Text-fig. 5. Influence of the level of pre-load with Ca^{2+} in high total [EGTA] on the latency before phasic contractions that were observed in low total [EGTA] (*A* and *B*) and on the contractions elicited by caffeine (*C*, *D* and *E*). A single skinned cell of 11 μm width and 38 μm length was used. Arrows indicate the change of perfusion medium. The pCa and total [EGTA] are indicated; pMg was 3·50 and pMgATP 2·50. Composition of the solutions with 4·0 mM total EGTA for pCa 8·0 was 0·180 mM total Ca, 4·04 mM Na_2ATP, and 3·53 mM-$MgCl_2$; and for pCa 9·20 the composition was 0·006 mM-$CaCl_2$, 4·04 mM-Na_2ATP, and 3·53 mM-$MgCl_2$. Composition of the solution in 0·050 mM total EGTA can be found in Text-fig. 4. The arrows indicate perfusion change.

perfusion medium with 4·0 mM total EGTA had a pCa 8·0, the first phasic contraction was observed 52 sec ± 9 sec (S.D. of fifteen observations) after the beginning of the perfusion with 0·050 mM total EGTA (Text-fig. 5*A*). When the same experiment was done with an initial perfusion at pCa 9·20, which resulted in the depletion of the Ca^{2+} of the internal stores, the delay between the beginning of the perfusion in 0·050 mM-EGTA and the first phasic contraction was 125 sec ± 12 sec (S.D. of fifteen observations) (Text-fig. 5*B*). These results suggest that the phasic contractions occurred when the intracellular stores were filled to a critical level. Accordingly, when these stores were emptied by a perfusion with 4·0 mM total EGTA and virtually no free Ca^{2+} (pCa 9·20) the delay required to reach this critical level for the filling of the internal stores was longer than when they were pre-loaded by a perfusion with pCa 8·0.

This hypothesis was supported by experiments using caffeine to measure the load of Ca^{2+} of the intracellular stores in accordance with the method of Endo *et al.* (1970). It can be assumed that 10 mM caffeine releases a large fraction of the Ca^{2+} contained in these stores (Weber & Herz, 1968). Accordingly, the amplitude and the duration of the phasic contraction elicited by 10 mM caffeine were used as indirect measurements of the Ca^{2+} contained in the stores at a specified time. In Text-fig. 5*C* and *D* a perfusion with 10 mM caffeine was introduced 37 sec after the beginning of the perfusion in 0·050 mM total EGTA. The resulting contraction was smaller in amplitude and shorter in duration in Text-fig. 5*D* (where the intracellular stores had been emptied by perfusion at pCa 9·20) as compared to Text-fig. 5*C* where they had been pre-loaded by a perfusion at pCa 8·0. Thus, at a specified time after the beginning of the perfusion in low total [EGTA], the content of Ca^{2+} of the intracellular stores was smaller when they had been emptied than when they had been pre-loaded by the previous perfusion in high total [EGTA]. The experiment represented in Text-fig. 5*E* was similar to that in Text-fig. 5*D* except that caffeine was introduced at a longer interval (96 sec) following the beginning of the perfusion in 0·050 mM total EGTA. The resulting contraction was longer in duration and larger in amplitude. This suggests that a progressive filling of the intracellular stores with Ca^{2+} takes place during the latency period separating the initiation of the perfusion in low total [EGTA] and the first phasic contraction. It was concluded that the triggering of phasic contractions was related to the filling of the internal stores to a critical level at which a fraction of the Ca^{2+} content is released. This conclusion is in agreement with data obtained in fragmented SR of skeletal muscle by Weber (1971*a*). This author demonstrated that the increase of intravesicular free [Ca^{2+}] resulted in a decrease of Ca^{2+} influx and that the overload with Ca^{2+} of the vesicles produced a Ca^{2+} outflux.

It may be assumed that the internal stores re-sequestrate Ca^{2+} after the contraction has been elicited by the release of Ca^{2+}. This re-sequestration would result primarily in a relaxation and secondarily in a new contraction (Text-fig. 5*A*) when the internal stores have again reached the critical level of $[Ca^{2+}]$. This hypothesis was supported by the experiment represented in Text-fig. 6. The skinned cell was permanently perfused at a pCa 7·0 in the presence of 0·050 mM total EGTA. Brief perfusions with 2 mM caffeine were applied at various intervals after the spontaneous contraction. The amplitude of the caffeine-induced contraction increased

