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EFFECTS OF CALCIUM, BARIUM AND LANTHANUM ON DEPOLARIZATION–CONTRACTION COUPLING IN SKELETAL MUSCLE FIBRES OF *RANA PIPIENS*

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SUMMARY

1. Voltage-clamp experiments were carried out to study the effect of Ca, Ba and La on the contractile responses of short (~ 1·5 mm) muscle fibres.

2. In the absence of external Ca or when 1·8 mM-Ca was substituted by 1·8 mM-Ba the contractile responses to short depolarizing pulses (400 ms) were not modified and only a minor shift in the tension–voltage relationship was observed.

3. At high concentration (76 mM) of Ba or Ca, or in the presence of 2 mM-La, shifts in the tension–voltage relationship of 30, 41 and 25 mV respectively were observed. In addition the steepness of the activation curve was decreased in high Ba and Ca solutions.

4. In the presence of 76 mM-Ba, the rate of the relaxation phase which follows membrane repolarization after a short pulse was diminished, possibly due to an intracellular action of Ba.

5. In the absence of Ca, or when Ca was substituted by 1·8 mM-Ba the area under the prolonged contractile responses (contractures) was reduced considerably. In the presence of 76 mM-Ba, the normal contracture time course was altered, showing a late slower relaxation phase, occasionally with a secondary tension development. In the presence of high Ca or La, the time course of the contractures was greatly prolonged.

6. The steady-state inactivation curve was shifted toward more negative potentials, in the absence of external Ca or when 1·8 mM-Ca was substituted by 1·8 mM-Ba.

7. In the presence of 76 mM-Ba the steady-state inactivation curve was not affected. In the presence of 76 mM-Ca or 2 mM-La the curve was shifted toward less negative potential, with a marked decrease in its steepness.

8. Ba at a concentration as high as 1 or 2 mM did not activate tension development in chemically skinned muscle fibres.

9. Ba at 1 or 2 mM did not appreciably alter the Ca uptake capacity of isolated sarcoplasmic reticulum vesicles. At 5 mM a decrease in the Ca uptake capacity was observed.

10. The results indicate that Ba at low (1·8 mM) concentration is not effective in substituting for Ca. The effects of divalent cations at high concentration, and of La, on the tension–voltage relationship and on the steady-state inactivation curve are

most probably mediated by interaction with the external surface of the fibre membrane.

11. While the effects of Ca, Ba and La, on depolarization–contraction coupling may be explained assuming an extracellular site of action, massive entry of Ba may elicit contractile activity by inducing a secondary release of Ca from the sarcoplasmic reticulum, or myoplasmic Ca buffer systems, as the parvalbumins.

INTRODUCTION

The question of whether Ca ions entering muscle fibres through voltage-dependent Ca channels participate directly in contractile activation is still controversial. While it is recognized that Ca currents develop too slowly to be of importance for activation during a twitch, it is thought that they may sustain the time course of prolonged responses such as tetani or contractures (Sánchez & Stefani, 1978). This idea is supported by the finding that some agents, like Mn ions (Chiarandini & Stefani, 1973) and D-600 (Eisenberg, McCarthy & Milton, 1983; Frank, 1984), which depress Ca currents, may also depress contractile responses. On the other hand there is also evidence indicating that extracellular Ca entering muscle fibres during membrane depolarization does not play a direct role in supporting contraction. The principal pieces of such evidence are the following: (a) the clear demonstration that contractile responses may be obtained in the virtual absence of external Ca (Armstrong, Bezanilla & Horowicz, 1972; Caputo & Gimenez, 1967; Lüttgau & Spiecker, 1979; Cota & Stefani, 1981); (b) the demonstration that K contractures of normal time course can be obtained when external Ca is replaced by Ni (Caputo, 1981), which does not permeate through Ca channels and blocks them (Almers & Palade, 1981).

