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EFFECTS OF MAGNESIUM ON CONTRACTILE ACTIVATION OF SKINNED CARDIAC CELLS

BY ALEXANDRE FABIATO AND FRANÇOISE FABIATO

From the Cardiovascular Research Laboratory, Harvard Medical School and Peter Bent Brigham Hospital, 721 Huntingdon Avenue, Boston, Massachusetts 02115, U.S.A.

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SUMMARY

1. In the presence of a slight buffering of the free [Ca^{2+}] with 0·050 mM total EGTA cyclic contractions were induced by a Ca^{2+}-triggered release of Ca^{2+} on skinned (sarcolemma-free) segments of single cardiac cells from rat ventricle. The threshold of the free [Ca^{2+}] trigger was elevated when the free [Mg^{2+}] was increased.

2. At a suprathreshold free [Ca^{2+}] increasing the free [Mg^{2+}] resulted in a decrease in frequency and in an increase in amplitude of the phasic contractions. Addition of caffeine at a specified interval after a cyclic contraction produced a larger contraction when free [Mg^{2+}] was higher. It was concluded that an increase of free [Mg^{2+}] increased the capacity and the rate of binding for Ca^{2+} by the sarcoplasmic reticulum (SR).

3. Small skinned fibres of skeletal muscle which were perfused with 10 mM caffeine yielded results similar to those obtained in skinned cardiac cells. It was concluded that the mechanism of action of free Mg^{2+} was similar in both preparations, but that the SR of skeletal muscle had a higher capacity and rate of binding for Ca^{2+} than the cardiac SR.

4. With a strong buffering of the free [Ca^{2+}] with 4·0 mM total EGTA, a smaller tonic tension was developed for a given pCa in the presence of a higher free [Mg^{2+}]. This result was nearly identical in skinned cells from cardiac and skeletal muscle tissue.

5. A decrease of the [$MgATP^{2-}$] produced a tension in the skinned cardiac cells that were perfused in Ca^{2+} free media. The maximum tension was observed for [$MgATP^{2-}$] $10^{-5\cdot50}$ M as in skinned fibres of skeletal muscle. A further decrease of [$MgATP^{2-}$] resulted in a decrease of tension.

INTRODUCTION

Cardiac tissue contains a large concentration of total Mg (Page & Polimeni, 1972). However, the exact role of free [Mg^{2+}] in excitation-contraction coupling is difficult to study in intact cells. Sensitive and reliable techniques for measuring the intracellular free [Mg^{2+}] are not available at the present time. Furthermore, the rate at which Mg^{2+} is transported into and out of the cell is much slower than that of Ca^{2+}. Consequently, isotopic flux studies on the intact tissue using radioactive ^{22}Mg are insensitive (Polimeni & Page, 1973).

Fragments of single cardiac cells from which the sarcolemma has been removed (skinned cardiac cells) permit the direct application of known concentrations of free Mg^{2+}, which are buffered with ATP or EDTA (ethylenediaminetetraacetic acid), on the intracellular structures involved in excitation-contraction coupling: myofilaments and sarcoplasmic reticulum (SR). In this preparation the tension recording provides information on the direct effect of the Ca^{2+} present in the solution on the myofilaments and on the effect mediated by a release of Ca^{2+} from the SR.

In the presence of a slight buffering of the free [Ca^{2+}] with 0·050 mM total EGTA (ethylene-bis (β-aminoethlylether) *N*,*N'*-tetraacetic acid) cyclic contractions were obtained that were attributed to a Ca^{2+}-triggered release of Ca^{2+} from the SR (Fabiato & Fabiato, 1975). This phenomenon is particularly well developed in skinned cells from rat ventricle. Accordingly, this species was used in the present study. The cyclic contractions were observed in this cardiac preparation at a much higher level of free [Mg^{2+}] than that inhibiting the Ca^{2+}-triggered release of Ca^{2+} in skinned fibres of frog skeletal muscle (Ford & Podolsky, 1972). Experimental protocols were developed to elucidate the cause of these differences between the two types of muscular tissues.

In the presence of a strong buffering of the free [Ca^{2+}] with 4·0 mM total EGTA movements of Ca^{2+} into and out of the SR cannot modify the free [Ca^{2+}] in the myofilament space. The sensitivity of the tension developed by the myofilaments to the free [Ca^{2+}] present in the buffer can then be directly studied. Results obtained in skinned fibres of skeletal muscle indicate that this tension is modulated by the free [Mg^{2+}] (Kerrick & Donaldson, 1972; Ashley & Moisescu, 1974) and by the concentration of the substrate MgATP (Reuben, Brandt, Berman & Grundfest, 1971; Brandt, Reuben & Grundfest 1972; Godt, 1974). The influence of these two parameters (free [Mg^{2+}] and [MgATP]) on the tension developed by skinned cells of rat ventricle is reported in the present study. A preliminary account has been reported in an Abstract (Fabiato & Fabiato, 1974*a*).

METHODS

Buffers

In all cases the buffers contained Tris-maleate 18 mM and dextrose 7 mM, pH was 7·0 and the temperature was 22° C ± 0·1° C. The concentrations of total Ca, Na_2 EGTA, Na_2ATP, and $MgCl_2$ were varied. A computer program developed by Reuben *et al.* (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 following apparent stability constants were used: CaEGTA, $4{\cdot}9 \times 10^6$ M^{-1}; MgEGTA, 40 M^{-1}; CaATP, 5×10^3 M^{-1}; MgATP, $11{\cdot}4 \times 10^3$ M^{-1}. The free [Mg^{2+}] depends primarily on the MgATP buffer and the free [Ca^{2+}] on the CaEGTA buffer; however, Ca^{2+} competes with Mg^{2+} for ATP. Free [Ca^{2+}], free [Mg^{2+}], [$MgATP^{2-}$], and free [ATP^{4-}] were expressed by their inverse logarithms: pCa, pMg, pMgATP, and pATP. However, to permit comparison with articles related to the same subject that give only the total concentrations of $MgCl_2$ and (Na_2ATP) (Ford & Podolsky, 1972) or use different stability constants (Kerrick & Donaldson, 1972; Godt, 1974), the total concentrations in mM of EGTA, Ca, ATP, and Mg used for most of the solutions are given in Table 1 in the order in which they are used in the presentation of the data.

Very low [$MgATP^{2-}$] was needed in some experiments, the achievement of which with the previously described buffer system would have required total ATP in the micromolar range. The myoplasmic [ATP] would have been uncertain because of the hydrolysis of ATP within the cell. This hydrolysis could have resulted in a gradient between the concentrations of ATP in the perfusion medium and in the centre of the cell (Reuben *et al.* 1971; Godt, 1974). To avoid this problem total [ATP] below 1·0 mM were not used. Instead, low free [Mg^{2+}] and [$MgATP^{2-}$] were obtained by the use of EDTA in the place of EGTA. The EDTA strongly chelates both Mg^{2+} and Ca^{2+}. The apparent stability constants at pH 7·0 were as follows: CaEDTA, $2{\cdot}4 \times 10^7$ M^{-1}; MgEDTA, $2{\cdot}3 \times 10^5$ M^{-1} (Portzehl *et al.* 1964). Table 2 gives the concentrations in mM of total Mg and of MgEDTA that were added to solutions containing 1 mM-ATP and 10 mM-EDTA to obtain the pMg and pMgATP used in the results. In these solutions no Ca^{2+} was added so that the pCa was always about 9·50.