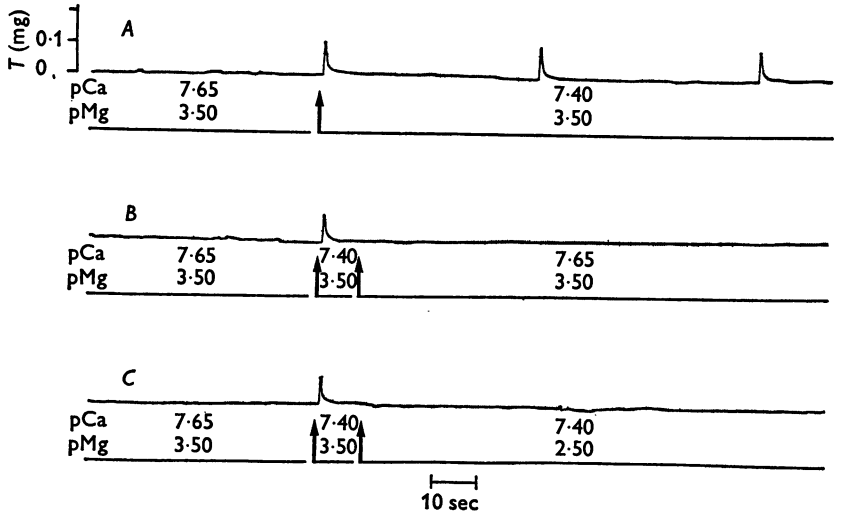


Text-fig. 6. Effect of the interval on the amplitude of the contraction elicited by 2 mM caffeine. The experiment was done on a segment of skinned cell of 13 μm diameter and 42 μm length. The perfusion medium contained 0·050 mM total EGTA, pCa 7·0, pMg 3·50, pMgATP 2·50 (0·017 mM total Ca, 4·04 mM-Na_2 ATP, and 3·48 mM-$MgCl_2$). The arrows indicate the beginning and the end of the perfusion with 2 mM caffeine added to the same medium.

when the interval was increased (Text-fig. 6*A*, *B*, *C*). The spontaneous cycle is shown in Text-fig. 6*D*. This experiment indicates that a progressive filling of the internal stores with Ca^{2+} occurs during the cycle. When repeated contractions were induced with caffeine at intervals shorter than the spontaneous cycle, a negative staircase was obtained (Text-fig. 6*E*).

Interruption of the cyclic contractions

During the physiological activation of intact cardiac muscle a single contraction is observed rather than cyclic contractions. Accordingly, experimental conditions capable of interrupting the cyclic contractions of skinned cardiac cells were studied (Text-fig. 7). As previously shown, cyclic contractions with a cycle of 50 sec ± 12 sec (S.D. of thirteen observations) were produced by changing the pCa from 7·65 to 7·40 (Text-fig. 7*A*).



Text-fig. 7. Interruption of cyclic contractions. A skinned cell of 9 μm width and 36 μm length was used. The total [EGTA] was 0·050 mM and pMgATP was 2·50 in all cases; pCa and pMg are indicated. The composition of the solution at pCa 7·40 and pMg 2·50 was 0·007 mM-$CaCl_2$, 3·25 mM-Na_2 ATP and 6·34 mM-$MgCl_2$. Composition of the other solutions can be found in Text-fig. 4. Arrows indicate perfusion change.

However, if the perfusion at pCa 7·40 was brief and was followed by a return to pCa 7·65 only a single contraction was observed (Text-fig. 7*B*). Therefore, in intact cardiac muscle a trans-sarcolemmal flux of Ca^{2+} producing a decrease of the myoplasmic pCa from 7·65 to 7·40 would be sufficient to induce a contraction by release of Ca^{2+} from the internal stores. Furthermore, a secondary increase of pCa to 7·65 would be necessary to prevent the cyclic recurrence of the contractions. Since cyclic contractions were observed in skinned cells the internal stores seem unable to lower the myoplasmic free [Ca^{2+}] sufficiently to prevent their recurrence. In intact muscle, a Ca^{2+} pump associated with the sarcolemma may be responsible for lowering the free [Ca^{2+}] below the threshold for cyclic contractions (Sulahke & Dhalla, 1971).

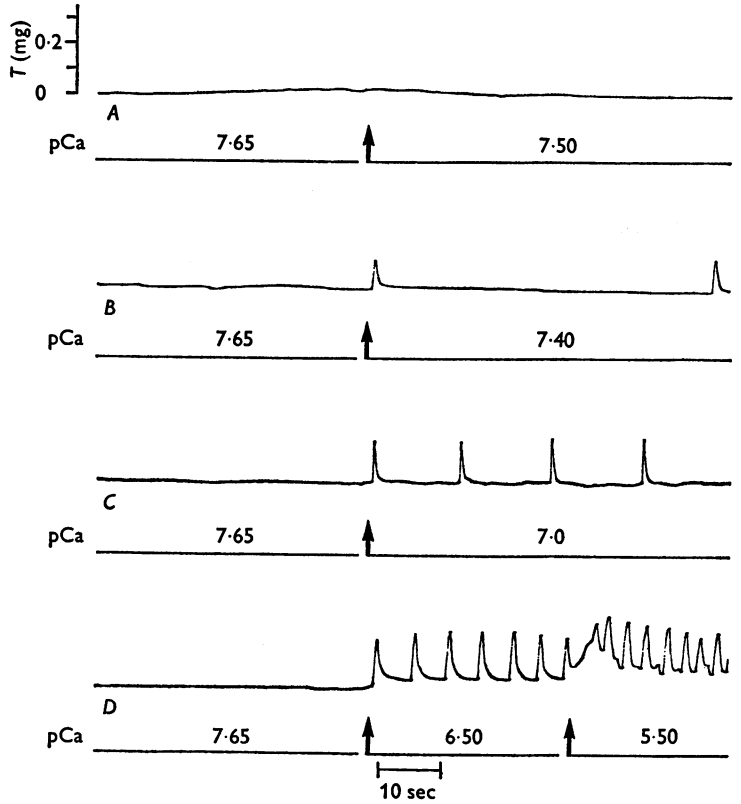
These results suggest that the release of Ca^{2+} and the re-binding by the internal stores amplify the variation of myoplasmic free [Ca^{2+}] produced by the trans-sarcolemmal influx and efflux of Ca^{2+}. However, the values of pCa given here cannot be used for quantitative calculation of the trans-sarcolemmal influx of Ca^{2+} necessary for activation of the contraction and of the subsequent trans-sarcolemmal efflux. In the presence of a low total [EGTA], the internal stores compete with the EGTA for Ca^{2+}, so that the free [Ca^{2+}] in the myofilament space may be lower than that indicated by the calculation of the pCa. Furthermore, the apparent stability constant of the Ca-EGTA complex is uncertain (Ogawa, 1968).

