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SLOW INWARD BARIUM CURRENT AND CONTRACTION ON FROG SINGLE MUSCLE FIBRES

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SUMMARY

1. Excitation–contraction coupling process in isolated frog muscle fibres, under conditions which allow the development of a Ba permeability, has been investigated by the simultaneous recording of electrical and mechanical activity.

2. The sustained contraction elicited by a long lasting Ba action potential depends on two mechanisms. The first is potential dependent, the second which is inhibited by $MnCl_2$ (10 mM), depends on the inward flux of Ba ions.

3. The relationship observed between the inward I_{Ba} and the peak tension resembles that which has been observed between I_{Ca} and the contraction on other muscular structures.

4. The relative tension progressively declines as the intracellular Ba concentration increases and the contractility ends after a series of depolarizing pulses (or Ba action potentials). This indicates that the Ba ions which enter the cell release Ca ions and replace them in the intracellular storage sites.

5. Following a pretreatment with caffeine, the inward I_{Ba} fails to induce a contraction. Moreover a muscle which has been loaded with barium until the contraction ceases, does not develop a contracture in presence of caffeine. These results show that the Ba induced Ca release is located at the level of the sarcoplasmic reticulum.

6. Calculations show that the amount of Ba ions necessary to abolish the contractility corresponds to the maximum ability of the sarcoplasmic reticulum for Ca binding.

7. Almost all the inward flux of Ba ions and the contraction are abolished by glycerol-treatment which suggests that the coupling occurs at the T-system level. The results are discussed in regard to the technical limitations of the voltage-clamp method.

INTRODUCTION

Alkali-earth cations can serve as charge carriers in excitable membranes and may participate in long-lasting regenerative depolarizations (for review see Reuter, 1973). The existence of a permeability to divalent cations was first shown in crustacean muscular fibres (Fatt & Ginsborg, 1958). Bernard, Cardinaux & Potreau (1976) found that frog muscle fibres bathed in a physiological solution containing $BaCl_2$ but without $CaCl_2$, developed heart-like action potentials due to an increased permeability to Ba ions during the excitation. On the same preparation, in chloride

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free solutions, Beaty & Stefani (1976*a*, *b*), Stanfield (1977) and Potreau & Raymond (1978) demonstrated the development of a membrane permeability to Ca ions. It is therefore possible that an inward flow of divalent cations can play a role in the excitation–contraction coupling process. In order to study this possibility, the electrical and mechanical activities of frog muscle fibre were simultaneously recorded when the permeability to Ba ions developed. Of the divalent cations, Ba ions were selected for three main reasons: (i) Hagiwara, Fukuda & Eaton (1974) showed, on barnacle muscle fibres, that Ba ions are more efficient than the other divalent cations to carry current; (ii) Ba ions inhibit drastically the K conductance (Sperelakis, Schneider & Harris, 1967) and this greatly facilitates the observation of a membrane permeability to divalent cations; (iii) on frog skeletal muscle fibres, intracellularly injected Ba ions induce a large contraction (Heilbrunn & Wiercinski, 1947).

In a previous paper (Raymond & Potreau, 1977), we showed that the contraction elicited by a long lasting action potential depends, in part, on the inward flow of Ba ions which enter the cell and release Ca ions from intracellular storage sites.

The present investigation gives a detailed analysis of the link between the inward barium current and the resulting mechanical response of frog muscle fibres. A cumulative effect of Ba ions on the contractility which leads to a progressive block of the contraction is shown. A preliminary account of this work has been already reported (Raymond, Potreau & Bernard, 1977).

METHODS

Muscular preparations

All the experiments were performed at room temperature (18–20 °C), on muscle fibres (80–120 μm in diameter) isolated from the semitendinosus muscle of the frog (*Rana ridibunda*).

The detubulated fibres were obtained by Howell & Jenden's method (1967) modified by Eisenberg, Howell & Vaughan (1971) in order to maintain the resting potential. The detubulation was considered to be achieved when (i) the action potential did not show a negative after potential and was unable to elicit a contraction, and (ii) under voltage clamp conditions, a 100 mV depolarizing pulse lasting 100 msec did not induce any mechanical tension.

Electrical measurements

The electrical measurements were done by means of a double sucrose gap device derived from that described by Rougier, Vassort & Stampfli (1968), with an experimental trough and electrodes as described by Léoty & Alix (1976). The direct measurement of the membrane currents was done by a current to voltage converter (Poindessault, Duval & Léoty, 1974). The sucrose channels were 250 μm wide and the test gap was 200 μm wide.

Tension measurements

The tension was estimated by means of a variable resistance transducer prolonged by a thin sharp needle whose tip was put tangentially to the fibre in the test node (Raymond & Potreau, 1977). A stereomicroscope allows the observation of the fibre in the central compartment at a high magnification (× 100).

Solutions

The composition of the solutions is shown in Table 1. In the Ba containing solution NaCl was replaced isotonically by $BaCl_2$. This prevents a possible 'contamination' of the electrical response by uncontrolled Na spikes. This solution was called Ba Ringer. The solutions were buffered to pH 7·4 with Tris-HCl. When used, $MnCl_2$ and caffeine were added in amounts which did not drastically alter the tonicity of the medium (respectively 10 and 5 mM).

The fibres were isolated as the standard experimental conditions described previously (Raymond & Potreau, 1977) and then equilibrated for 15 min in normal Ringer, and then the fibre was placed in Ba Ringer.

TABLE 1. Composition of solutions

Solution...	NaCl (mM)	KCl (mM)	$CaCl_2$ (mM)	$BaCl_2$ (mM)	Buffer (mM)	Glucose (mM)
Normal Ringer	115	2·5	1·8	—	10	5·6
Ba Ringer	—	2·5	—	76	10	5·6

Isotonic sucrose solution: 266 mM

Nomenclature

V (mV): variations of membrane potential from its resting value considered as zero reference. The holding potential was progressively increased until the amplitude of the fast inward current, induced by any depolarization, reaches its maximum (i.e. $h_\infty \simeq 1$; holding potential $\simeq -90$ mV).

I (A): membrane currents whose values are negative for inward current and positive for outward current.

The values of membrane currents reported here were generally obtained by subtracting the leakage current (assumed to be linear from currents induced by small depolarizations) from the total current.

T (μN): mechanical 'tension' developed by the portion of fibre in the test node.

A.E.: absolute error estimated by assuming that the error done in the measurement of the dimensions of the fibre in the test node is < 5 μm and that for the measurement of the surface area delimited by the inward Ba current is $< 0{\cdot}01$ cm^2. For a product ($C = A \times B$) or a quotient ($C = A/B$), A.E. is given by $\Delta C = C(\Delta A/A + \Delta B/B)$.

Control of the tension recording method

The fibre is held on each side of the test node and there is no contraction in the sucrose chambers (as viewed under the stereomicroscope). The contraction in the central compartment is therefore recorded under approximately isometric conditions. However, for large contractions it is possible that a small displacement of the Vaseline partitions occurs without variation of the surface of the fibre in the test node. In this case, the mechanical activity recording represents a compromise between isometric and isotonic conditions.