It is known that Ba ions may carry charge through voltage-dependent Ca channels in several types of excitable cells (Hagiwara & Byerly, 1981; Potreau & Raymond 1980*a*). For frog skeletal muscle fibres, this property appears to be of particular interest in view of its possible relevance in the process of depolarization–contraction coupling. Several years ago, Heilbrunn & Wiercinski (1947) reported that Ba ions, injected at a relatively high concentration into frog skeletal muscle fibres, induced a strong contractile response. More recently, it has been proposed that Ba ions entering muscle fibres through voltage-dependent calcium channels (Sánchez & Stefani, 1978) may activate contraction, by releasing Ca from the sarcoplasmic reticulum (Potreau & Raymond, 1980*a*), through a mechanism analogous to the Ca-induced Ca release (Endo, 1977). This proposal is based on the dependency of a component of the contractile response on the inward flow of Ba currents in the absence of extracellular Ca and in the presence of high (76 mM) external Ba (Potreau & Raymond, 1980*a*). Such a mechanism would constitute an alternative mode of contractile activation, through the action of an extraneous divalent cation, which reportedly does not directly interact with muscle contractile proteins. The operation of such a mechanism could be indicative that extracellular Ca might act in a similar way (Potreau & Raymond, 1980*b*).

In the present work we have compared the effects of Ca, Ba and La on depolarization–contraction coupling, using a two micro-electrode voltage-clamp technique applied to short ($\sim$ 1·5 mm) muscle fibres of the frog. We have tested the

effects of these cations on the shape of contractile responses to short (< 1 s) and prolonged (> 4 s) depolarizing pulses, on the relationship between tension development and membrane potential, and on the relationship between steady-state inactivation and membrane potential. In addition, experiments were carried out to test whether Ba ions could directly interact with contractile proteins using chemically skinned fibres, and whether they could affect the Ca uptake capacity of isolated sarcoplasmic reticulum.

METHODS

Voltage-clamp experiments

Bundles of six to twenty fibres were dissected from the m. lumbricalis IV digiti of the hind limb of *Rana pipiens*. These fibres have a short length (about 1·5 mm), and allow the application of a two micro-electrode voltage-clamp technique for the study of a relatively slow process such as contractile activation. The details and justification of the technique, as well as the experimental procedure have been published previously (Caputo & Fernández de Bolaños, 1979; Caputo, Bezanilla & Horowicz, 1984). Contractile tension was measured with a Cambridge force transducer (Cambridge Technology series 400, Cambridge, MA, U.S.A.). The fibres were clamped at a resting holding potential of −100 mV. The normal Ringer solution had the following composition in mM: NaCl, 115; KCl, 2·5; $CaCl_2$, 1·8; Tris buffer (pH 7·4), 10. Some experiments were carried out using a solution prepared without added Ca (with a contamination level of less than 10 μM), in the presence of 5 mM-Mg and 1 mM-EGTA. Using association constants given by Sillén & Martell (1971) the calculated free Ca and Mg concentration are 10^{-8} M and 4·8 mM respectively. Tetrodotoxin was added at a concentration of 10^{-6} g/ml. High Ca or Ba solutions were prepared by substituting for NaCl. All the experiments were performed at room temperature 20–22 °C. Some experiments with La were carried out at pH 6·5 to avoid precipitation of La salts, when Tris buffer was used, or at pH 7·3 in other experiments in which HEPES buffer was used. Some experiments were carried out with fibres bathed in tetraethylammonium-(TEA)-SO_4 Ringer; in this case no difference was noticed in the contractile responses.

Skinned fibre experiments

Single muscle fibres dissected from the m. semitendinosus were mounted for tension measurement in a chamber which allowed quick solution changes. Once mounted the fibres were chemically skinned, following the procedure and using the same solutions described by Julian (1971). The final pCa and pBa were calculated using association constants with EGTA of $5·01 \times 10^6$ and $1·26 \times 10^4$, respectively.

Calcium uptake capacity

Sarcoplasmic reticulum vesicles were prepared from leg muscle of *Rana catesbiana*, following the procedure described by Ikemoto, Sreter & Gergely (1971). Ca uptake rates were measured by the Millipore filtration technique (Martonosi & Feretos, 1964) in a medium containing 0·1 M-KCl, 10 mM-$MgCl_2$, 5 mM-ammonium oxalate, 40 mM-Tris maleate, at pH 6·6 in order to prevent Ba salt precipitates, when this ion was present. To study the interference of Ba on Ca uptake capacity, $BaCl_2$ was added at different concentrations from 0·1 to 5 mM. ^{45}Ca uptake was studied in the presence of 0·1 mM-Ca.