Concentrations of contaminant total Ca and Mg were measured by atomic absorption spectrophotometry (Perkin Elmer 303, Palo Alto, Calif., U.S.A.) in each chemical and were controlled in each buffer. The solutions were completed with a variable [KCl] so that the ionic strength was kept constant at 0·16 M. Caffeine, tris-maleate, dextrose, Na_2ATP (low Ca^{2+} content), EGTA, EDTA, disodium creatine phosphate (99% purity), creatine phosphokinase and azide were obtained from Sigma Chemical Company (St Louis, Mo., U.S.A.). All inorganic chemicals were reagent grade.

Preparation of skinned cells

The technique of preparation of skinned cardiac cells from the ventricle of adult rat was identical to that previously described (Fabiato & Fabiato, 1975). The medium used for homogenization and micro-dissection contained 0·20 mM total EGTA, pCa 8·40, pMg 3·50, and pMgATP 2·50. The pH was always 7·0 and the temperature was maintained at 22 ± 0·1° C. These skinned cardiac cells were 8–14 μm in width and 35–60 μm in length.

Small fragments of skinned fibres of frog semitendinosus, which were 8–12 μm in width and 30–60 μm in length, were prepared with exactly the same technique as that used for cardiac muscle. The semi-tendinosus of the frog was minced into small fragments in the medium of homogenization. The fragments were rinsed in the same medium and were homogenized. The dissection into small bundles of

TABLE 1. Composition of the solutions used for Ca^{2+} activation

pCa	pMg	pMgATP	pATP	Total EGTA (mM)	Total Ca (mM)	Total ATP (mM)	Total Mg (mM)
8·40	3·50	2·50	3·06	0·20	0·004	4·04	3·48
7·65	3·50	2·50	3·06	0·05	0·005	4·04	3·48
7·40	3·50	2·50	3·06	0·05	0·008	4·04	3·48
7·40	2·50	2·50	4·06	0·05	0·007	3·25	6·34
7·0	3·50	2·50	3·06	0·05	0·017	4·04	3·48
7·0	2·50	2·50	4·06	0·05	0·015	3·25	6·34
9·0	3·50	2·50	3·06	4·0	0·019	4·04	3·53
6·75	3·50	2·50	3·06	4·0	1·85	4·04	3·51
6·50	3·50	2·50	3·06	4·0	2·42	4·04	3·50
6·25	3·50	2·50	3·06	4·0	2·93	4·04	3·50
6·0	3·50	2·50	3·06	4·0	3·32	4·04	3·49
5·75	3·50	2·50	3·06	4·0	3·59	4·05	3·48
5·50	3·50	2·50	3·06	4·0	3·77	4·05	3·48
5·0	3·50	2·50	3·06	4·0	3·97	4·08	3·48
4·50	3·50	2·50	3·06	4·0	4·0	4·10	3·48
9·0	4·50	2·50	2·06	4·0	0·019	11·91	3·20
7·0	4·50	2·50	2·06	4·0	1·32	11·92	3·20
6·75	4·50	2·50	2·06	4·0	1·87	11·92	3·20
6·50	4·50	2·50	2·06	4·0	2·44	11·93	3·20
6·25	4·50	2·50	2·06	4·0	2·96	11·94	3·20
6·0	4·50	2·50	2·06	4·0	3·36	11·96	3·20
5·75	4·50	2·50	2·06	4·0	3·67	11·99	3·20
5·50	4·50	2·50	2·06	4·0	3·90	12·05	3·19
5·0	4·50	2·50	2·06	4·0	3·98	12·10	3·19
4·50	4·50	2·50	2·06	4·0	4·0	12·21	3·19
9·0	4·50	3·50	3·06	4·0	0·019	1·19	0·35
8·50	4·50	3·50	3·06	4·0	0·061	1·19	0·35
8·0	4·50	3·50	3·06	4·0	0·19	1·19	0·35
7·50	4·50	3·50	3·06	4·0	0·53	1·19	0·35
7·0	4·50	3·50	3·06	4·0	1·31	1·19	0·35
6·75	4·50	3·50	3·06	4·0	1·86	1·19	0·35
6·50	4·50	3·50	3·06	4·0	2·43	1·19	0·35
6·25	4·50	3·50	3·06	4·0	2·93	1·19	0·35
6·0	4·50	3·50	3·06	4·0	3·33	1·20	0·35
5·75	4·50	3·50	3·06	4·0	3·60	1·20	0·35
5·50	4·50	3·50	3·06	4·0	3·77	1·20	0·35
5·0	4·50	3·50	3·06	4·0	3·97	1·23	0·35
4·50	4·50	3·50	3·06	4·0	4·0	1·25	0·35
9·0	2·50	2·50	4·06	4·0	0·017	3·25	6·78
6·50	2·50	2·50	4·06	4·0	2·31	3·25	6·52
6·25	2·50	2·50	4·06	4·0	2·84	3·25	6·46
6·0	2·50	2·50	4·06	4·0	3·25	3·25	6·42
5·75	2·50	2·50	4·06	4·0	3·54	3·25	6·38
5·50	2·50	2·50	4·06	4·0	3·73	3·25	6·36
5·0	2·50	2·50	4·06	4·0	3·92	3·25	6·34
4·50	2·50	2·50	4·06	4·0	3·98	3·26	6·33

myofibrils was completed by microdissection under a Biovert inverted microscope (Reichert, Vienna, Austria).

For both cardiac and skeletal muscle preparations of larger dimensions were used in some experiments to study the effects of the propagation of the contraction through the preparation. These preparations were obtained by a homogenization of shorter duration than usual. For the cardiac tissue these fragments were pluricellular and the sarcolemma that was remaining after homogenization was not skinned.

TABLE 2. Composition of the solutions used for MgATP activation. The pCa was about 9·50 in all cases. The solutions contained 1·0 mM-ATP and 10·0 mM-EDTA in addition to the added MgEDTA. The Table gives the total Mg and added MgEDTA necessary to obtain the desired pMgATP and pMg

Total Mg (mM)	MgEDTA (mM)	pMgATP	pMg
0·02	0·020	7·0	8·0
0·06	0·060	6·50	7·60
0·20	0·198	6·0	7·10
0·40	0·395	5·75	6·80
0·70	0·696	5·50	6·60
1·10	1·09	5·25	6·30
2·0	1·99	5·0	6·10
6·0	5·97	4·50	5·50
20·0	19·90	4·0	5·0

Recording of contraction

Tension developed by the skinned cells was recorded with a photo-diode force transducer as previously described (Fabiato & Fabiato, 1975). Cyclic contractions observed in slight EGTA buffering could be localized or could propagate within the cell. Accordingly, they might not be studied accurately by the simple tension measured from the ends of the cell. Therefore, two additional techniques of contraction recording were used in some experiments. (1) Propagation velocity of the contraction was measured with a closed circuit television. (2) Localized contractions were recorded with a photo-multiplier (A. O. Reichert spectrophotometer, Reichert, Vienna, Austria) that was attached to the microscope. The photo-multiplier recorded the variation of light transmittance occurring during the contraction of the 2 μm diameter area encompassed by the photomultiplier diaphragm (Fabiato & Fabiato, 1972). The electrical outputs of both the photo-multiplier and the transducer were filtered above 50 Hz and were displayed on a DC linear penwriter (Hewlett-Packard, Inc., Cupertino, Calif., U.S.A.) and on an oscilloscope. Additionally, the first derivative of the tension was recorded. The screen of the oscilloscope was viewed with a television camera. The images of the microscope camera and of the oscilloscope camera were mixed by a special effects generator and were stored on a tape recorder. The details of the television system were described previously (Fabiato & Fabiato, 1972). Skinned cells were stretched to a sarcomere length of 2·3 μm, corresponding to the maximal developed tension. The length and the width of the preparations were always measured when the cell was stretched to this sarcomere length. All values have been expressed as mean $\pm$ S.D. of an observation. Statistical significance of a difference between two means was determined by the Student t test.