The force (R) measured by means of the transducer is the resulting force of the linear force (F) developed by the fibre on each side of the transducer needle. If the needle is at the middle of the central compartment then

$$R = 2F \cos \alpha$$

and F can be estimated from R

$$F = \frac{R}{2 \cos \alpha}.$$

So, provided that the angle α formed by the fibre axis and the displacement plane of the needle is $\simeq 60°$, $R \simeq F$.

A similar technique has already been used by Vassort & Rougier (1972), Caillé, Ildefonse & Rougier (1978), however some control experiments have been performed. Fig. 1*A* shows a typical simultaneous recording of an action potential and the twitch; the twitch starts 4 msec later than the action potential, the maximum tension (90 μN) is reached in 20 msec and the full relaxation occurs after 70 msec. The latency relaxation which is seen in this record occurred in at least 50 % of the tested fibres. The amplitude of the twitch is of the same order of magnitude as those recorded in a double sucrose gap device by Nakajima & Bastian (1974), Caillé *et al.* (1978). Therefore the twitch recorded is similar to those obtained with other methods at the same temperature, except for the relaxation which is often shorter than if recorded in strict isometric conditions.

The curve of Fig. 1*B* was plotted from an experiment (panel *a*) where, under voltage-clamp

conditions, increasing depolarizing steps (> 1 sec) were applied to the preparation in normal Ringer solution. The threshold of the mechanical response lies between +30 and +40 mV from the resting potential and the maximum tension is obtained near a potential of +80 mV. Both these values and the S-shape of the curve are in good agreement with those obtained under voltage-clamp conditions by Lüttgau (1963), Bezanilla, Caputo & Horowicz (1971). The contractures obtained with our device in the presence of 5 mM-caffeine (see Fig. 10) also have

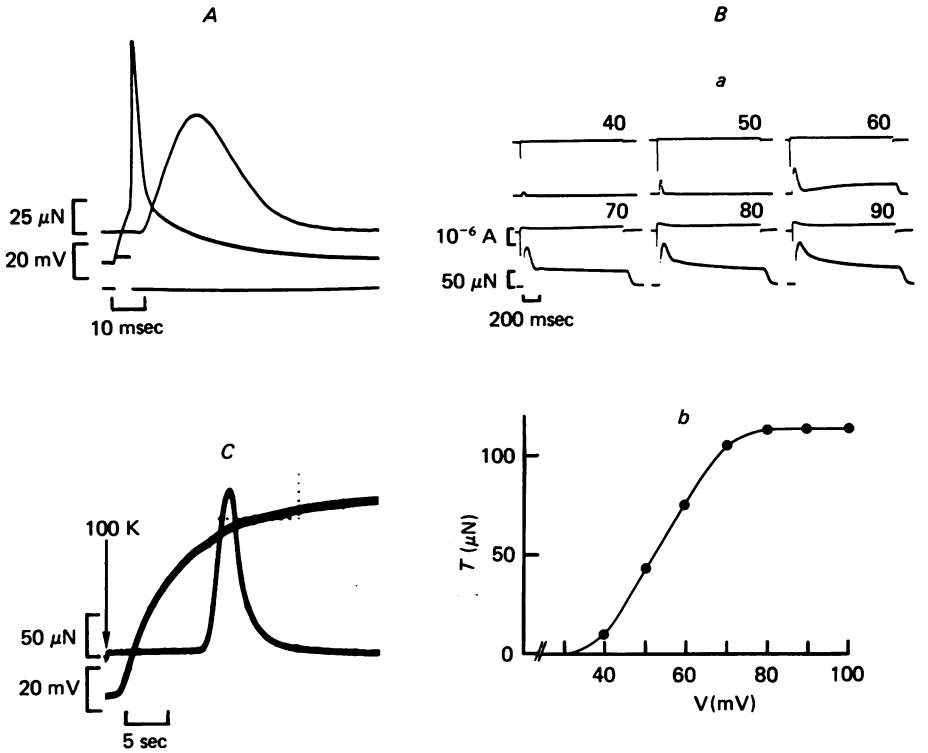

Fig. 1. *A*, simultaneous typical recording of an action potential (middle trace) and of the corresponding twitch (upper trace) in normal Ringer. Fibre diameter: 80 μm. *B*, panel *b*: relationships between the membrane potential (*V*) and the peak tension (*T*) plotted from the experiment illustrated. Panel *a*: simultaneous recordings of the current (upper traces) and of the contraction (lower traces) induced by depolarizing steps from 40 to 90 mV. Fibre diameter: 105 μm. *C*, typical contracture induced by a potassium depolarizing solution containing (mM): K glutamate: 100; NaCl: 3; Na glutamate: 14·5; Ca glutamate: 1·8; HEPES: 10; glucose: 5·6. Flow rate: 7·5 ml./min. Fibre diameter: 110 μm.

a time course similar to those reported by Lüttgau & Oetliker (1968). Furthermore, provided that the rate of perfusion in the test gap is faster than 5 ml./min, contractures can be recorded in presence of depolarizing K solutions (Fig. 1*C*). For slower flow rates the variation of the potential in the central compartment appears to be too slow to induce a contracture. Compared to the experiments of Hodgkin & Horowicz (1960), the low rate of perfusion (7·5 ml. instead of 60–360 ml./min) could account for the absence of plateau in our contractures.

From these results it appears that the tension recording method used in the present investigation can serve for a qualitative study of the contraction.

Measurement of the Ba influx during a depolarizing step

The Ba influx was estimated by the graphical integration of the surface area delimited by the inward Ba current (Fig. 2). This area was obtained by subtracting the total inward current obtained in Ba Ringer (76 Ba) from the outward current recorded at the same potential in presence of 10 mM-$MnCl_2$ (76 Ba, 10 Mn). This manganese concentration was found to inhibit completely the Ba permeability as shown in the Results section. The tail current area was included in the measurement because an inward flow of Ba occurs on repolarization.

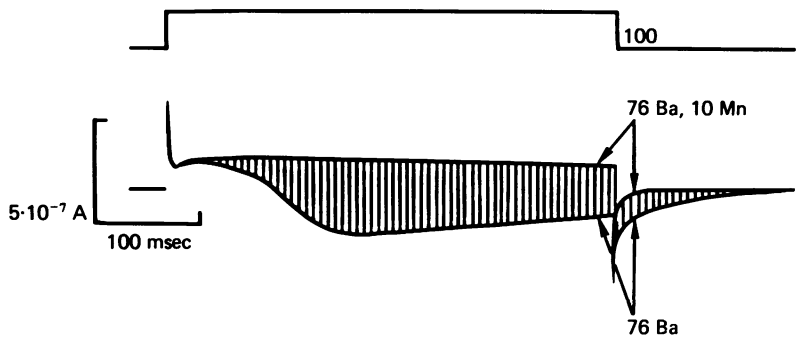

Fig. 2. An example of the estimation of the inward Ba flux. For a depolarizing pulse of 100 mV (upper trace) superimposed currents (lower traces) obtained in Ba Ringer (76 Ba) and in presence of 10 mM-$MnCl_2$ (76 Ba, 10 Mn). The hatched area corresponds to the surface delimited by the inward Ba current. Redrawn from the experiment illustrated in Fig. 5. Fibre diameter: 100 μm.