RESULTS

Short voltage-clamp pulses

It has been clearly demonstrated that in the virtual absence of extracellular Ca, frog muscle fibres can twitch in response to electrical stimulation for relatively long periods (Armstrong, Bezanilla & Horowicz 1972; Lüttgau & Spiecker, 1979). Under these conditions potassium contractures, of maximal peak tension but shortened time course, can also be obtained (Caputo, 1981). These considerations indicate that under

voltage clamp, and for the case of relatively short depolarizing pulses, indistinguishable contractile responses should be obtained irrespective of the presence or absence of Ca or Ba ions. In agreement with this, Fig. 1 shows that contractile responses to voltage-clamp depolarizing pulses of relatively short duration (400 ms) and varying amplitude, obtained with fibres bathed in solutions containing either 0 Ca,

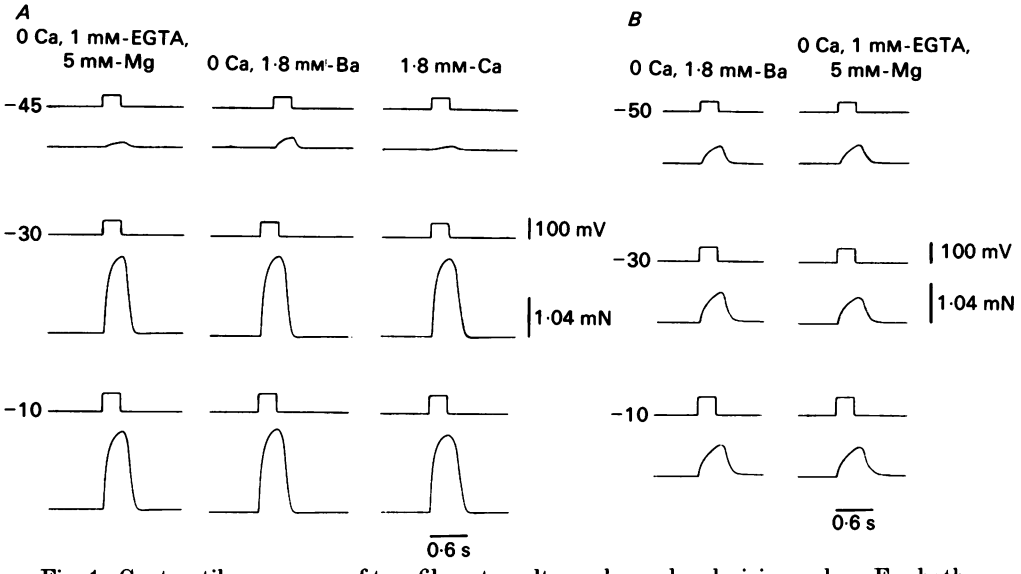

Fig. 1. Contractile responses of two fibres to voltage-clamp depolarizing pulses. For both fibres the holding membrane potential was −100 mV. The records in *A* show the results obtained with one fibre exposed first to a 0 Ca, 1 mM-EGTA, 5 mM-Mg solution (left), later to a solution prepared with 0 Ca, 1·8 mM-Ba (middle), and finally to a normal Ringer solution (right). In each record the upper trace shows the membrane potential, with the value of the membrane potential indicated for each pulse, and the lower trace shows the contractile response. Notice how the responses obtained with different pulses are rather similar, independent of the external medium. The records on the right (*B*) show similar results obtained with another fibre exposed first to 0 Ca, 1·8 mM-Ba medium and later to a solution prepared with 0 Ca, 1 mM-EGTA and 5 mM-Mg. The difference in the contractile responses of the fibres is due to different fibre diameters.