RESULTS

Effects of Mg on cyclic contractions induced by a Ca-triggered release of Ca from the sarcoplasmic reticulum

In the presence of 0·050 mM total EGTA with pMg 3·50 and pMgATP 2·50 a skinned cardiac cell was quiescent at pCa 7·65. A phasic contraction was induced by decreasing pCa from 7·65 to 7·40. This phasic contraction was cyclically repeated (Fig. 1*A*). Decreasing pMg from 3·50 to 2·50 (while keeping pCa at 7·40 and pMgATP at 2·50) after the initial phasic contraction prevented its cyclic repetition (Fig. 1*B*). Caffeine (3 mM) was injected 45 sec after a phasic contraction in the presence of pMg 3·50 (Fig. 1*C*) and pMg 2·50 (Fig. 1*D*). The resulting contraction was

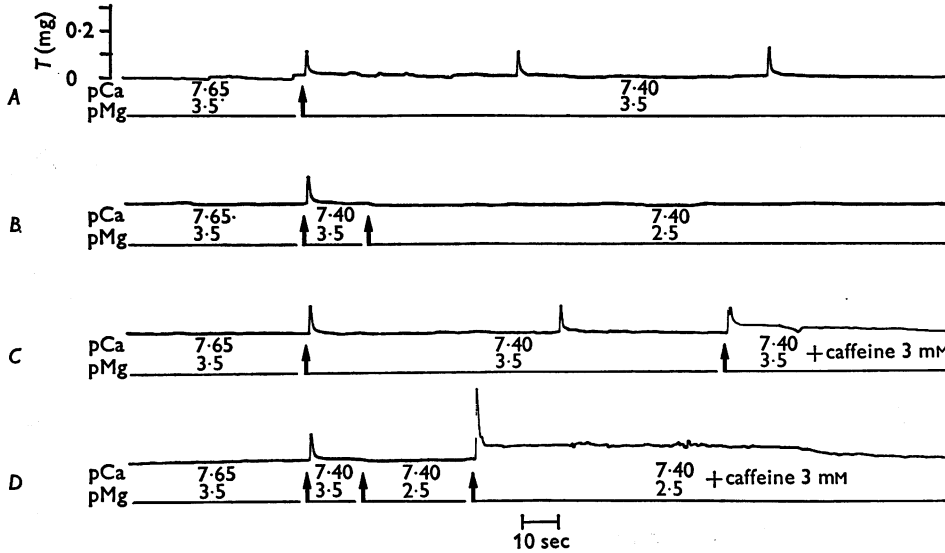

Fig. 1. Tension recording in a single skinned cell of 9 μm width and 44 μm length. While obtained from the same cell, the tracings are not continuous. Changes of perfusion medium are indicated by the arrows. In all cases total [EGTA] was 0·050 mM and pMgATP 2·50. Variations of pCa and pMg and additions of caffeine are indicated on the Figure.

much larger in amplitude and longer in duration in the presence of pMg 2·50 than in the presence of pMg 3·50. Since caffeine releases a fraction of the Ca^{2+} contained in the SR, it may be concluded that decreasing pMg produces an increase in rate and capacity of Ca^{2+} binding by the SR. This method of evaluating the Ca^{2+} content of the intracellular stores by measuring the contraction that was induced by caffeine was developed by Endo, Tanaka & Ogawa (1970). The cyclic

contractions were triggered by the overloading of the SR with Ca^{2+} to a critical level at which it released a portion of the Ca^{2+} it contained (Fabiato & Fabiato, 1975). Thus, the inhibition of the cyclic contractions that were produced by decreasing pMg would be due to an enhancement of the capacity of the SR for Ca^{2+}. Consequently, a pCa of 7·40 would represent too low a free [Ca^{2+}] to permit the filling of the SR to the level at which Ca^{2+} is released.

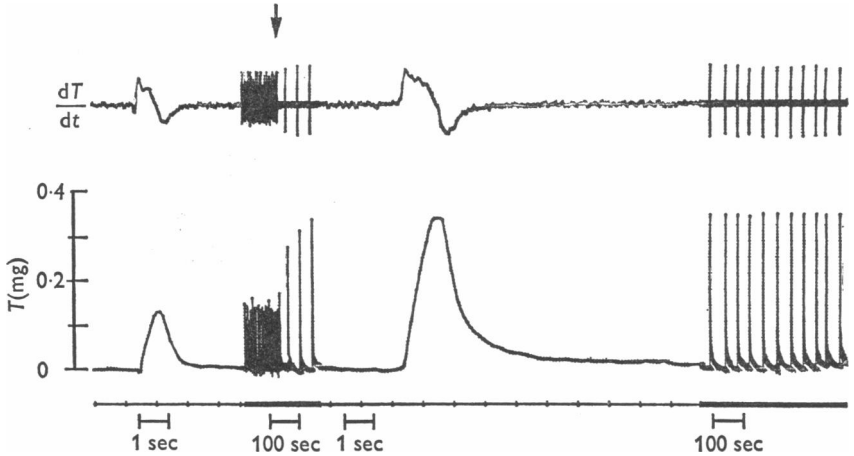

Fig. 2. Recording of tension (T) and of its first derivative (dT/dt) from a skinned cardiac cell of 11 μm width and 49 μm length. Two different recording speeds were used as indicated by the timer trace. Initially, the perfusion medium contained 0·050 mM total EGTA with pCa 7·0, pMgATP 2·50, and pMg 3·50. At the time indicated by the arrow, a medium of the same composition but with pMg 2·50 was introduced.

If the preceding hypothesis is correct, cyclic contractions should be obtained in the presence of pMg 2·50 but with a pCa lower than 7·40. In the experiment represented in Fig. 2 the pCa was maintained constant at 7·0 and pMgATP at 2·50. The decrease of pMg from 3·50 to 2·50 did not inhibit the cyclic contractions but decreased their frequency from 11 c/min to 1·4 c/min, increased their amplitude from 0·13 to 0·35 mg, and increased their duration from 1·5 to 4·0 sec. The duration was measured from the beginning of the contraction to 90 % of the relaxation. This result is consistent with the hypothesis that an enhancement of Ca^{2+} binding by the SR is produced by decreasing pMg. A longer time would be required to overload the SR to a level at which a fraction of Ca^{2+} is released, thus explaining the increase of cycle duration. When Ca^{2+} is eventually released, the large amplitude of the contraction observed in low pMg would be due to the release of a large amount of Ca^{2+} since the Ca^{2+} stores were more loaded than in high pMg.