For a given potential the Ba influx is given by

$$\Delta[\mathrm{Ba}] = \frac{1}{2F}\int I_{\mathrm{Ba}}\,dt.$$

In the example of Fig. 2:

$$\int I_{\mathrm{Ba}}\,dt = 1{\cdot}075 \times 10^{-7}\ \mathrm{C}$$
$$\Delta[\mathrm{Ba}] = 5{\cdot}58 \times 10^{-13}\ \mathrm{mole}.$$

The volume of the fibre included in the test node ($1{\cdot}57 \times 10^6\ \mu^3$) was estimated by assuming that the fibres were cylindrical (those whose section did not seem cylindrical under microscope were discarded). It was thus possible to approximate the amount of Ba which entered the cell per unit of volume: 0·36 mM.

RESULTS

The relationships between the electrical activity and mechanical tension. Under our experimental conditions, more than 80 % of the fibres developed a permeability to barium ions. Fig. 3 (*B*, *C*) shows a long lasting action potential and the accompanying contraction. The electrotonic depolarization is followed by a maintained plateau which induced a large contraction. Compared to the control response of the record *A*, a twofold increase in the amplitude of the mechanical response is observed (according to the fibres, the amplitude of the contraction is generally between 2 and 4 times larger). The duration of the contraction can exceed that of the action potentials (as in record *C*) but for longer action potentials (up to 1 sec) the relaxation often occurred before the full repolarization of the membrane.

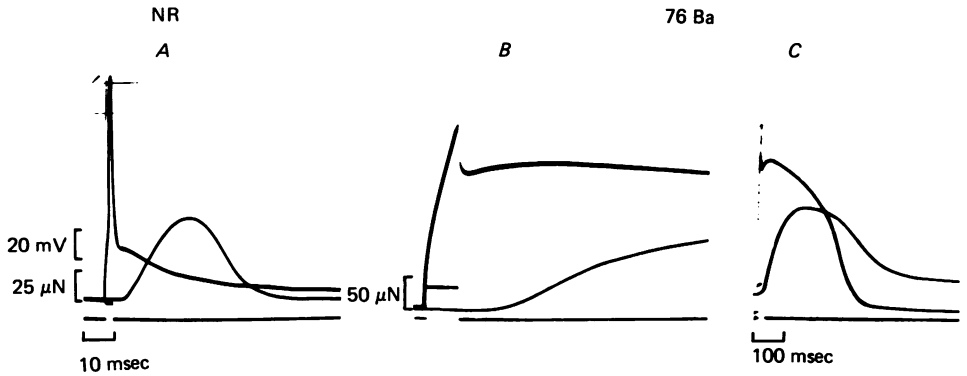

Fig. 3. Simultaneous recordings of AP and contraction. *A*, control response in normal Ringer. *B* and *C*, on the same fibre, a long lasting barium regenerative response (recorded at two different speeds) and the large contraction elicited. Note that for the contraction, the magnification is lower in *B* and *C* than in *A*. Fibre diameter: 90 μm.

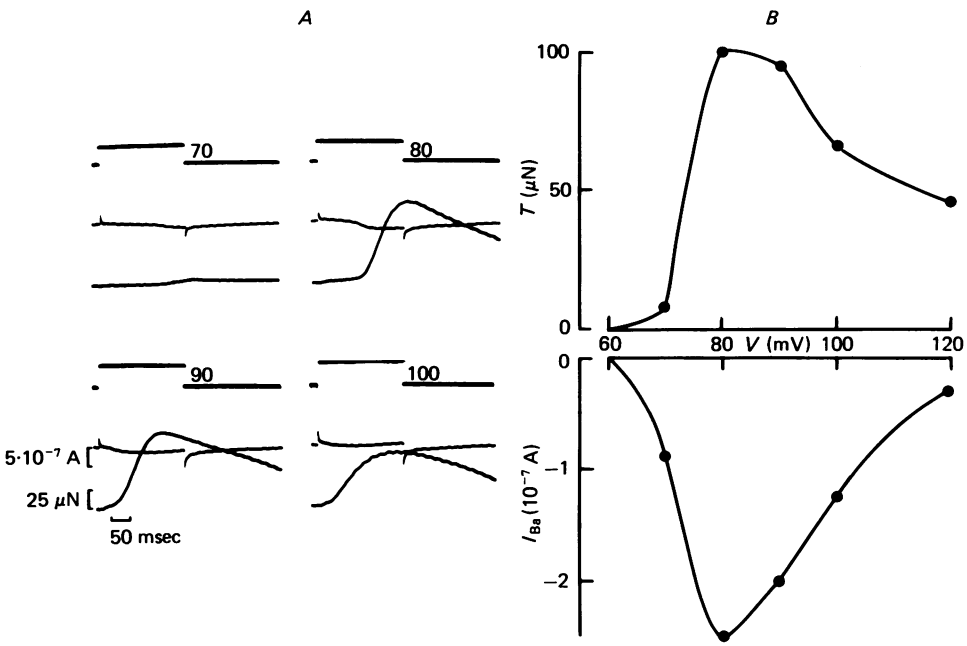

Fig. 4. Relation between the inward barium current and the tension. *A*, simultaneous recordings of the total current (middle traces) and of the contraction (lower traces) for different depolarizing pulses (upper traces). *B*, the relationship between the membrane potential (V) and the peak tension (T) and the inward Ba current (I) corrected from the leakage current. These curves were plotted from the data illustrated in *A*.

The records of Fig. 4 obtained under voltage clamp conditions illustrate the effects of depolarization on I_{Ba} and on the corresponding contraction. For depolarizations larger than 70 mV, the small outward current is followed by an inward current most likely carried by Ba ions. On the repolarization, an inward tail current, due to the incomplete inactivation of I_{Ba} is observed. The simultaneous contraction develops in two phases: the first, which starts as soon as the pulse is applied is very small in this experiment, the second whose peak tension reaches 100 μN develops simultaneously with I_{Ba}.

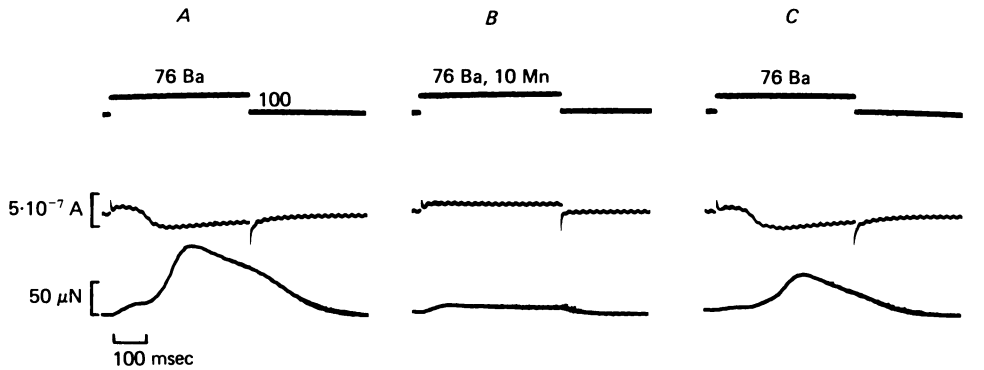

Fig. 5. Effect of $MnCl_2$ (10 mM) on the Ba permeability. Simultaneous recordings of the total current (middle traces) and of the contraction (lower traces) for a given potential (+100 mV). *A*, control response in Ba Ringer. *B*, after 3 min in presence of Ba Ringer + 10 $MnCl_2$. *C*, 5 min after the removal of $MnCl_2$. Fibre diameter: 110 μm.