1 mM-EGTA, 5 mM-Mg; 0 Ca, 1·8 mM-Ba; or 1·8 mM-Ca are practically indistinguishable. The Figure shows the results obtained with two fibres, one (Fig. 1*A*) depolarized to −45, −30 and −10 mV, in the three media mentioned above successively; while the second fibre (Fig. 1*B*) was depolarized to −50, −30 and −10 mV first in a medium containing 0 Ca, 1·8 mM-Ba and later in a medium containing 0 Ca, 1 mM-EGTA and 5 mM-Mg. In all cases it can be seen that rather small differences exist between records obtained at the same membrane potential but in different solutions.

Fig. 2 shows the results obtained using high (76 mM) concentrations of divalent cations, Ca or Ba, in the external medium. The upper set of records compares the results obtained with one fibre in the presence of 1·8 mM-Ca or 76 mM-Ba. The lower records were obtained with another fibre in the presence of 1·8 mM-Ca or 76 mM-Ca. It is clear that the main effect of increased divalent cation concentration is a shift toward less negative potentials. Furthermore, for the case of the records obtained

in the presence of 76 mM-Ba the rate of relaxation following the end of the pulse is markedly slowed. This effect, is better illustrated in Fig. 3, which compares the results obtained with two other fibres. The graphs show plots of the fractional tension, during the rapid relaxation phase which follows membrane repolarization, in logarithmic scale against time. In all cases it can be observed that most of the

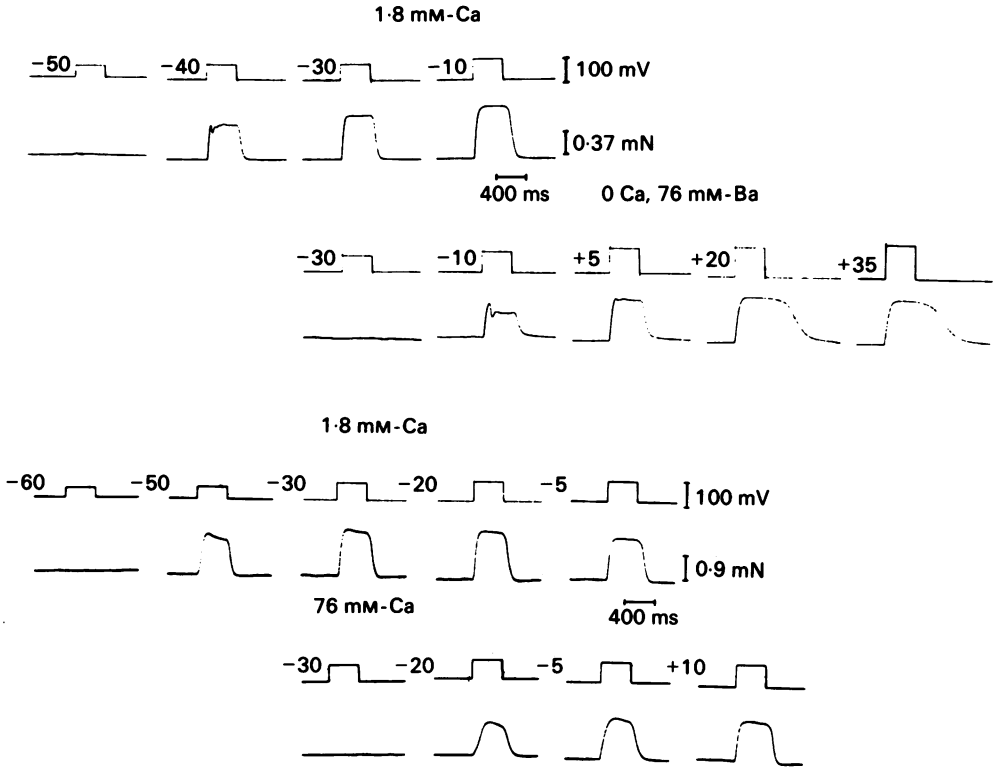

Fig. 2. Contractile responses of two fibres to voltage-clamp depolarizing pulses. The upper set of records shows the results obtained with one fibre exposed first to a 1·8 mM-Ca (upper row) and later to a 0 Ca, 76 mM-Ba medium (second row). In each record the upper trace shows the voltage clamp pulse, with an indication of the membrane potential during the pulse; the lower trace shows the tension. The lower set of records shows the results obtained with a second fibre exposed first to a 1·8 mM-Ca and later to 76 mM-Ca medium.