These results are quite different from those reported in skinned fibres of skeletal muscle by Ford & Podolsky (1972). They observed that decreasing pMg (with simultaneous variation of pMgATP) produced a dramatic decrease in amplitude of the phasic contraction that was produced by a Ca^{2+}-triggered release of Ca^{2+}, instead of the increase in amplitude observed in skinned cardiac cells. The mechanism of this effect of pMg on skinned fibres of skeletal muscle was assumed, on indirect evidence (Stephenson & Podolsky, 1973), to be related to an acceleration of Ca^{2+} uptake by the SR. As previously shown, this mechanism is consistent with the results obtained in skinned cardiac cells. Thus, it was assumed that the mechanism of action of free Mg^{2+} may be the same in the two types of muscular tissue, but that the quantitative differences in the Ca^{2+}-triggered release of Ca^{2+} and in its propagation through preparations of different dimensions were responsible for the divergence of experimental results: i.e., the production by a decrease of pMg of an increase of contraction amplitude in cardiac cells and of a decrease in amplitude in skeletal muscle cells. To test this hypothesis experimental modifications were introduced in an attempt to obtain in skinned cardiac cells results similar to those usually obtained in skinned fibres of skeletal muscle and vice versa.

To explain their results Ford & Podolsky (1972) proposed the hypothesis that the Ca^{2+} released from one vesicle of the SR not only activates the myofibrils in the immediate vicinity, but also triggers a release of Ca^{2+} from other vesicles. This autocatalytic process would propagate the activation within the fibre. Decreasing pMg would inhibit this propagation by decreasing release of Ca^{2+} or by increasing the rate of Ca^{2+} binding so that Ca^{2+} release would not be triggered in the depth of the skinned fibre. Diffusion is much faster in single skinned cardiac cells with a diameter of 10 μm than it is in skinned fibres of skeletal muscle with a diameter equal to or larger than 100 μm. Therefore, the centre of the cardiac cell is rapidly submitted to the same pCa as the surface. Accordingly, no propagation of the cyclic contractions within a single skinned cell (less than 15 μm width and 60 μm length) was visible on the television recording. There was good synchronism between the contractions recorded from the whole cell with the transducer and the localized contraction recorded with a photomultiplier from a 2 μm diameter area in the centre of the cell (Fig. 3). In the presence of pCa 7·20 the amplitude of both contraction recordings increased when the pMg was decreased and decreased when the pMg was increased.

In some experiments larger preparations of cardiac tissue were used. These consisted of groups of a few cells with disrupted but not skinned sarcolemmas. The width of these preparations was 25–30 μm and their length was 100–150 μm. In these pluricellular preparations a propagation

of the cyclic contractions was observed with a velocity of 80–100 μm/sec, as measured on the video-tape recording in the presence of pMg 3·50 and pCa 7·0. Lowering pMg from 3·50 to 2·50 resulted in a decrease in the velocity of this propagation to 10–30 μm/sec. The photomultiplier recording was more sensitive than the tension recording in the detection of the asynchrony of contraction. In low pMg the photo-multiplier recording showed each beat to have several components (Fig. 4). Some corresponded to contractions of the area encompassed by the photomultiplier, while

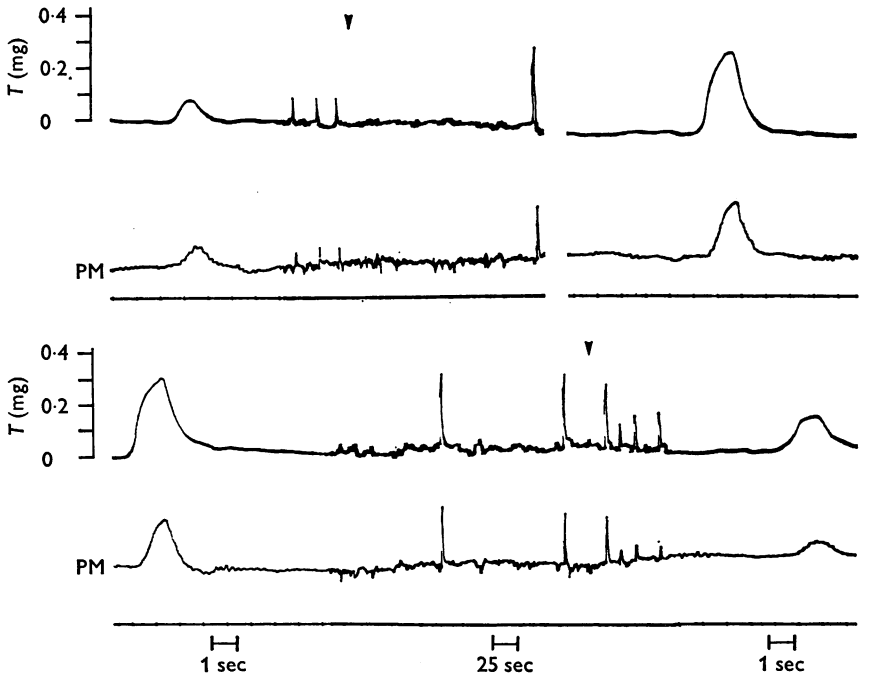

Fig. 3. Recording of the tension (T) from the ends of a skinned cardiac cell of 10 μm width and 41 μm length. Simultaneously, a variation of light transmittance (PM) was obtained with a photomultiplier from a 2 μm diameter area situated in the centre of the preparation. Two different recording speeds were used as indicated by the timer trace. Initially, the perfusion medium contained 0·050 mM total EGTA, pCa 7·20, pMgATP 2·50, and pMg 3·50. At the time indicated by the first arrow this medium was replaced by a similar one except that pMg was 2·50. At the time indicated by the second arrow the perfusion with pMg 3·50 was resumed.

others reflected asynchronous contractions of other areas. This asynchrony of contractions was deemed responsible for the slightly smaller amplitude of the tension recorded from the ends of the skinned cell in low pMg as compared to that obtained in high pMg. Thus, the effect of the decrease

of pMg on the conduction masked its effects on the contraction amplitude. These results were comparable to those obtained in skinned fibres of skeletal muscle. However, the reduction of amplitude of contraction in low pMg was never as dramatic as in skeletal muscle.