Another mechanism capable of interrupting the cyclic contractions was a decrease of pMg. A decrease of pMg from 3·50 to 2·50 was sufficient to inhibit the cyclic contractions induced at pCa 7·40 (Text-fig. 7*C*). This mechanism has been described in skinned fibres of skeletal muscle by Ford & Podolsky (1972*b*) and attributed to an enhancement by Mg^{2+} of the binding of Ca^{2+} by the SR. Similar observations were made in skinned fibres of cardiac muscle (Fabiato & Fabiato, 1975). An increase of free [Mg^{2+}] could occur during ATP break-down since ADP binds less Mg^{2+} than ATP does. The apparent stability constant of MgADP at pH 7·0 is 2×10^3 M^{-1} (O'Sullivan & Perrin, 1964) versus $11{\cdot}4 \times 10^3$ M^{-1} for MgATP (Nanninga, 1961). However, experiments, which will be presented later in this paper, indicated that substituting ADP for ATP with constant total Mg^{2+} concentration resulted in a release of Ca^{2+} rather than in an enhancement of Ca^{2+} binding. Therefore, it is unlikely that variation of free [Mg^{2+}] represents the physiological mechanism preventing the cyclic repetition of contractions during Ca^{2+} activation of intact cardiac muscle.

Gradation of the amplitude of the contractions with the concentration of trigger-free Ca

The induction of the cyclic contractions by a slight variation of the free [Ca^{2+}] in the solution suggests a regenerative process. Being a positive feed-back a regenerative process would imply an all-or-nothing response (Costantin & Taylor, 1973). In skinned fibres of skeletal muscle and in pluricellular fragments of cardiac tissue with disrupted sarcolemmas, the contraction elicited by a Ca^{2+}-triggered release of Ca^{2+} was propagated along the preparation at a velocity of 50–100 μm/sec (Fabiato & Fabiato, 1972). The hypothesis proposed by Ford & Podolsky (1972*b*) to explain this propagation is that the release of Ca^{2+} from one vesicle of the SR not only produces a contraction of the myofilaments in the vicinity but also triggers a release of Ca^{2+} from other vesicles of the SR. This autocatalytic release results in a propagation of the regenerative process along the preparation. Accordingly, a gradation of the amplitude of the contraction in these large preparations could be due to a modification of the conduction in the regenerative process, which would result in a variation of the degree

of synchronism in the contraction of the myofilaments. This would not rule out the possibility of an all-or-nothing response in the release of Ca^{2+} at the level of an individual vesicle of the SR. In contrast, in a segment of a single skinned cardiac cell the diffusion of externally applied solutions within the cell was very rapid and no conduction of the contraction was observed. Therefore, this preparation permitted a simple approach to the

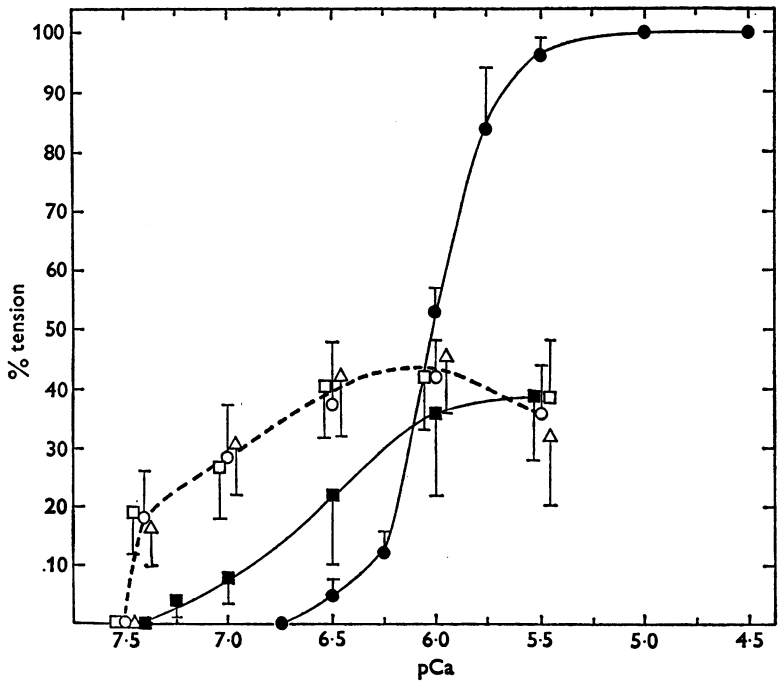


Text-fig. 8. Effect of decreasing trigger pCa on the amplitude and the frequency of cyclic contractions. A single skinned cell of 12 μm width and 42 μm length was used. In all cases total [EGTA] was 0·050 mM, pMg 3·50, pMgATP 2·50, and pATP 3·0. The arrows indicate perfusion change.

study of the effect of the concentration of trigger-free Ca^{2+} on the amount of Ca^{2+} released, as indirectly measured by the amplitude of the phasic contractions.