relaxation phase can be described by a single exponential, in agreement with a previous report (Caputo & Fernández de Bolaños, 1979). For the case of the fibre on the left the rates of relaxation were 7·7 s^{-1} in normal Ringer and 7·1 s^{-1} in 76 mM-Ca solution, while for the fibre on the right the rates were 7·3 s^{-1} in the presence of 1·8 mM-Ca and 2·7 s^{-1} in the presence of high Ba. In addition to the slowing of the relaxation rate, high Ba also causes a delay in the onset of the fast relaxation phase.

Fig. 4*A* summarizes the results obtained with five fibres exposed to solutions containing 1·8 mM-Ca and six fibres exposed to solutions containing 1·8 mM-Ba. The graph shows the tension–voltage relationship obtained plotting the peak tension, expressed as fraction of the maximum value, against the fibre membrane potential

during the pulse. In the graph each symbol corresponds to a different fibre. The empty and filled symbols represent the results obtained in the presence of 1·8 mM-Ca or 0 Ca, 1·8 mM-Ba respectively. The continuous line was drawn according to the equation:

$$T/T_{max} = \frac{1}{1+\exp[-(V-\bar{V})]/K} \quad (1)$$

where T/T_{max} represents the fractional tension (relative to the maximal tension), V is the membrane potential, $\bar{V}$ is the potential at which half-maximal tension is

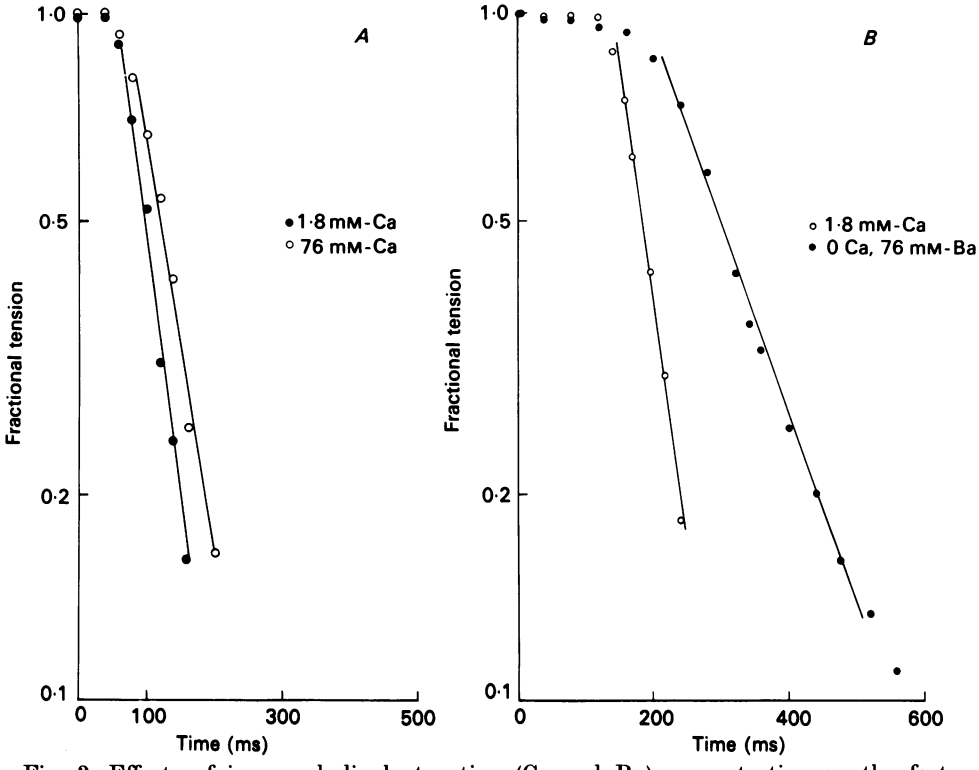

Fig. 3. Effects of increased divalent cation (Ca and Ba) concentration on the fast relaxation phase following the trailing edge of the depolarizing pulse. The graphs show semilogarithmic plots of fractional tension *versus* time of relaxation of maximal responses obtained with two fibres under different conditions. *A* shows that the relaxation phase is not affected by high Ca concentration. *B*, obtained with another fibre shows that high Ba decreases the relaxation phase. The results presented in this Figure were obtained with different fibres than those shown in Fig. 2.