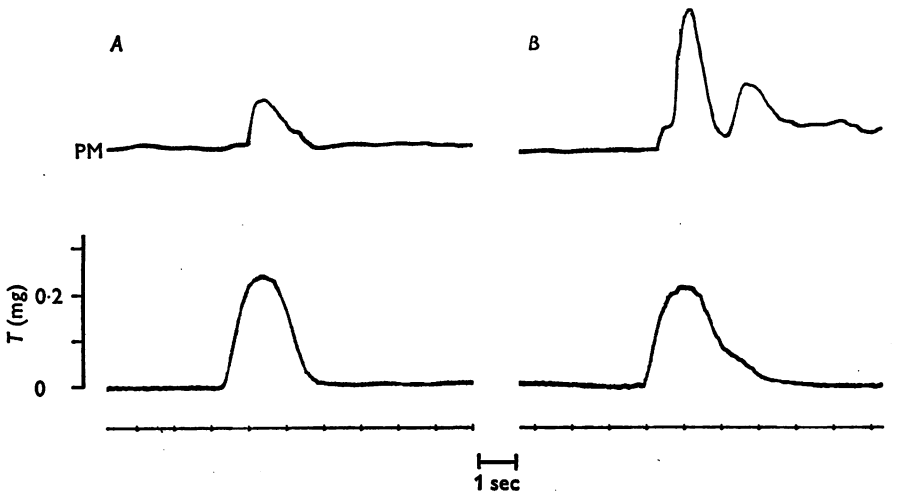

Fig. 4. Recording of tension (T) from the ends of a pluricellular cardiac fibre with disrupted (but not skinned) sarcolemma of 30 μm diameter and 102 μm length. Simultaneously, a variation of light transmittance (PM) was monitored with a photomultiplier from a 2 μm diameter area situated in the centre of the preparation. In A the preparation was perfused with a medium containing 0·050 mM total EGTA, pCa 7·0, pMgATP 2·50 and pMg 3·50. In B the composition of the perfusion medium was similar except that pMg was 2·50.

To reduce the influence of the conduction on the effects of decreasing pMg, fragments of skinned fibres of skeletal muscle were obtained by homogenization and were dissected in small bundles. These small bundles of myofibrils with attached SR were 8–12 μm in width. Phasic contractions by a Ca^{2+}-triggered release of Ca^{2+} were evoked by decreasing pCa from a value of 8·40 (obtained with 0·050 mM total EGTA) to a value of 4·0 (obtained in the absence of EGTA). Decreasing pMg from 3·50 to 2·50 resulted in a nearly complete disappearance of the phasic contractions, which were reduced to one or several small oscillations superimposed on a slow increase of tension. The amplitude of these oscillations was less than 5% of the amplitude of the phasic contraction at pMg 3·50 (data not shown). This result was similar to that obtained by Ford & Podolsky (1972) in skinned fibres of about 100 μm width. Thus, the results observed in skeletal muscle are not significantly modified by variations of the size

of the preparation. Therefore they are not mainly due to a decrease of the conduction of the regenerative process throughout the preparation by decreasing pMg. No phasic contractions were observed in skinned fibres of skeletal muscle at any pMg when the pCa was greater than 5·0. Thus, no direct comparison between skinned cells of cardiac and skeletal tissue was possible, since cyclic contractions were induced in skinned cardiac cells at pCa 7·40 when the pMg was 3·50 and at pCa 7·0 when the pMg was 2·50.

Another difference may be that the Ca^{2+} uptake is more important and more rapid in the SR of skeletal muscle than in the cardiac SR. Fragments of skinned fibres of skeletal muscle of 8–12 μm width were perfused with 10 mM caffeine which reduced the rate and amount of the uptake of Ca^{2+}.

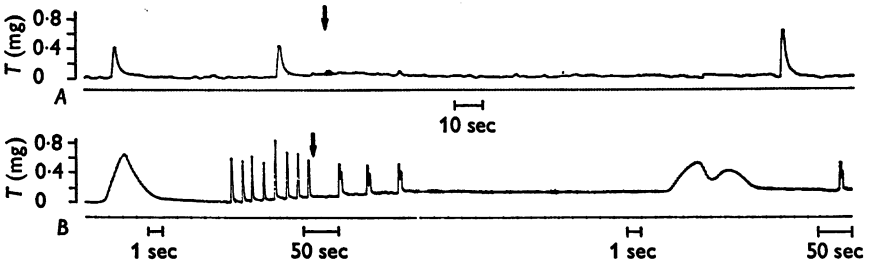

Fig. 5. In *A* recording of tension from a fragment of skinned fibre of skeletal muscle with 10 μm width and 32 μm length. In *B* recording of tension from a fragment of skinned fibre of skeletal muscle with 28 μm width and 60 μm length. In both *A* and *B*, the preparation was initially perfused with a medium containing 0·050 mM total EGTA, pCa 7·0, pMg-ATP 2·50 and pMg 3·50. At the time indicated by the arrow a perfusion with a medium of the same composition but with pMg 2·50 was introduced. Various speeds of recording were used as indicated by the timer trace.

Under these conditions, cyclic contractions were observed in the presence of pCa 7·0 with 0·050 mM total EGTA, pMg 3·50 and pMgATP 2·50. Decreasing pMg from 3·50 to 2·50 (while keeping the other parameters constant) resulted in an increase in the duration of the cycles and in the amplitude and the duration of cyclic contractions (Fig. 5*A*). This result is similar to that obtained in skinned cardiac cells. When the same experiment was done in skinned fibres of skeletal muscle of 25 to 40 μm width, the decrease of pMg from 3·50 to 2·50 resulted in a decrease in amplitude of the contractions (Fig. 5*B*). Two or more components were apparent in this contraction of smaller amplitude, which was evidence of an asynchrony of contraction due to the depression of the conduction of the process of regenerative release of Ca^{2+} throughout the fibre. Therefore, reduction in the dimensions of the skinned fibres of skeletal muscle and depression of the Ca^{2+} binding of their SR by caffeine permitted the

decrease of pMg to have an effect similar to that observed on skinned cardiac cells.

Addition of 10 mM azide to the solution did not modify the effects of increasing pMg on the cyclic contractions obtained in skinned cardiac cells. Hence, these effects seem to be mediated by the SR alone and do not seem to involve the mitochondria.

Varying pMgATP from 3·50 to 2·50 while keeping pMg constant at 3·50 had no significant effect on the frequency and the amplitude of the cyclic contractions induced by a Ca^{2+} triggered release of Ca^{2+} (data not shown). This decrease of (pMgATP) from 3·50 to 2·50 was accompanied by a decrease of pATP from 4·06 to 3·06. Hence, this negative result rules out the hypothesis of a major role of either [MgATP] or free [ATP] on Ca^{2+} release and uptake by the SR.

Effects of Mg and of MgATP of the tonic tension evoked by Ca

In the presence of a strong buffering with 4·0 mM total EGTA, cyclic contractions were inhibited, and the tonic tension evoked by decreasing pCa in the buffer corresponded to the direct effect of Ca^{2+} on the myofilaments (Fabiato & Fabiato, 1975). The tension increased when the pCa was decreased, and a curve of tension as a function of pCa was established. The effects of varying pMg, pMgATP, or both (Table 1) on this curve were studied. The effects of the variations of free [ATP] alone were not studied since studies in skeletal muscle have shown that free [ATP] has no effect on the tension (Reuben *et al.* 1971; Kerrick & Donaldson 1972).

At the beginning of each experiment, the skinned cardiac cell was in relaxing solution at pCa 9·0, pMg 3·50, and pMgATP 2·50, and the tension recorded was defined as zero. Contracting solutions with various pCa, pMg and pMgATP were applied until a plateau of tension was obtained. Generally, applications of the contracting solutions were separated by a return to the relaxing solution (Fig. 6*A* and *B*). In some cases, however, two contracting solutions were applied without intermediary return to the initial solution (Fig. 6*C*). Each series of contracting solutions applied on a given skinned cell was begun and ended by the application of the same solution. When the two resulting tensions differed by more than 15%, the experiment was discarded. In addition, a contracting solution at pCa 5·0, pMg 3·50 and pMgATP 2·50 was applied to each skinned cell. The resulting tension was defined as 100% to permit the relative measurement of the other tensions developed (Fig. 7). Since only the width of the cells was measured in these experiments, it was impossible to express the tension developed per cross-section area.