As the electrical event always precedes the development of tension, the inward Ba current cannot be a movement artifact. (This is also confirmed by the observation of a large inward current without an accompanying contraction, see Fig. 8.)

The curves *B* show that the threshold of the two phenomena is situated between +60 and +70 mV. Their amplitude is maximum for a potential of +80 mV and then decreases at higher potentials. At first sight, a close relation would seem to exist between these two phenomena. However for the equilibrium potential of I_{Ba} (which lies between +130 and +150 mV), a noticeable tension is still observed. This suggests that the contraction is not only dependent on this current. A conclusion which is reinforced by the results illustrated in Fig. 5, which show that $MnCl_2$ inhibits the inward I_{Ba} and the contraction observed in *A*. This effect is fully reversible for the current and partly for the contraction as shown in *C*. Nevertheless, in absence of I_{Ba}, a small contraction, starting at the beginning of the pulse, is still present. This indicates that a part of the contraction is not current dependent.

Cumulative effect of Ba on the contractility. The preceding set of experiments suggests a close correlation between I_{Ba} and the contraction. However, such a conclusion becomes less obvious when analysing the results obtained in the following experiments.

Fig. 6 shows five successive recordings of a long lasting action potential and of the corresponding twitch. The amplitude of the twitch decreases progressively without any change in the action potential irrespective of the frequency of stimulation and,

in this experiment the fifth electrical response fails to generate mechanical tension (according to the fibres, four to ten successive action potentials are necessary to abolish the contraction).

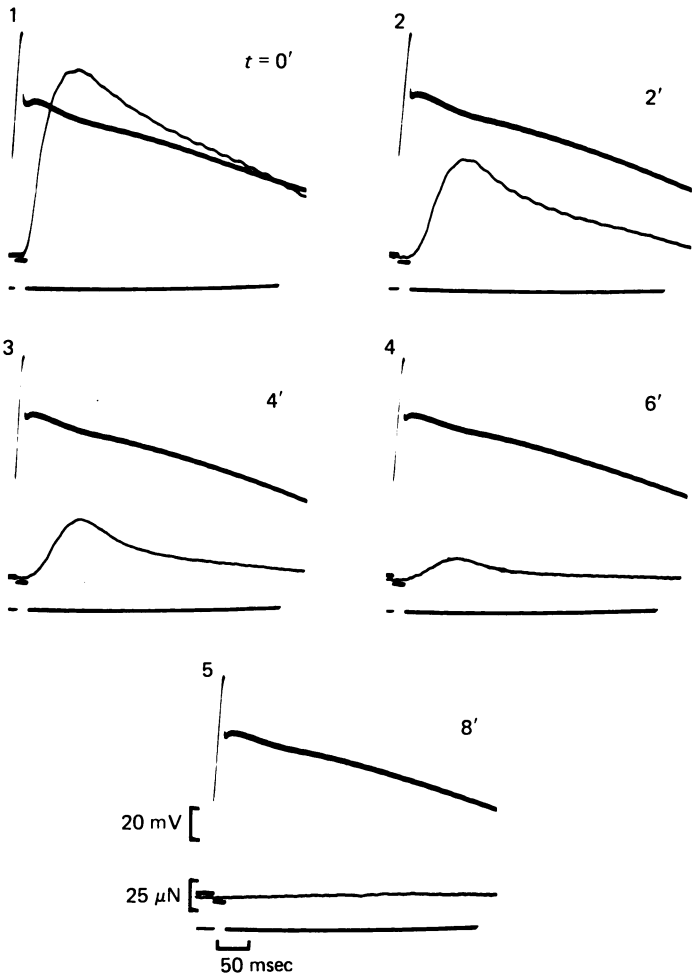

Fig. 6. Successive recordings of five long lasting barium APs (upper traces) and the contraction (middle trace). Note the progressive decline of the tension though the action potential time course is unchanged. Frequency 0·5 min^{-1}. Fibre diameter: 80 μm.

This decrease in the contractility is not due to fatigue caused by the force exerted by the lever since this can be observed, under microscope, without mechanical recording of the tension. Moreover the fibres remain quite transparent with contrast with a fibre which is running down which turns opaque.

The same kind of mechanical behaviour is also observed in voltage-clamp experiments. Fig. 7 shows simultaneous recordings of inward Ba current and of the contraction elicited by a 80 mV depolarizing pulse lasting more than 1 sec. The amplitude of the successive contraction decreases continuously without a noticeable

change in the time course of the current (Figs. 7 and 8), whereas the amplitude of the 16th response (record 6) has fallen to 1% of its initial value. This block of the contractility is irreversible, even after return to normal Ringer for 10 min as shown in another fibre in Fig. 8 (record 4). In Fig. 9*A*, the relative tension has been plotted against the intracellular Ba concentration estimated from the graphical integration of the surface delimited by the successive I_{Ba} (seven fibres).