A segment of skinned cell was quiescent at pCa 7·65 in 0·050 mM total EGTA, and cyclic contractions were induced by perfusion in lower pCa (Text-fig. 8). No contractions occurred at pCa 7·50 (Text-fig. 8 *A*) but they

were induced at pCa 7·40 (Text-fig. 8*B*). The amplitude and frequency of the cyclic contractions increased when pCa was lower (Text-fig. 8*C* and *D*). However, at a very low pCa an increase in resting tension was observed, and the amplitude of the cyclic contractions decreased (Text-fig. 8*D*). The results of similar experiments are presented in Text-fig. 9. The amplitude of the cyclic contractions was plotted as a function of the pCa which



Text-fig. 9. Amplitude of the cyclic contractions obtained in the presence of 0·050 mM total EGTA as a function of the pCa set in the perfusion medium. The phasic tension developed by a given cell at a given pCa was expressed by the percentage of the tonic tension developed by the same cell at pCa 5·0 in the presence of 4·0 mM total EGTA. The tension 0% corresponds to the tension developed at pCa 9·0 with 4·0 mM total EGTA. For △, ○, □ and ■ total [EGTA] was 0·050 mM. For △ the segments of skinned cells had 5 ± 1 μm width and 15 ± 3 μm in length; for ○ 10 ± 2 μm in width and 40 ± 6 μm in length; for □ 14 ± 2 μm in width and 65 ± 10 μm in length. For ■ pluricellular fibres with disrupted sarcolemma of 30 ± 5 μm width and 110 ± 26 μm were used. Each point represents the mean of twenty measurements and vertical bars the standard deviations. For clarity, the standard deviation is shown in one direction only and some symbols have been slightly offset horizontally. The curve of tonic tension developed in the presence of 4·0 mM total EGTA (●—●) as a function of the pCa (same curve as in Text-fig. 2) has been represented for comparison (see Discussion). In all cases, pMg was 3·50, pMgATP 2·50 and pATP 3·06.

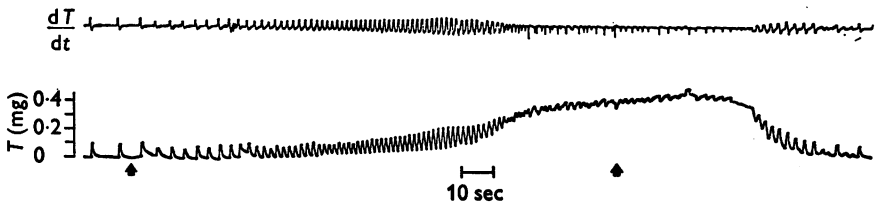
triggered it in preparations of various dimensions. The absence of significant differences among the results obtained in fragments of skinned cells of 5, 10 and 14 μm width respectively suggests that the possible conduction of the Ca^{2+} release process within these small preparations does not play a major role in the observed gradation of the contraction. In contrast, in pluricellular fragments of cardiac tissue with disrupted sarcolemmas of 30 μm width, the gradation of the amplitude of contraction with the trigger pCa was more pronounced because of the modification of the propagation within the preparation. In these preparations decrease of the pCa resulted in a more rapid propagation of the contraction and consequently in a more homogeneous contraction of the whole fibre. These experiments indicate that even in the absence of any major role of the conduction, there is a gradation of the contraction with the free [Ca^{2+}] that triggers it. This result suggests that the process of Ca^{2+}-triggered release of Ca^{2+} is graded with the concentration of free [Ca^{2+}] that induces it.

Modulation of the release of Ca

A non-testable hypothesis to explain the gradation of the contraction with the free [Ca^{2+}] trigger is that the increase of free [Ca^{2+}] outside the membrane of the vesicles of the SR increased the critical level at which they released Ca^{2+}. A larger amount of Ca^{2+} would then be released, resulting in a contraction of greater amplitude. This hypothesis is consistent with the data of Weber (1971*b*) which show that the efflux of Ca^{2+} from a fragmented SR of skeletal muscle is dependent on both the content of Ca^{2+} of the vesicles and the free [Ca^{2+}] outside the vesicles.

Another explanation for the gradation of contraction would be that Ca^{2+} may trigger the release of Ca^{2+} from storage sites with different affinities and capacities for Ca^{2+}. A slight increase of the free [Ca^{2+}] in the medium would overload the sites that have a high affinity or small capacity and would induce a release of Ca^{2+} from them only. A larger increase of free [Ca^{2+}] would also overload storage sites with a lower affinity or larger capacity and would result in a greater release of Ca^{2+}. In the absence of sarcolemma, two major storage sites of Ca^{2+} may exist in cardiac cells: the SR and the mitochondria. They are known to present different affinities and capacities for Ca^{2+} (Winegrad, 1973). The cyclic contractions of skinned cardiac cells were inhibited by pre-treatment with 10 mM caffeine, which inhibits the sequestration of Ca^{2+} by the SR (Weber & Herz, 1968), and by 20 min of pre-treatment with the surfactant deoxycholate (1 mM) (Hellam & Podolsky, 1969) or with the detergent Brij 58 (0·5 %), both of which destroy the SR. It was concluded that the SR played a major role in the cyclic binding and release of Ca^{2+} responsible for the cyclic contractions. In contrast, neither azide (10 mM) nor low concentrations of ruthenium red (1 μM) modified the cyclic contractions nor the resting tension when pCa was greater than 6·50. Azide

inhibits the oxidative phosphorylation and the energy-linked transport of Ca^{2+} by the mitochondria. Ruthenium red inhibits passive binding of Ca^{2+} by the mitochondria (Gillis, 1972). Oligomycin could not be used in skinned cardiac cells because of the contracture produced by the ethanol that is used as its solvent. Thus, it was concluded that the mitochondria played no role in the initiation of the cyclic contractions and that they were generated by the SR. However, after some Ca^{2+} has been released from the SR the myoplasmic pCa may become lower than 6·50. In the absence of EGTA at pCa $\simeq$ 5·80 rapid cyclic contractions were observed (Text-fig. 10). Addition of 5·0 mM azide resulted in an increase in their frequency and in the development of a contracture. This effect was reversible after return to the initial medium. This result, which was similar to the effect