Decreasing pMg from 4·50 to 2·50 while keeping the pMgATP constant at 2·50 resulted in a shift to the right of the tension–pCa curve so that a smaller tension was developed for a given pCa (Fig. 7: open symbols). Statistical analysis demonstrated a more significant difference when the pMg was varied from 3·50 to 2·50 (Fig. 7: filled circles and open triangles,

$P < 0{\cdot}001$ for all pairs of points of sub-maximal tension), that when the pMg was varied from 4·50 to 3·50 (Fig. 7: open squares and filled circles, $P < 0{\cdot}02$ for the pairs of points corresponding to pCa 6·0 and 5·75, $P < 0{\cdot}01$ for pCa 6·25 and $P < 0{\cdot}001$ for all other pairs of points of sub-maximal tension). The maximum tension developed at pCa 5·0 was not modified. In contrast, the threshold of pCa for tension development was shifted to the right by decreasing pMg.

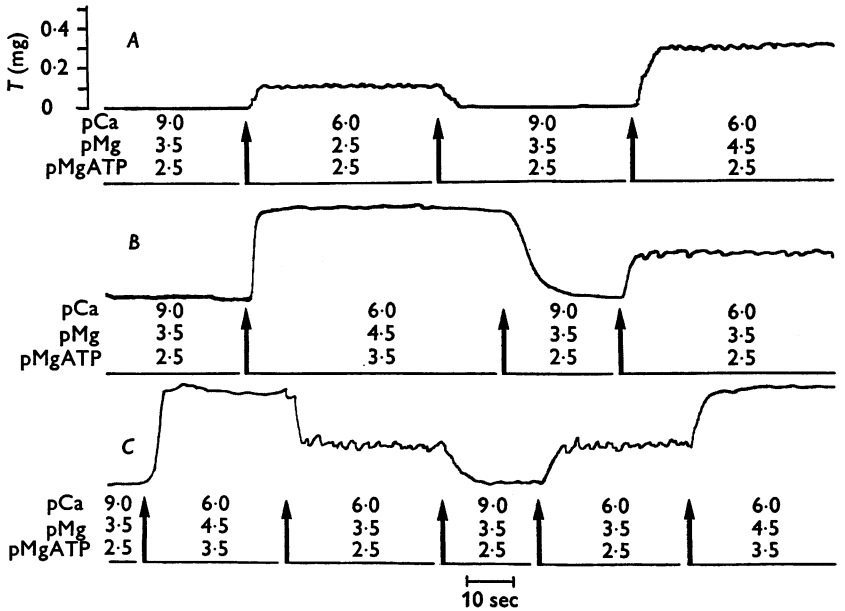

Fig. 6. Effects of varying pMg and pMgATP on the tonic tension induced by Ca^{2+}. For tracing *A* and *B* a skinned cardiac cell of 10 μm width and 40 μm length was used. For tracing *C* a skinned cardiac cell of 11 μm width and 42 μm length was used. The total [EGTA] was 4·0 mM in all cases; pCa, pMg and pMgATP are indicated in the Figure. The arrows indicate the perfusion changes.

Similar effects of varying pMg at constant pMgATP were found on fragments of skinned fibres of frog skeletal muscle of 8–12 μm width and 30–60 μm length. Only the tension developed at pCa 6·0 was studied for this preparation, and it was expressed relative to the tension developed at pCa 4·50 with pMg 3·50 and pMgATP 2·50. The relative tension at pCa 6·0 was $66\% \pm 13\%$ (S.D. for twenty observations) at pMg 4·50, $58\% \pm 4\%$ (S.D. for fifty observations) at pMg 3·50 and $18\% \pm 11\%$ (S.D. for twenty observations) at pMg 2·50. These results are consistent with those reported by Kerrick & Donaldson (1972) on skinned fibres of frog semi-tendinosus and by Ashley & Moisescu (1974) on bundles of myofibrils

of barnacle. They are also consistent with the original observations of Ebashi & Endo (1968) on skinned fibres of frog semi-tendinosus, although these authors did not separate the effects of pMg from those of pMgATP.

In skinned cardiac cells, increasing pMgATP from 2·50 to 3·50 while keeping the pMg at 4·50 resulted in a significant ($P < 0{\cdot}001$ for all points)

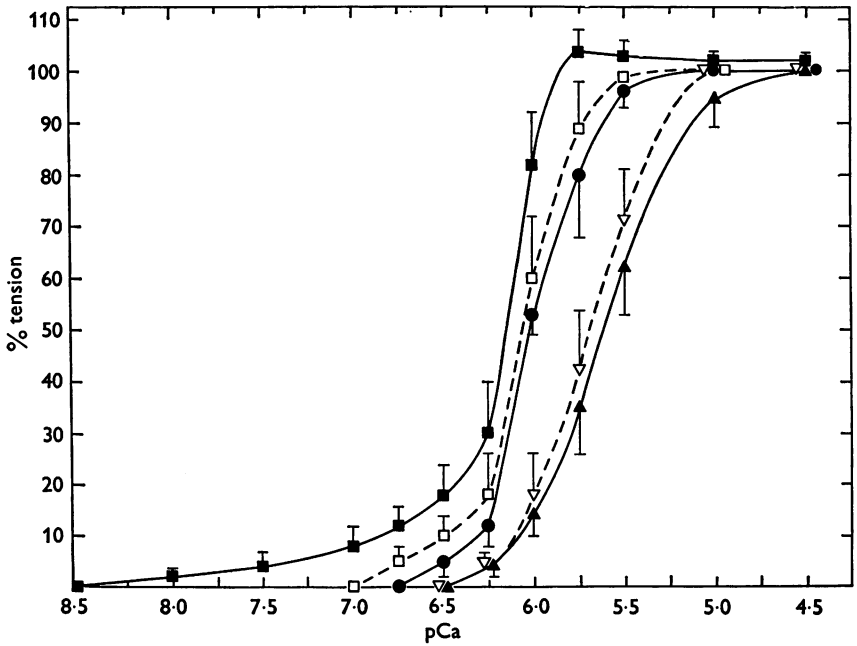

Fig. 7. Curves of the tension developed by skinned cardiac cells of 9–13 μm width and 35–60 μm length as a function of pCa for various pMg and pMgATP (▲—▲, pMg 2·50, pMgATP 1·80; ▽—▽ pMg 2·50, pMgATP 2·50; ●—● pMg 3·50, pMgATP 2·50; □—□ pMg 4·50, pMgATP 2·50; ■—■ pMg 4·50, pMgATP 3·50). The total [EGTA] was always 4·0 mM. For each medium applied to a given cell, the tension is expressed by the percentage of the tension developed by the same cell at pCa 5·0, pMg 3·50 and pMgATP 2·50. The zero percent of tension is defined by the tension developed at pCa 9·0, pMg 3·50 and pMgATP 2·50. Each point represents the mean of twenty measurements except for the point corresponding to pCa 6·0, pMg 3·50, pMgATP 2·50, which corresponds to the mean of fifty measurements. Vertical bars represent the standard deviations. For clarity, the S.D. is shown in one direction only.

and a pronounced shift to the left of the tension–pCa curve (Fig. 7: open and filled squares). Additionally, the maximum tension was slightly but significantly increased, and the threshold of free [Ca^{2+}] for tension development was considerably decreased. A significant tension was observed

at a pCa as great as 8·0 with pMgATP 3·50. These effects of varying pMgATP on the tension developed at constant and high pMg are similar to those reported by Brandt *et al.* (1972) in crayfish skinned fibres.