obtained, and K is a constant related to the steepness of the tension–voltage relationship. In this Figure, as well as in the others which show the tension–voltage curve obtained in the presence of 1·8 mM-Ca, $\bar{V}$ has a value of -37 mV, while K has a value of 4. Thus all the curves fitted to the data obtained with 1·8 mM-Ca are identical and superimposable. Eqn. (1), which describes a two-state Boltzmann distribution, has been widely used (Chandler, Rakowsky & Schneider, 1976; Shlevin, 1979) to describe the voltage dependency of the intramembrane charge movement,

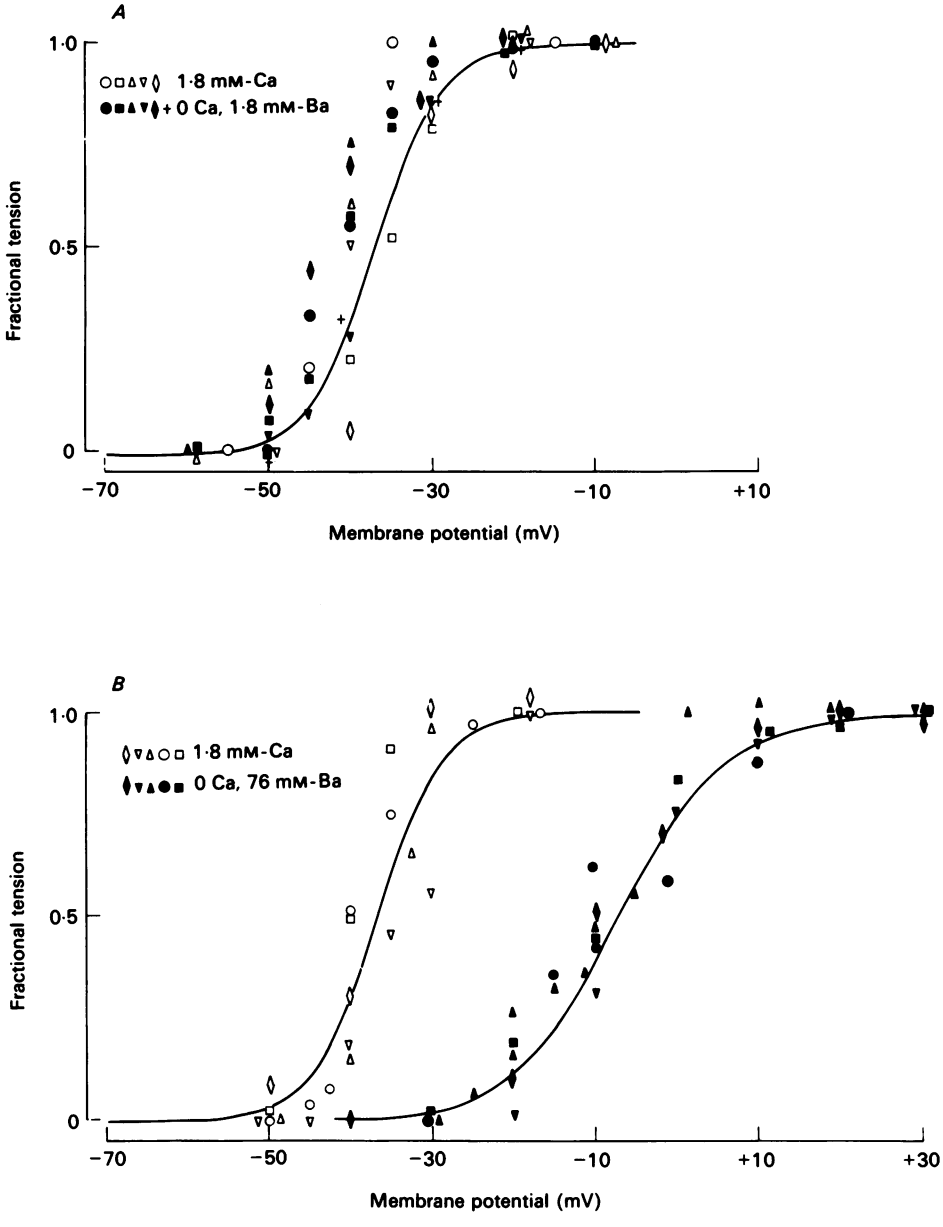

Fig. 4. Tension–voltage relationships of different fibres. In this and the next Figure, the relationship is expressed as the fraction of the maximum fibre tension, and the membrane potential during the depolarizing pulse. In each graph, each symbol represents the results obtained with the same fibre. *A* shows the results obtained with one group of fibres tested with 1·8 mM-Ca and 0 Ca, 1·8 mM-Ba. The continuous line was drawn as described in the text, and is the same in the other graphs in which the results obtained with other fibre groups in the presence of 1·8 mM-Ca are shown. *B* shows the results obtained with another group of fibres exposed to 1·8 Ca and later to 0 Ca, 76 mMBa.