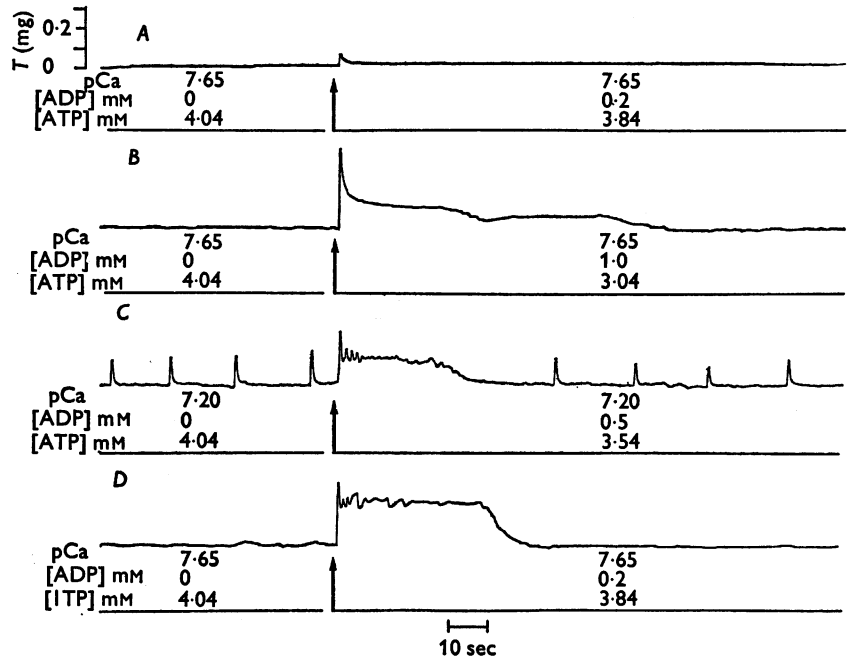


Text-fig. 10. Effects of azide on the cyclic contractions observed in the absence of EGTA with pCa $\simeq$ 5·80, pMg 3·50, and pMgATP 2·50 (0·003 mM total Ca, 4·04 mM-Na_2 ATP, and 3·48 mM-$MgCl_2$) on a single skinned cardiac cell of 10 μm width and 39 μm length. At the time indicated by the first arrow, 5 mM azide was added to the same perfusion medium. The second arrow indicates return to the initial perfusion medium.

of a simple decrease of pCa in the solution, suggests that azide released Ca^{2+} from the mitochondria. However, this does not demonstrate that mitochondria participate in the transport of Ca^{2+} generating each contraction. On the contrary, quantitative data indicated that transport of Ca^{2+} by the mitochondria was much too slow to participate in the Ca^{2+} movements during individual contractions (Scarpa & Graziotti, 1973). Therefore, it seems unlikely that the participation of the mitochondria in the cellular metabolism of Ca^{2+} in the presence of a high myoplasmic free [Ca^{2+}] would explain the gradation of the cyclic contractions.

Another hypothesis to explain this gradation is the presence of additional processes modulating the Ca^{2+}-triggered release of Ca^{2+} from the S.R. As an example of such a process, it was observed that phasic contractions can be induced by slight variations of [ADP] in the solution. A single skinned cell was quiescent at pCa 7·65 in 0·050 mM total EGTA. The introduction of a perfusion with the same medium but with 0·20 mM-ADP replacing 0·20 mM-ATP resulted in the induction of a small phasic contraction (Text-fig. 11*A*). Although the perfusion was continued, this

contraction was not cyclically repeated, unlike the contractions produced by a Ca^{2+}-triggered release of Ca^{2+}. Perfusion with 1 mM-ADP resulted in a single contraction of large amplitude and very slow relaxation (Text-fig. 11*B*). In Text-fig. 11*C* cyclic contractions were obtained at pCa 7·20 with 0·050 mM total EGTA. The introduction of a perfusion with the same medium plus 0·50 mM-ADP resulted in a contraction of greater amplitude than without ADP followed by a sustained contraction. The amplitude of the subsequent cyclic contractions returned to the initial level. Since ADP



Text-fig. 11. Effect of ADP on skinned cardiac cells. Three different single skinned cells were used. Their dimensions were as follows: for *A* and *B* (same cell) – 10 μm diameter and 47 μm length; for *C* – 9 μm diameter and 48 μm length; for *D* – 11 μm diameter and 48 μm length. In all cases total [EGTA] was 0·050 mM, $MgCl_2$ 3·48 mM; the pCa, total [ADP], and total [ATP] are indicated. In *D*, ATP is replaced by ITP. Arrows indicate perfusion change.

binds less Mg^{2+} than ATP does (O'Sullivan & Perrin, 1964), an increase of both the free [Mg^{2+}] and the [ADP] was produced in these experiments. However, the induction of the contractions can be attributed to the increase of [ADP] since increase of free [Mg^{2+}] alone inhibits the release of Ca^{2+}. Experiments were done in which the free [Mg^{2+}] was kept constant. Contractions induced by a given [ADP] were larger than when the free