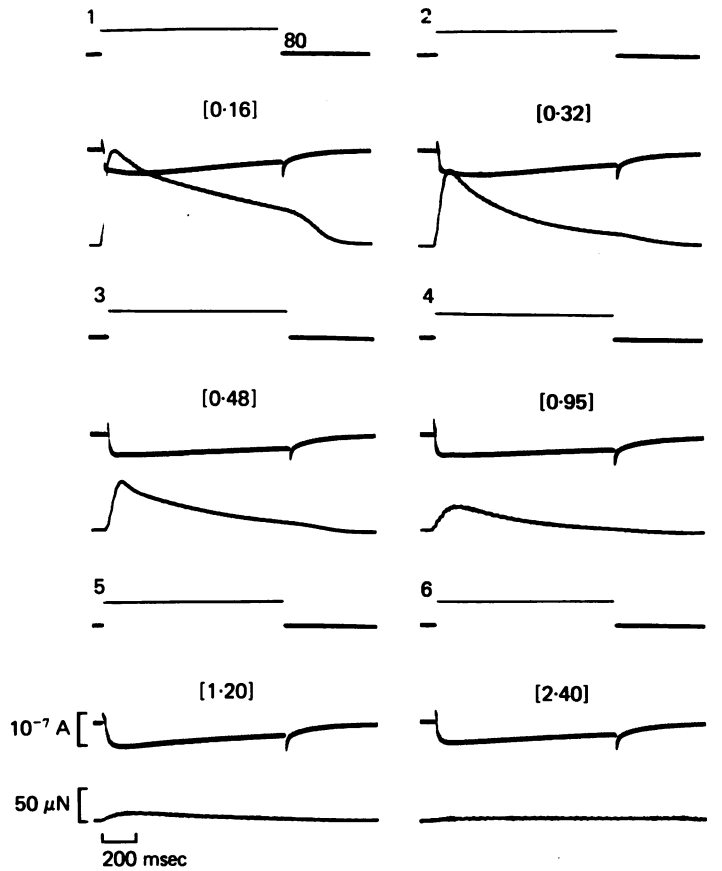

Fig. 7. Effect of repetitive pulsing. Successive simultaneous recordings of the total current (middle trace) and of the contraction (lower trace) elicited by a 80 mV depolarizing pulse (upper trace) at a frequency of 1/min. Records 1, 2, 3 correspond respectively to the 1st, 2nd and 3rd response, record 4 to the 6th response, record 5 to the 8th response and record 6 to the 16th response. For each record the number in brackets corresponds to the estimated intracellular Ba concentration (mM). Fibre diameter: 115 μm.

The curve through the triangles corresponds to the mean of the seven experimental curves determined by the filled circles. The plot of this curve on logarithmic coordinates suggests the existence of two processes which are both apparent up to a concentration of 10^{-3} M. At higher concentrations, only one process seems to be involved and might be related to the change in the rate of rise of the tension development observed over this range of barium concentrations (Fig. 7). These results suggest

that the amount of the activator of contraction available wanes as Ba ions enter the cell. As the contractile proteins have a very low affinity for Ba ions (Ebashi & Endo, 1968) they are unlikely to be this activator.

Study on caffeine treated fibres. Fig. 10*A* shows a typical contracture induced by 5 mM-caffeine. The time to peak is reached in 10–15 sec and then the tension falls

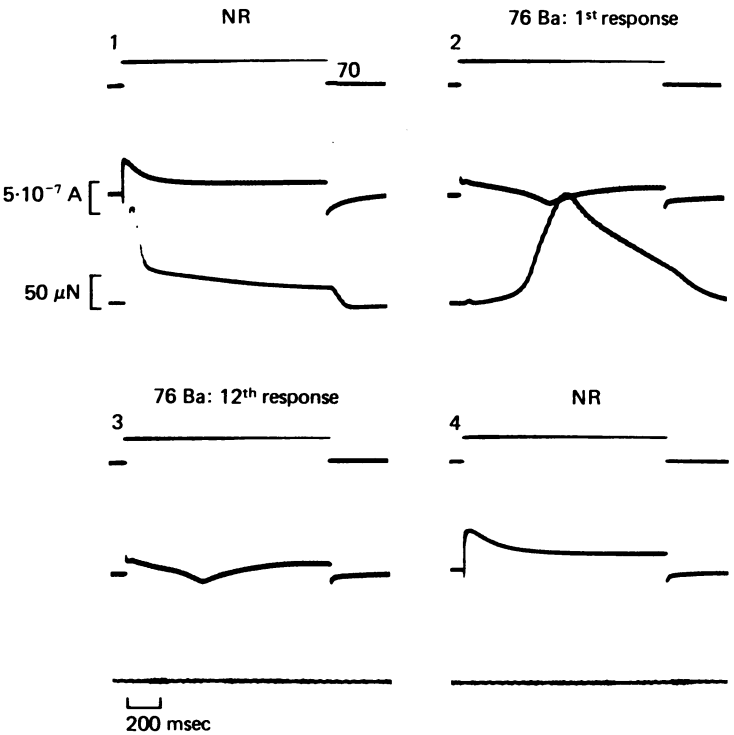

Fig. 8. Irreversibility of the contractile block. Simultaneous recordings of the total current (middle trace) and of the contraction (lower trace) elicited by a 70 mV depolarizing pulse (upper trace). Record 1: normal Ringer (NR). Record 2: 1st response after 5 min in Ba Ringer. Record 3: 12th response after 16 min in Ba Ringer. Record 4: after 10 min in normal Ringer. Frequency of stimulation: 1/min. Fibre diameter: 90 μm.

in two phases. After the full relaxation, the Ba Ringer replaces the Ringer caffeine and an inward I_{Ba} can then be elicited by depolarizing pulses (Fig. 10*B*). When, irrespective of the strength of the pulse, no contraction could be elicited (controlled under stereomicroscope) and the return to normal Ringer did not restore the contraction. The application of caffeine after the ending of the contractility failed to elicit a contracture. These results can be interpreted if Ba ions substitute irreversibly Ca ions in the intracellular storage sites. Thus, the amount of Ba ions necessary to abolish the contractility would be expected to be similar in different fibres. Such an estimation was done and the results summarized in Tables 2 and 3.

Allowing for absolute errors, the internal Ba concentration (estimated from the seven experiments where successive identical pulses were applied, see Figs 7 and 9*A*)

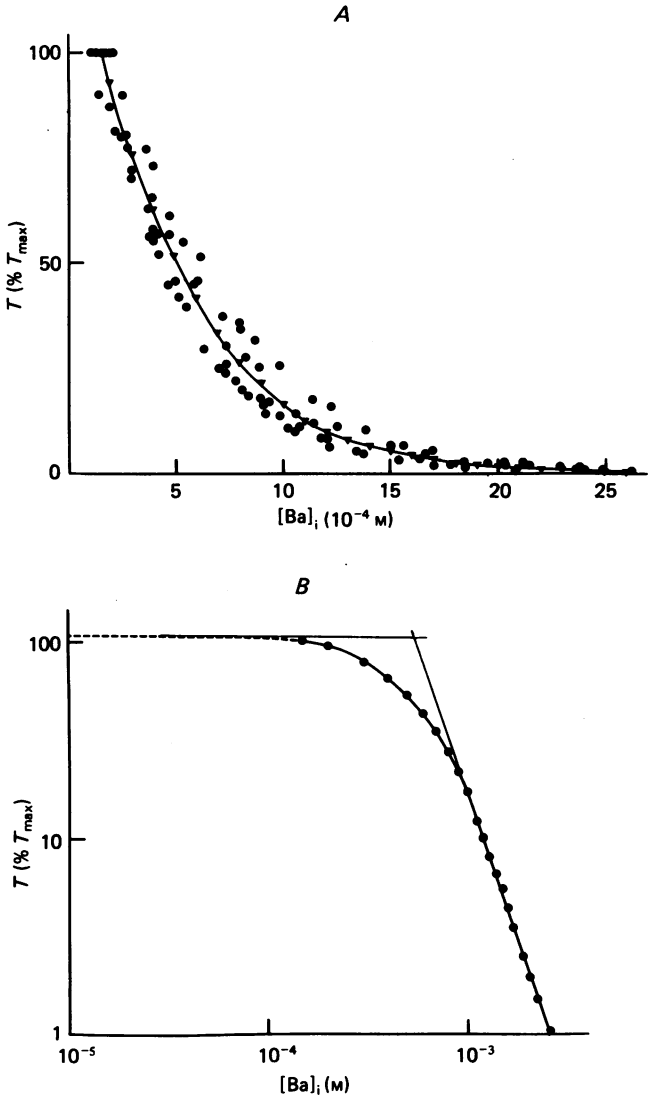

Fig. 9. *A*, relationship between the relative tension (T) and the estimated intracellular Ba concentration ($[Ba]_i$). Pooled data from seven experiments. *B*, logarithmic plot of the same data. The dashed line has been drawn by eyes. The fact that it starts for $T > 100\%$ could indicate that the 1st contraction elicited by I_{Ba} is lower than the maximum available tension.

was found to range between 1·82 and 3·26 mM. The same kind of calculation was done on eleven fibres on which depolarizing pulses of different amplitude and duration were successively applied. The calculated values, ranging between 1·52 and 2·95 mM are not significantly different from those obtained previously.