Decreasing pMgATP from 2·50 to 1·80, while keeping pMg constant at 2·50 (Fig. 7: open and filled triangles) also resulted in a shift of the curve of tension as a function of the pCa. However, statistical analysis demonstrated that this shift was less significant than when the pMgATP was varied from 3·50 to 4·50 (Fig. 7: open and filled triangles, $P < 0{\cdot}001$ for the pairs of points corresponding to pCa 5·0, $P < 0{\cdot}01$ for pCa 5·5, $P < 0{\cdot}05$ for pCa 5·75 and non-significant difference for pCa 6·0). The weakness of this effect of varying pMgATP on the tension–pCa relation obtained in the presence of a low pMg is consistent with the findings of Kerrick & Donaldson (1972) in skinned fibres of frog semitendinosus.

In a recent paper (kindly communicated to us before publication), Godt (1974) demonstrated that the hydrolysis of ATP within skinned fibres of skeletal muscle rendered necessary the use of an ATP regenerating system to study the effects of low pMgATP. Accordingly, a curve of tension as a function of the pCa was obtained in skinned cardiac cells bathed in a medium containing 14·5 mM creatine phosphate and creatine phosphokinase 1 mg/ml. with pMg 4·50 and pMgATP 3·50. No significant difference was found between this curve and that obtained in the absence of the ATP regenerating system. For two reasons, this result could have been anticipated: (1) the total [ATP] used (Table 1) was at a level where the fraction of ATP that was hydrolysed was small in the study of Godt (1974); and (2) the radius of the skinned cardiac cells is ten times smaller than that of the skinned fibres of frog semitendinosus (the rate of hydrolysis is directly proportional to the square of the radius).

Tonic tension evoked by varying pMgATP in the virtual absence of free Ca^{2+}

Increasing pMgATP above 3·50 in the virtual absence of free Ca^{2+} (pCa 9·50) resulted in the development of a tension in skinned cardiac cells (Fig. 8). Very high pMgATP were obtained by using EDTA instead of EGTA. Total [ATP] was always 1·0 mM (Table 2). The increase of pMgATP was associated with an increase of pMg. However, on the basis of a demonstration provided by Reuben *et al.* (1971) on skinned fibres of crayfish muscle, the resulting effect was attributed to the variation of pMgATP. When the pMgATP was increased from 4·0 to 5·50, the tension that was developed at pCa 9·50 increased (Figs. 8 and 9). The maximum tension activated by MgATP was $52 \pm 8\%$ of the previously defined maximum tension activated by Ca^{2+} that was obtained at pMgATP 2·50. When pMgATP was further increased from 5·50 to 7·0, the tension decreased. The tension that was induced by varying [MgATP] appeared several seconds after the application of the solution, and the rate of development of the tension was slow as compared with that observed in the Ca^{2+} activated tension (Fig. 8*A*).

A similar bell-shaped curve of tension as a function of pMgATP at pCa 9·50 was obtained in segments of skinned fibres of skeletal muscle of 8–12 μm width and 30–60 μm length (Fig. 9). Similar observations were made on skinned fibres of crayfish (Reuben *et al.* 1971), on glycerinated fibres of skeletal muscle (Watanabe, Sargeant & Angleton 1964; Filo, Bohr & Ruegg, 1965) and on glycerinated insect flight muscles (White, 1970). In contrast, no tension was evoked in smooth muscle by increasing pMgATP in glycerinated smooth muscle (Filo *et al.* 1965).

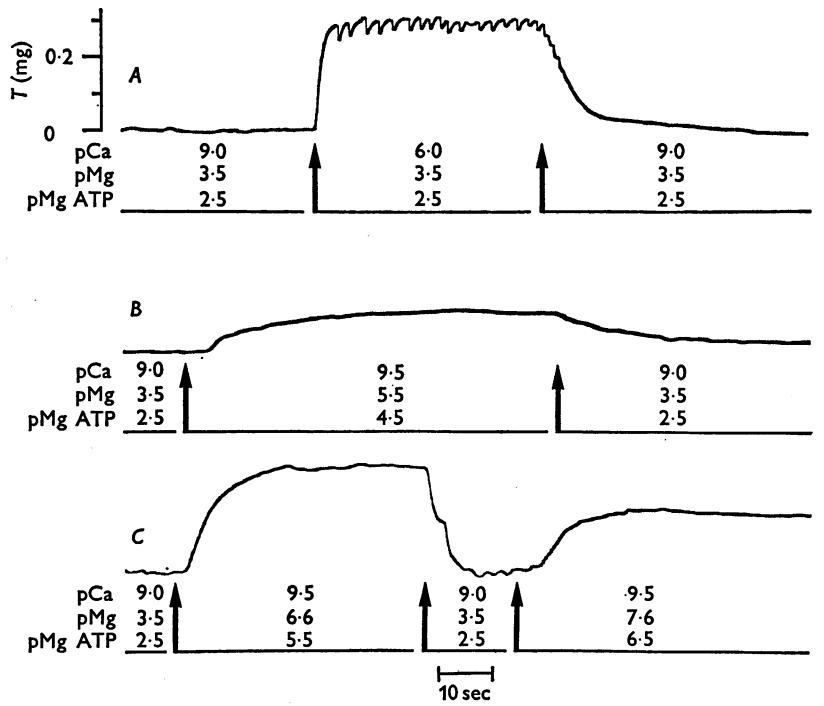

Fig. 8. Tension induced in the virtual absence of free Ca^{2+} by varying pMgATP in a skinned cardiac cell of 10 μm width and 39 μm length. The pCa, pMg and pMgATP are indicated in the figure; the composition of the solutions is given in Table 2. The arrows indicate the perfusion changes. In *A* the tension developed by the same cell at pCa 6·0 with 4·0 mM total EGTA, pMg 3·50 and pMgATP 2·50 is shown for comparison. The small oscillations observed during the plateau of tension in tracing *A* of this Figure as well as in some of the tracings of Fig. 6 were described in another study (Fabiato & Fabiato 1974*b*).