[Mg^{2+}] was increased. Furthermore, when other parameters were maintained at a constant level, the amplitude of the ADP-induced contraction was increased when total [ATP] was decreased. Skinned cells were pretreated for 1 hr with 10 mM azide and 0·50 mM iodacetate to block ATP synthesis and were bathed in a medium where ATP was replaced by ITP. These cells did not exhibit cyclic contractions in 0·050 mM total EGTA at any pCa. However, a large contraction with slow relaxation was induced by 0·20 mM-ADP (Text-fig. 11*D*). In isolated microsomes and in conditions different from those used in the present report, an increase of [ADP] in the extravesicular medium produced a release of Ca^{2+} (Yamada & Tonomura, 1972; Makinose, 1972; Panet & Selinger, 1972). In these studies synthesis of ADP into ATP was associated with a release of Ca^{2+} from the SR. The reaction was dependent upon the free [Ca^{2+}] in the bathing medium. Since the experimental conditions were very different it cannot be ascertained that this mechanism was responsible for the results reported here. However, these results indicate that Ca^{2+} released from the SR is very sensitive to the [ADP] in the solution and suggest that this mechanism might play a physiological role in the modulation of the amplitude of the Ca^{2+}-triggered release of Ca^{2+}.

A transient increase of [ADP] may be produced by a break-down of ATP. Taylor, Lymn & Moll (1970) suggested that during the contraction of the myofilaments ATP becomes attached to the myosin and is then hydrolysed, which would result secondarily in the liberation of ADP. Thus, it might be proposed that a contraction of the myofilaments is induced by a Ca^{2+}-triggered release of Ca^{2+} from the SR and then the [ADP] increases transiently, which would result in an enhancement of the release of Ca^{2+} from the SR. This assumption would explain that the phasic contraction induced by a free [Ca^{2+}] trigger slightly above the threshold for direct activation of the myofilaments was larger than that produced by a free [Ca^{2+}] trigger lower than this threshold. This gradation would continue when more ADP is produced by a more complete activation of the myofilaments, which is induced by increasing the free [Ca^{2+}]. The absence of *net* ATP break-down during the tetanic contraction of skeletal muscle (Gilbert, Kretzschmar, Wilkie & Woledge, 1971) does not exclude the possibility of a transient break-down of ATP which would be resynthesized in a reaction that is coupled with the release of Ca^{2+} from the SR (Makinose, 1972; Yamada & Tonomura, 1972; Panet & Selinger, 1972). In Text-fig. 11 only the transient increase of [ADP] produced a contraction, while the continuation of the perfusion with high [ADP] had no further effect. Thus, situations such as hypoxia, where the ratio of [ADP]/[ATP] is permanently elevated (Scheuer, 1967), would not result in an enhancement of the release of Ca^{2+} from the SR.

DISCUSSION

Differences between the Ca-triggered release of Ca in skinned cells of cardiac and skeletal muscle

The reproducibility of the cyclic contractions in skinned cardiac cells permitted a quantitative study of the factors influencing their generation

and the variation of their amplitude. Their induction by a Ca^{2+}-triggered release of Ca^{2+} has been inferred from indirect evidence, such as the effects of caffeine, and from correlations between similar contractions and release of Ca^{2+}, which were directly demonstrated in skinned fibres of skeletal muscle (Ford & Podolsky, 1972*b*; Endo & Blinks, 1973). This inference may be justified by the extreme difficulty in doing similar studies at the present time in small skinned cardiac cells. For instance, the intensity of the luminescence of aequorin is directly proportional to the number of ions of Ca^{2+} that are released, which is itself directly proportional to the size of the preparation. Using a volume of tissue which was about 5000 times larger than a skinned cardiac cell, Endo & Blinks demonstrated by the bioluminescence of aequorin variations of myoplasmic free [Ca^{2+}] which were 10 times larger than those triggering the cyclic contraction in skinned cardiac cells.

The contractions presumably induced by a Ca^{2+}-triggered release of Ca^{2+} from the SR of skinned cardiac cells, which are described in the present study, differ from those observed in skinned fibres of skeletal muscle (Endo *et al.* 1970; Ford & Podolsky, 1970, 1972*b*; Endo & Blinks, 1973) by the following characteristics. (1) In cardiac cells the trigger free [Ca^{2+}] was much lower than the threshold for direct activation of the myofilaments (Text-fig. 9), while it was higher in skeletal muscle fibres (Ford & Podolsky, 1970, 1972*b*). (2) In cardiac cells the resulting tension transient was 20–40 % of the maximum tension developed by the directly activated myofilaments (Text-fig. 9), while it was nearly as large as the maximum tension in skeletal muscle (Ford & Podolsky, 1970, 1972*b*). (3) A pre-load with a large amount of Ca^{2+} was not required to obtain a Ca^{2+}-triggered release of Ca^{2+} in skinned cardiac cells while it was necessary in skinned skeletal muscle fibres. All of these differences may be explained by a smaller binding capacity for Ca^{2+} by the cardiac SR as compared to the SR of skeletal muscle (Solaro & Briggs, 1974) since the release of Ca^{2+} seems to be triggered by an overload of the SR with Ca^{2+}. When the binding capacity for Ca^{2+} by the SR of skinned fibres of skeletal muscle was depressed by 10 mM caffeine, cyclic contractions, which were attributed to a cyclic release of Ca^{2+} from the SR, were found under conditions similar to those used for skinned cardiac cells (Endo *et al.* 1970).