The results therefore show a cumulative blocking effect of intracellular Ba ions on the contractility. Such an effect is independent of potential and of pulse duration.

The downward trend of the curve, relating the amplitude of the contraction to the potential (Fig. 4) at high depolarizations, can be explained in one of two ways: either it is due to a decrease in I_{Ba}, or it is related to the effect of the intracellular Ba accumulation. The latter could obviously affect this downward deflexion of the curve but it is unlikely to be entirely responsible for the decline because similar

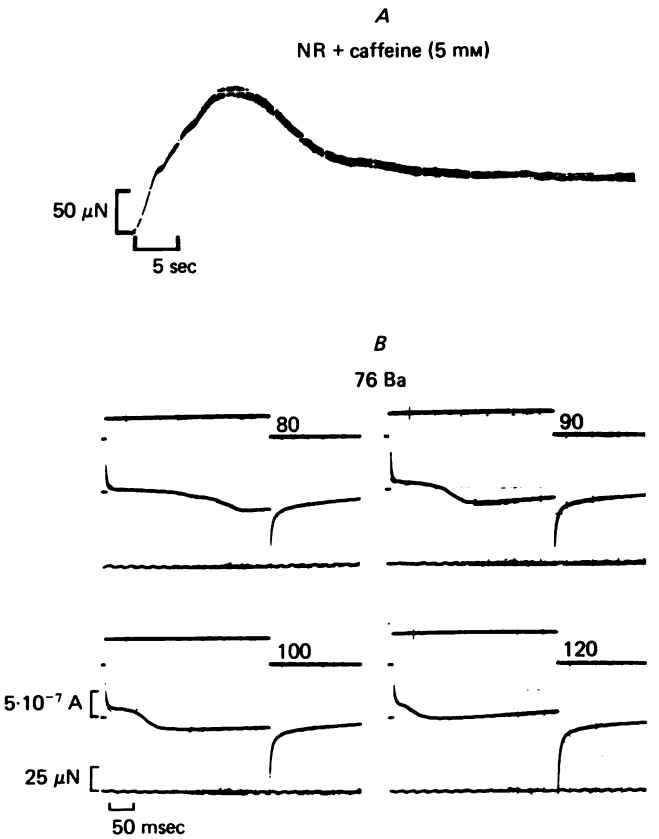

Fig. 10. Experiments performed on caffeine treated fibres. *A*, typical contracture induced by adding 5 mM-caffeine to Normal Ringer. *B*, after the caffeine contracture is fully relaxed, simultaneous recordings of the total current (middle traces) and of the tension (lower traces) for different depolarizing pulses (upper traces) in Ba Ringer. Note that a large inward I_{Ba} does not elicit a contraction. Fibre diameter: 120 μm.

curves to those illustrated in Figs. 4 and 11 are obtained when the inward current is carried by Ca ions which have not a cumulative blocking effect on the contractility (Potreau & Raymond, 1978). This assumption is reinforced by the experiment illustrated by Fig. 11. This experiment was selected because the time course of the mechanical response shows two distinct humps (panel *A*): the first (arrows) starts as soon as the pulse is applied, the second develops simultaneously with I_{Ba} (arrowheads). The amplitude of each part of the contraction (panel *B*, upper curves) and that of I_{Ba} (panel *B*, lower curve) have been plotted against the membrane potential. The S-shape of curve relating the amplitude of the initial hump (triangles) suggests

a potential dependence. The correlation previously described (Fig. 4) can be observed between the amplitude of I_{Ba} and that of the second component of the contraction (filled circles) against the potential. Panel *A* shows that, for potentials higher than 100 mV, the amplitude of the first hump is larger than that of the second. As the intracellular Ca source is likely the same for the two components of the contraction, if the decrease in the amplitude of the second was only due to a cumulative entry of Ba the amplitude of the first would decrease too. As it does not occur, the decrease in I_{Ba} is likely the cause of the decline of the amplitude of the last component of the mechanical response.

TABLE 2. The quantity of Ba which enters the cell in the test node before the relative tension becomes < 1%. The successive identical pulses were applied at a frequency of 1/min

Fibre	Diameter (μm)	Volume ± A.E. (10^{-9} l.)	$\Delta[Ba]_i$ ± A.E. (10^{-12} mole)	$\Delta[Ba]_i$/Volume ± A.E. (10^{-3} M)
1	95	1·42 ± 0·33	3·73 ± 0·01	2·64 ± 0·62
2	125	2·45 ± 0·46	5·58 ± 0·02	2·28 ± 0·46
3	120	2·26 ± 0·43	5·48 ± 0·02	2·40 ± 0·47
4	100	1·57 ± 0·35	3·94 ± 0·01	2·55 ± 0·57
5	120	2·26 ± 0·46	5·55 ± 0·02	2·48 ± 0·51
6	110	1·90 ± 0·39	4·52 ± 0·02	2·38 ± 0·52
7	125	2·45 ± 0·46	5·96 ± 0·02	2·49 ± 0·50

TABLE 3. The estimated amount of Ba which enters the cell in the test node before the relative tension is < 1%. The successive pulses, different in amplitude and duration were applied at a frequency of 1/min

Fibre	Diameter (μm)	Volume ± A.E. (10^{-9} l.)	$\Delta[Ba]_i$ ± A.E. (10^{-12} mole)	$\Delta[Ba]_i$/Volume ± A.E. (10^{-3} M)
1	110	1·90 ± 0·39	4·43 ± 0·02	2·33 ± 0·49
2	125	2·45 ± 0·46	6·08 ± 0·02	2·48 ± 0·47
3	85	1·13 ± 0·29	2·32 ± 0·01	2·05 ± 0·53
4	100	1·57 ± 0·35	3·16 ± 0·01	2·01 ± 0·45
5	95	1·42 ± 0·33	3·02 ± 0·01	2·13 ± 0·49
6	125	2·45 ± 0·46	5·14 ± 0·02	2·09 ± 0·40
7	110	1·90 ± 0·39	3·91 ± 0·01	2·06 ± 0·43
8	100	1·57 ± 0·35	3·24 ± 0·01	2·06 ± 0·46
9	80	1·00 ± 0·27	2·21 ± 0·01	2·21 ± 0·60
10	90	1·27 ± 0·31	2·94 ± 0·01	2·31 ± 0·57
11	110	1·90 ± 0·39	4·16 ± 0·01	2·19 ± 0·45

Study on glycerol treated fibres. A series of experiments was done on six glycerol treated fibres in order to determine the site of development of the permeability to Ba ions. From the records of Fig. 12*A*, it is evident that an inward current faster than on intact fibres, occurs in detubulated ones, especially for the potentials of +90 and +100 mV where a net inward current is observed. This inward current fails to induce a detectable contraction and is itself inhibited by $MnCl_2$. This suggests that it is carried by Ba ions.