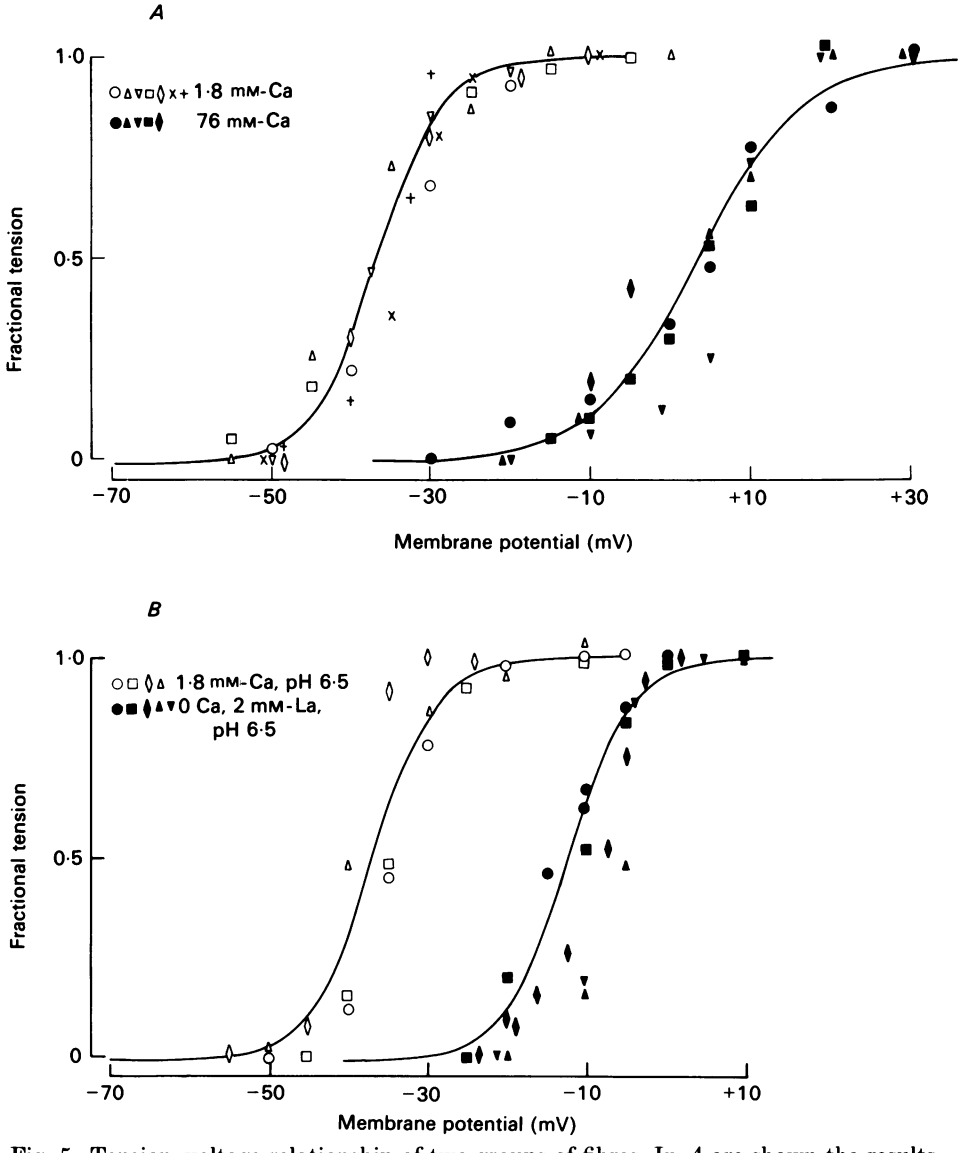

Fig. 5. Tension–voltage relationship of two groups of fibres. In *A* are shown the results obtained with fibres exposed to 1·8 mM-Ca and/or to 76 mM-Ca media. *B*, shows the results obtained with other fibres exposed either to a Ringer solution at pH 6·5 or to a solution prepared without Ca but with 2 mM-La at the same pH value.

which is thought to provide the required voltage sensitivity to contractile activation (Schneider & Chandler, 1973; Schneider, 1981). Here we have used this equation empirically and for comparative purposes only, since as will be shown, high Ca solutions induce qualitatively similar changes in the tension–voltage and charge–voltage relationships (Shlevin, 1979). The results of Fig. 4*A* indicate a small shift of the tension–voltage relationship toward more negative potentials in the presence of 0 Ca, 1·8 mM-Ba.

Fig. 4*B* shows the effect of 76 mM-Ba on the tension–potential relationship. In the graph, the open symbols represent the results obtained in the presence of 1·8 mM-Ca, while the filled symbols show the results obtained in the presence of high Ba. Each symbol represents the results obtained with the same fibre. In this and in the graphs of Fig. 5 the curves representing the results obtained in normal Ringer solution, are identical to the curve shown in Fig. 4*A*, which was drawn according to eqn. (1), using a value of $K = 4$. The curve fitting the points obtained in the presence of 76 mM-Ba, was drawn with $K = 6{\cdot}5$ and $\bar{V} = 6{\cdot}8$ mV. This represents a shift of 30 mV in the tension–voltage relationship, and a decrease in steepness.