Physiological nature of the Ca-triggered release of Ca in cardiac muscle

The following arguments have been raised against the physiological role of the Ca^{2+}-triggered release of Ca^{2+} in the excitation–contraction coupling of the skeletal muscle. (1) In intact skeletal muscle contractions were observed for several minutes during bathing at a pCa larger than 8·0 (Armstrong, Bezanilla & Horowicz, 1972). Thus, a Ca^{2+}-trigger appeared

to be unnecessary for the production of a release of Ca^{2+} from the internal stores. (2) In intact muscle fibres the contraction of individual myofibrils was graded with the amplitude of localized depolarization of the sarcolemma (Costantin & Taylor, 1973). This gradation was deemed incompatible with a simple positive feed-back. (3) A regenerative release was only produced when the SR had been heavily pre-loaded with Ca^{2+}.

All of the preceding arguments can be criticized and it cannot be ascertained that the Ca^{2+}-triggered release of Ca^{2+} plays no role in the activation of the intact skeletal muscle. However, this discussion will be limited to cardiac muscle. (1) In intact cardiac muscle contraction disappears within a few minutes after the removal of Ca^{2+} from the bathing medium (Ringer, 1883). This argument, which has been used to demonstrate the role of superficial stores of Ca^{2+} in cardiac excitation–contraction coupling (Langer, 1973), may also be used to support the requirement of a Ca^{2+}-trigger to produce a release of Ca^{2+} from the SR. (2) The contraction produced by a release of Ca^{2+} from the SR was found to be graded with the free $[Ca^{2+}]$ used to trigger it in the present study. However, a complex system with several feed-backs is compatible with a gradation of the response with the amplitude of the input signal (Grodins, 1963). For example, the Ca^{2+}-triggered release of Ca^{2+} may be enhanced by transient increase of [ADP] and hindered by an increase of free $[Mg^{2+}]$. Obviously, other regulatory mechanisms may still be found. (3) In skinned cardiac cells the load of the Ca^{2+} in the SR which is necessary to obtain a Ca^{2+}-triggered release of Ca^{2+} is small as compared to that required in skeletal muscle. However, this does not completely rule out the possibility of an overload of Ca^{2+} in the cardiac SR since it has a smaller binding capacity for Ca^{2+} than the SR of skeletal muscle. Preliminary electron-microscopic studies of skinned cardiac cells have shown the SR vesicles to be largely dilated. The dilation may have corresponded to an overload of the SR with Ca^{2+}, resulting in an osmotic attraction of water within the vesicles. However, the SR vesicles remained dilated in skinned cardiac cells that were homogenized and stored at a pCa larger than 9·0 with 4·0 mM total EGTA. Therefore, it is unlikely that the dilation of the SR vesicles is related to an overload of Ca^{2+}. Alternatively, this dilation of the SR may be associated with the swelling of the myofilament lattice that is produced by Donnan-osmotic forces which occur in skinned fibres bathed in saline solution (Elliott, 1973; Maughan & Godt, 1974). Maughan & Godt (1974) obtained a shrinking of the skinned fibres of skeletal muscle with return to the cross-section observed in the cells bathed in oil by addition to the saline solution of polyvinylpyrrolidone (mol. wt. 4×10^4) 1 mg/ml. Using the same concentration in skinned cardiac cells a $25 \pm 4\%$ reduction (S.D. of twelve observations) in the width of the cells was observed. Cyclic contractions induced

by a Ca^{2+}-triggered release of Ca^{2+} were obtained on these shrunken cells with the same level of free Ca^{2+} as was in the usual swollen preparations. Electron-microscopic studies of the SR vesicles in these shrunken cells are in progress.

These experimental data permit the conclusion that there is no steadfast argument against a physiological role of a Ca^{2+}-triggered release of Ca^{2+} from the SR in the excitation–contraction coupling in intact cells of rat ventricle. This process may amplify the effects of the trans-sarcolemmal flux of Ca^{2+} (Bassingthwaighte & Reuter, 1972) on the myoplasmic free $[Ca^{2+}]$, thereby permitting activation of the myofilaments.

The rapid relaxation found in the intact ventricular tissue of the rat suggests a rapid sequestration of Ca^{2+} by a well developed SR. During activation a Ca^{2+}-triggered release of Ca^{2+} from the SR would enhance the amount of Ca^{2+} available to the cross-bridges, thereby helping to explain the rapid rate of force development observed in the rat ventricular tissue (Henderson, Brutsaert, Forman & Sonnenblick, 1974). Furthermore, well developed stores of Ca^{2+} within the SR might account for the negative staircase observed in rat ventricle, as proposed by Hajdu (1969). Treatment with 10 mM caffeine, which prevents the induction of cyclic contractions in skinned cardiac cells, also slows the rate of tension development and relaxation and suppresses the negative staircase in the intact tissue of the rat ventricle (Henderson *et al.* 1974).