From this experiment, the Ba influx (mole/cm^2) was calculated for each potential and the values plotted against the membrane potential (Fig. 12*B*). The maximum Ba influx, calculated from similar experiments performed on five fibres are listed in

Table 4 (upper part). Allowing for absolute errors, the values range between 2 and 7×10^{-11} mole/cm^2. They are about 20 times lower than that obtained on 11 intact fibres (0·63 to $1{\cdot}90 \times 10^{-9}$ mole/cm^2; Table 4, lower part).

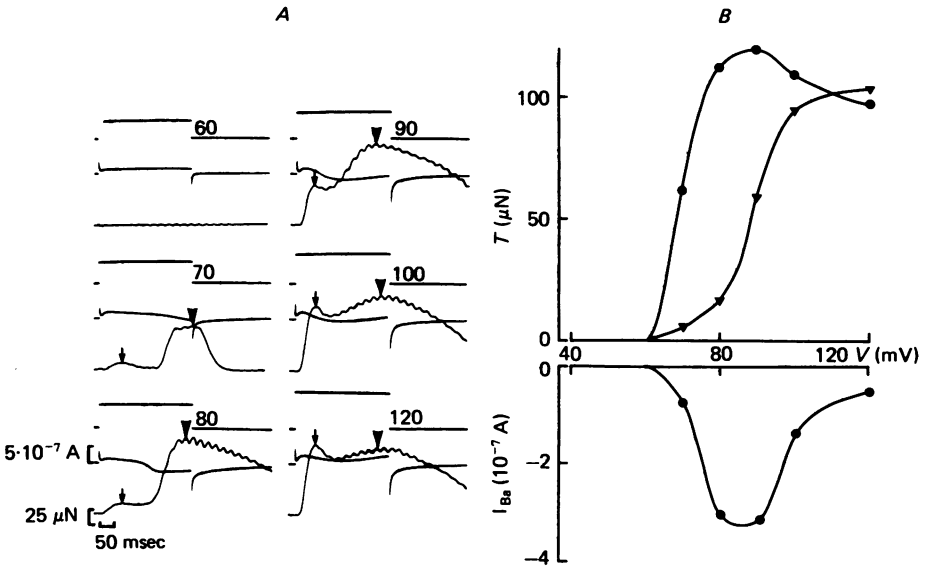

Fig. 11. Existence of two components in the contraction. *A*, simultaneous recordings of the total current (middle trace) and of the contraction (lower trace) for different depolarizing pulses (upper trace). Note that for potentials higher than +90 mV the first part of the contraction (arrows) increases while the second one (arrowheads) decreases. *B*, the relationship between the membrane potential (*V*) and the amplitude of each component of the contraction (*T*) (first component: triangles; second component: circles) and of the net inward Ba current (*I*). Fibre diameter: 95 μm.

DISCUSSION

The voltage-clamp experiments have confirmed the previous observation that a correlation exists between the inward I_{Ba} and the tension since both show a similar dependence on potential. This relationship resembles that which has been described between the inward I_{Ca} and the contraction in several muscular structures: frog skeletal muscle (Potreau & Raymond, 1978), heart muscle (Léoty & Raymond, 1972; Vassort & Rougier, 1972) and smooth muscle (Mironneau, 1973). But the contraction is not only dependent on the inward barium current, because it develops in two phases. The relationship between the amplitude of the first and the membrane potential has the classical S-shape of the potential dependent contraction of voltage-clamped muscle fibres (Heistracher & Hunt, 1969; Bezanilla *et al.* 1971) while the second, which is inhibited by $MnCl_2$ depends on the current. Such a current component could be due either to a direct activation of contractile proteins by Ba ions or to a Ba induced Ca release mechanism. The first possibility can be discarded since Ebashi & Endo (1968) have shown that skeletal muscular contractile proteins have a very low affinity for Ba ions (600 times lower than for Ca). Furthermore

electron microscope localizations of intracellular Ba, according to Somlyo, Somlyo, Devine, Peters & Hall (1974), performed on fibres fixed in the test node after the contractility has ended, have never shown any Ba salt precipitation in the myoplasm (D. Gros, personal communication). Thus it is likely that Ba ions release Ca ions

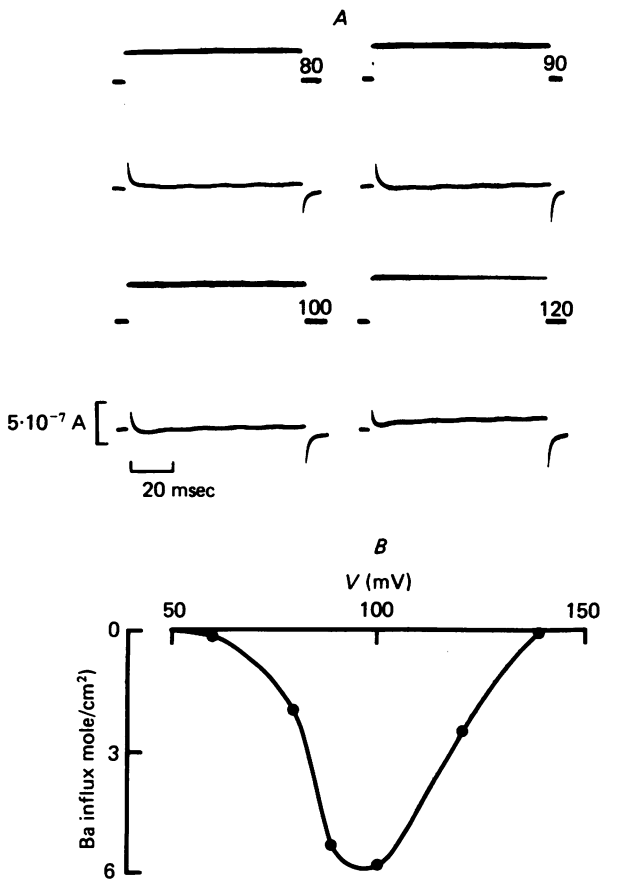

Fig. 12. Experiments performed on glycerol treated fibres. *A*, in Ba Ringer, total current (lower traces) elicited by different depolarizing pulses (upper traces) on detubulated fibres. *B*, relationship between the Ba influx (ordinate) and the membrane potential (*V*) in the experiment illustrated in *A*. Fibre diameter: 100 μm.