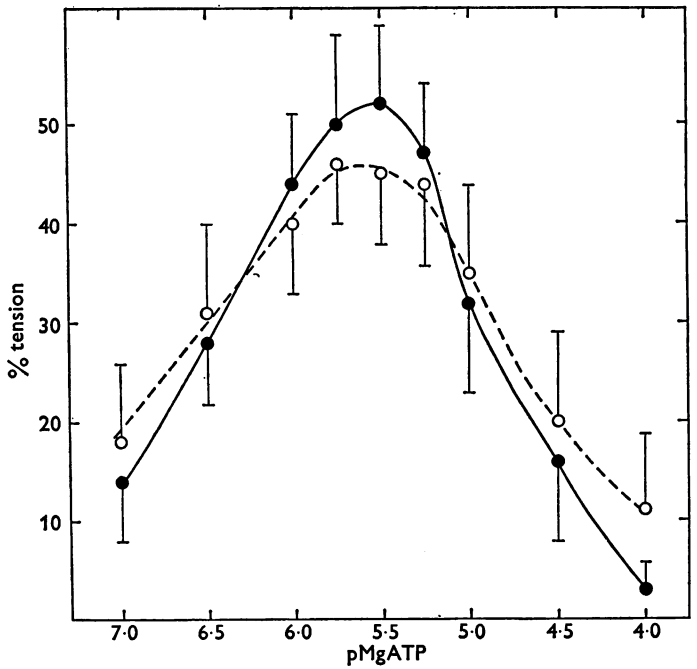

Fig. 9. Curve of tension developed by skinned cardiac cells of 9–13 μm width and 35–60 μm length (●—●) and by segments of skinned fibres of frog semi-tendinosus of 8–12 μm width and 30–60 μm length (○ - - ○) as a function of pMgATP at pCa 9·50. For each medium applied to a given cell the tension is expressed by the percentage of the tension developed by the same cell at pCa 5·0 (cardiac cell) or 4·50 (skeletal muscle fibre), with 4·0 mM total EGTA, pMg 3·50 and pMgATP 2·50. The zero percent of tension is defined by the tension developed at pCa 9·0 with 4·0 mM total EGTA, pMg 3·50 and pMgATP 2·50. Each point represents the mean of twenty measurements. Vertical bars represent the S.D. For clarity, the S.D. is shown in one direction only.

DISCUSSION

These results show a similarity between cardiac and skeletal muscle in the effects of pMgATP and pMg on tension development. In the presence of much greater pMg and pMgATP than those corresponding to physiological levels (Polimeni & Page, 1973), increase of pMgATP produced a tension in the virtual absence of free Ca^{2+}. Reuben *et al.* (1971) interpreted the bell-shaped curve of tension as a function of pMgATP as a phenomenon of substrate inhibition (Levy & Ryan, 1965). The MgATP would interact with the globular part of the myosin molecule, resulting in the formation of cross-bridges in the presence of a high pMgATP. Consequently, the tension increases when pMgATP decreases from 7·0 to 5·50.

In this reaction the myosin (or acto-myosin) is an enzyme which catalyses the hydrolysis of its substrate MgATP. When pMgATP decreases from 5·50 to 4·0, the excess of substrate inhibits the enzymatic reaction. Accordingly, a relaxation is observed.

Bremel & Weber (1972) proposed a different mechanism to explain the effect of MgATP on Ca^{2+}-activation of the myofibrillar ATPase from skeletal muscle. This mechanism assumes cooperation among the molecules of the thin filaments. At high pMgATP, the binding of MgATP to the myosin would result in the formation of 'rigor complexes' between the actin molecules of the thin filaments and the myosin molecules of the thick filament. The formation of rigor complexes would modify the affinity of the troponin molecules to Ca^{2+} by causing a cooperative transformation of the low affinity sites of the troponin into high affinity sites. The contraction would then be initiated at a higher pCa than in the presence of a physiological pMgATP, where there are practically no rigor complexes. This hypothesis has been derived from experiments done on isolated myofibrils (Bremel & Weber, 1972) and confirmed by tension measurements in skinned fibres of skeletal muscle (Godt, 1974).

Under physiological conditions the pMgATP and the pMg are probably in the millimolar range (Polimeni & Page 1973). The curve of tension as a function of the pCa obtained at pMg 3·50 and pMgATP 2·50 (Fig. 7, filled circles) is the most likely to correspond to physiological conditions. According to the hypothesis of Bremel & Weber (1972), and Godt (1974) rigor complexes are practically absent at this level of pMgATP. According to the hypothesis of Reuben *et al.* (1971), the excess of substrate inhibits the reaction between MgATP and myosin, and the role of Ca^{2+} is the suppression of this inhibition (Brandt *et al.* 1972). At this level, variations of pMgATP have little effect, but decrease of pMg decreases the sensitivity of the myofilaments to Ca^{2+}. However, variations of free $[Mg^{2+}]$ in the millimolar range result in variations in the sensitivity of the myofilaments to the free $[Ca^{2+}]$ in a micromolar range. This suggest that the affinity of the binding sites for Mg^{2+} is three orders of magnitude lower than the affinity of the binding sites for Ca^{2+}. This observation supports the conclusion derived from studies on isolated proteins from skeletal muscle that Mg^{2+} interacts with troponin by binding to sites different from those binding Ca^{2+} (Fuchs, Reddy & Briggs, 1970).

The similarity in the effects of pMg and pMgATP on the tonic contractions in skinned cells of skeletal and cardiac muscle contrasts with the dissimilarity between the two tissues in the effects of pMg on the cyclic contractions induced by Ca^{2+}-triggered release of Ca^{2+} from the SR. The effects of a decrease in pMg on the cyclic contractions produced by Ca^{2+}-triggered release of Ca^{2+} in cardiac cells can be explained by an increase

in capacity and rate of binding for the Ca^{2+} of the SR. This hypothesis is consistent with the observation obtained from isolated SR fragments of skeletal muscle that an increase of free Mg^{2+} produced an increase in capacity for Ca^{2+} (Ebashi & Lipman, 1962; Carvalho & Leo, 1967) and in rate of Ca^{2+} binding (Tonomura, 1973; Horgan, 1974). Similar observations were recently reported in fragmented SR of cardiac muscle (Entman, Snow, Freed & Schwartz, 1973). Therefore the mechanism of effect of free Mg^{2+} on the SR is similar in cardiac and skeletal muscle. However, the SR of skeletal muscle has a larger capacity for Ca^{2+} and rate of Ca^{2+} binding than the SR of cardiac muscle (Solaro & Briggs, 1974). Consequently, cyclic contractions by Ca^{2+}-triggered release of Ca^{2+} are observed in cardiac muscle in the presence of free $[Mg^{2+}]$ which inhibits almost completely the Ca^{2+} release process in skeletal muscle (in the absence of caffeine). Two hypotheses are possible: (1) the intracellular free $[Mg^{2+}]$ is physiologically different in skeletal muscle than it is in cardiac muscle, and (2) the Ca^{2+}-triggered release of Ca^{2+} is a physiological process in cardiac muscle and probably not in skeletal muscle. The first hypothesis is unlikely because the free $[Mg^{2+}]$ appears to be relatively constant in a broad variety of tissues (Veloso *et al.* 1973), and because the sensitivity of the myofilaments to free $[Mg^{2+}]$ appears to be strikingly similar in cardiac and skeletal muscle. The second hypothesis is supported by other arguments, which are set forth in another article (Fabiato & Fabiato, 1975). It is reasonable to assume that the physiological intracellular free $[Mg^{2+}]$ is between $10^{-4.0}$ and $10^{-3.0}$ M (Page & Polimeni, 1972). Even in the presence of a free $[Mg^{2+}]$ of $10^{-2.50}$ M, a contraction by release of Ca^{2+} from the SR can be triggered in skinned cardiac cells from rat ventricle by a free $[Ca^{2+}]$ much smaller than that required to directly activate the myofilaments.

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