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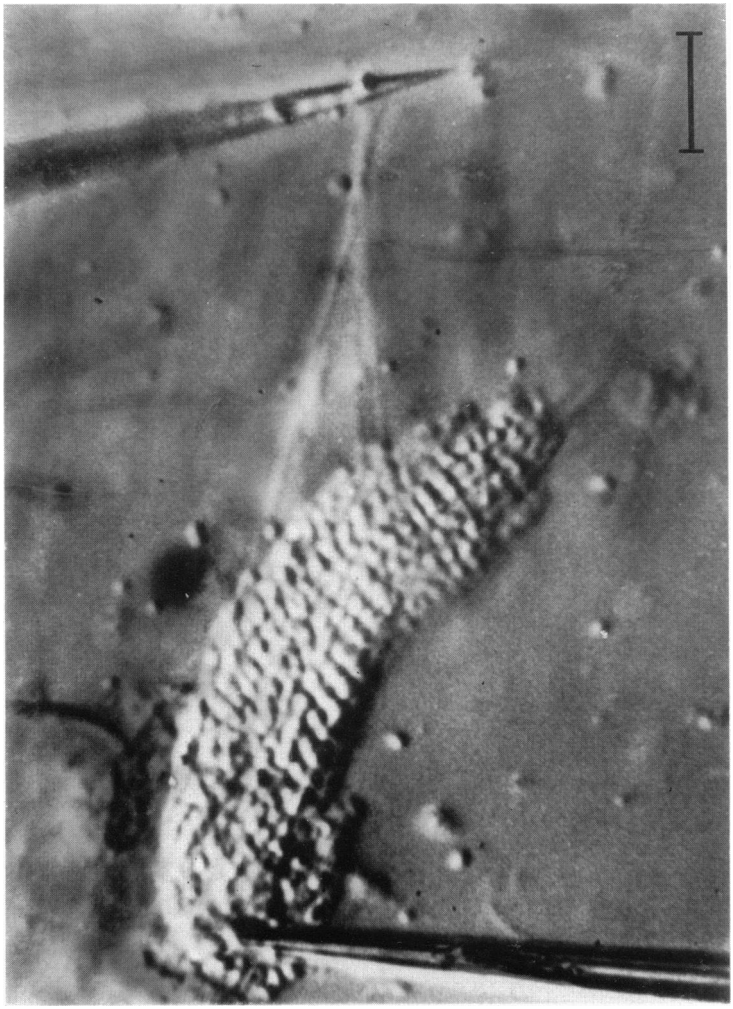

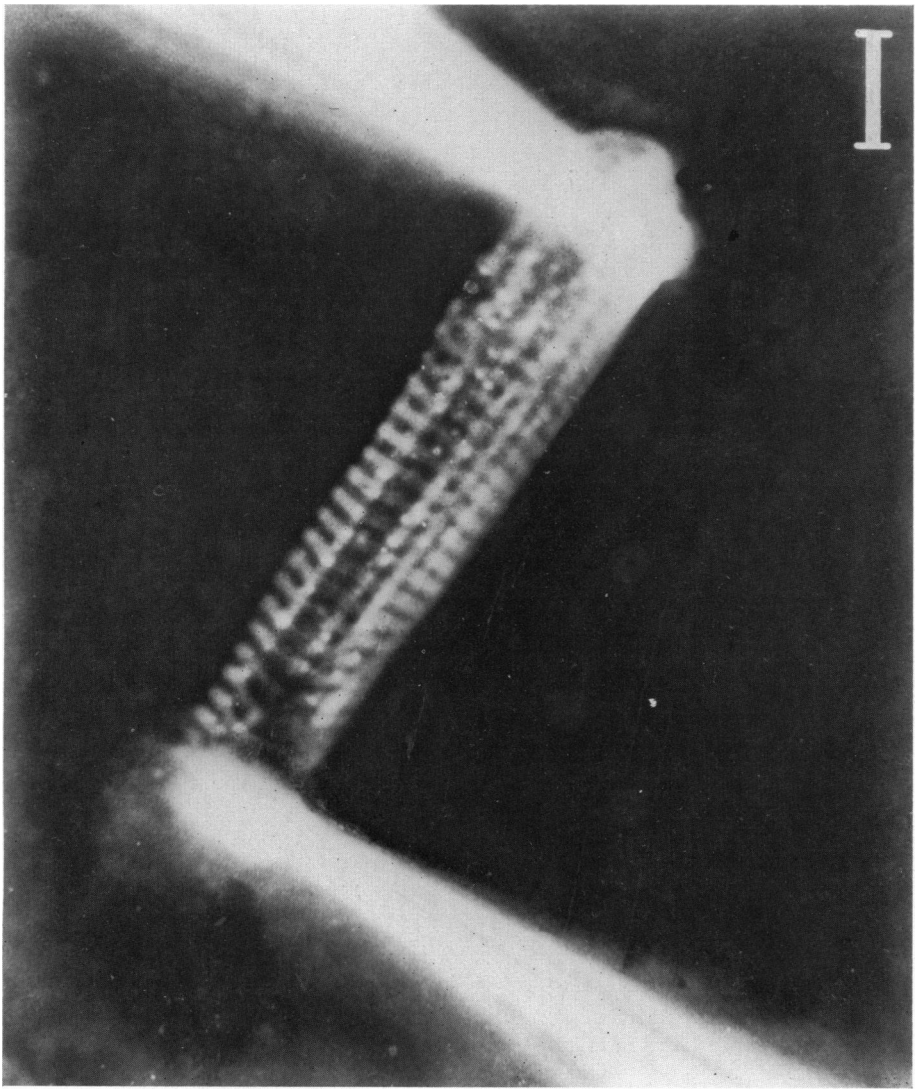
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EXPLANATION OF PLATES

PLATE 1

Skinning of a single broken cell of rat ventricle. The micrograph was obtained in interference type Nomarski. The sarcolemma is pulled by the upper glass micro-needle, while the lower glass micro-needle immobilizes the broken cell. The vertical bar indicates 10 μm.

PLATE 2

Skinned cardiac cell of rat ventricle attached with two glass micro-needles for tension recording. The lower micro-needle was attached to the transducer while the upper one was immobilized. The micrograph was obtained in anoptral phase contrast. Consequently, the *A* band appears clear and the I band dark. The sarcomere length was 2·3 μm. The vertical bar indicates 10 μm.