from their intracellular sites of storage. In skeletal muscle there are two main intracellular storage sites for Ca^{2+}; namely the terminal cisternae of the sarcoplasmic reticulum (Costantin, Franzini-Armstrong & Podolsky, 1965) and, to a lesser degree, the mitochondria (Patriarca & Carafoli, 1969). It is generally agreed that the intramitochondrial Ca does not play a role in the excitation–contraction coupling process (for review see Endo, 1977). Somlyo *et al.* (1974) have shown a preferential compartmentalization of Ba into the mitochondria and it is not impossible that Ba ions could release intramitochondrial Ca. This hypothesis has been rejected for the following reasons: (i) the absence of delay between the onset of I_{Ba} and that of the

contraction and (ii) the cessation of contraction which indicates that the sarcotubular Ca becomes uneffective. There is the possibility that Ba ions act on sarcotubular rather than on intramitochondrial calcium for Weber, Herz & Reiss (1966) and Edwards, Lorkovic & Weber (1966) assume that the sarcoplasmic reticulum can bind Ba. According to this assumption the progressive block of the contraction can be explained if Ba ions which enter the cell, and release intracellular Ca, irreversibly replace the Ca ions at their storage sites and thereby lead to a progressive decline in the amount of Ca available for contraction. This hypothesis is supported by the fact that all the fibres show the same dependence of the relative tension upon the intracellular Ba accumulation.

TABLE 4. Estimation of the maximum amount of Ba carried by the inward current in detubulated and normal fibres

Fibre	Diameter (μm)	Surface ± A.E. (10^{-4} cm^2)	Maximum amount of Ba carried by I_{Ba} ± A.E. (mole/cm^2)
Detubulated			
1	100	6·3 ± 0·63	$5{\cdot}73 \times 10^{-11} \pm 0{\cdot}62$
2	120	7·5 ± 0·69	$4{\cdot}47 \times 10^{-11} \pm 0{\cdot}54$
3	90	5·7 ± 0·60	$3{\cdot}36 \times 10^{-11} \pm 0{\cdot}44$
4	110	6·9 ± 0·66	$4{\cdot}86 \times 10^{-11} \pm 0{\cdot}57$
5	120	7·5 ± 0·69	$5{\cdot}21 \times 10^{-11} \pm 0{\cdot}59$
Normal			
1	110	6·9 ± 0·66	$1{\cdot}72 \times 10^{-9} \pm 0{\cdot}13$
2	110	6·9 ± 0·66	$1{\cdot}21 \times 10^{-9} \pm 0{\cdot}12$
3	125	7·9 ± 0·71	$1{\cdot}29 \times 10^{-9} \pm 0{\cdot}12$
4	85	5·3 ± 0·58	$1{\cdot}09 \times 10^{-9} \pm 0{\cdot}13$
5	100	6·3 ± 0·63	$0{\cdot}70 \times 10^{-9} \pm 0{\cdot}07$
6	95	6·0 ± 0·61	$0{\cdot}84 \times 10^{-9} \pm 0{\cdot}09$
7	125	7·9 ± 0·71	$1{\cdot}37 \times 10^{-9} \pm 0{\cdot}13$
8	100	6·3 ± 0·63	$1{\cdot}63 \times 10^{-9} \pm 0{\cdot}17$
9	80	5·0 ± 0·57	$1{\cdot}32 \times 10^{-9} \pm 0{\cdot}13$
10	90	5·7 ± 0·60	$1{\cdot}54 \times 10^{-9} \pm 0{\cdot}16$
11	110	6·9 ± 0·66	$0{\cdot}92 \times 10^{-9} \pm 0{\cdot}09$

Another argument for the site of action of intracellular Ba comes from the experiments performed in presence of caffeine. This drug is known to liberate specifically Ca from the sarcoplasmic reticulum (Weber, 1968; Thorpe & Seeman, 1971) and to induce contractures in skeletal muscle. Our results show that after a caffeine contracture, an intracellular flow of Ba ions does not elicit any contraction and that caffeine fails to elicit a contracture when applied after the ending of the contractility. The first observation may mean that the Ca which is taken up during the relaxing phase of the contracture is not immediately available for contraction, as suggested by Chapman & Léoty (1976). The sarcoplasmic reticulum loading with Ba is very rapid because there is no competition between Ca and Ba, the former being absent from the bathing fluid. Thus, as the intracellular stores are full of Ba the return to normal Ringer cannot restore the contractility. The second observation indicates that intracellular Ca ions are stored in sites and/or under a form which are insensible

to caffeine and confirms that Ba acts at the sarcoplasmic reticulum level. This conclusion is reinforced by the calculation of the amount of Ba which has entered the fibre before the completion of the contraction. The values obtained (1·52 to 3·26 m-mole/l. of fibre) correspond to the maximum capacity with which the sarcoplasmic reticulum can be loaded with Ca: 2–3 mM for Ford & Podolsky (1972) and 1·5 to 3·9 mM as calculated by Endo (1977).

Detubulation of fibres shows that more than 95% of the inward flux of Ba ions is lost and the contraction is abolished. The small remaining current is inhibited by $MnCl_2$ and is probably carried by Ba ions but is earlier to peak than I_{Ba} observed in intact fibres. This could suggest that this early part of the current, located at the surface membrane, is masked on intact fibres by the part of the outward K current which develops at T system level (Freygang, Goldstein & Hellam, 1964; Adrian, Chandler & Hodgkin, 1970). This small amount of Ba ions that cross the surface membrane plays no role in the excitation–contraction coupling process. The coupling between I_{Ba} and the contraction can be located at the T-system level.

At the moment, a precise relationship between tension and current cannot be formulated, because calculations and results (Adrian, Chandler & Hodgkin, 1969; Adrian & Peachey, 1973; Hille & Campbell, 1976) show that the potential of the tubular membrane is not well controlled. By contrast Caillé *et al.* (1978) have suggested that this membrane can be better clamped than previously supposed. In the present experiment it is possible that due to the slowness of the current, a fairly good control is obtained since the tubular potential is no longer contaminated by the tubular capacitive current at the onset of the inward Ba current. As the physiological solution is free of NaCl the only source of disturbance for the potential is the flow of I_{Ba} across the input resistance of the tubular system. As the maximum amplitude of I_{Ba} is about 10 times smaller than that of a typical I_{Na}, the tubular potential may well be better controlled than predicted for Na current.

In conclusion the present investigation has shown that the contraction elicited by a long lasting Ba response depends on two mechanisms: a potential dependent mechanism and a mechanism which is related to the entry of Ba ions in the cell through the tubular membrane. The latter resembles the Ca dependent component previously described on other muscular structures. In the presence of Ba the activation of the contractile proteins occurs by means of a Ba induced Ca release mechanism analogous to the 'calcium induced calcium release' mechanism described by Endo, Tanaka & Ogawa (1970) and Ford & Podolsky (1970). Such a mechanism could be involved in the excitation–contraction coupling process as suggested by Chiarandini & Stefani (1976). In order to develop this hypothesis further investigations will be done in the presence of a more 'physiological' cation than Ba^{2+}.

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