Fig. 5*A* shows the effect of 76 mM-Ca on the tension–voltage relationship. The curve through the points obtained in the presence of high Ca, was drawn using a $K = 6{\cdot}5$, and $\bar{V} = +4$ mV, indicating a shift of 41 mV.

Finally, Fig. 5*B* shows the effect of 2 mM-La, indicating a shift of 25 mV without a change in steepness. The results shown in Figs. 4 and 5 demonstrate that the shift of the tension–potential relationship toward less negative potential values, is not specific for divalent cations which can permeate the muscle fibre membrane through voltage-dependent calcium channels, since it is also produced by La. Interestingly, in the presence of high Ca or Ba, but not in the presence of 2 mM-La, a decrease in the steepness of the tension–potential relationship is observed. This effect is qualitatively similar to that reported by Shlevin (1979) for charge movement in the presence of high Ca solutions.

Contractures and steady-state inactivation

The time course of K and voltage-clamp contractures can be affected by a variety of experimental conditions. Thus it is known that in the absence of external Ca, the contracture time course is shortened, mainly due to the disappearance of its characteristic plateau phase, while in the presence of high Ca concentrations and to a lesser degree in the presence of 0·5–2 mM-La, the contracture duration is greatly prolonged. After the spontaneous relaxation from a contracture, repolarization of the fibre membrane potential to a critical value, is necessary for repriming the contractile capacity. While the relationship between the fractional reprimed tension and the membrane potential during the repriming period represents the restoration of contractility, it may be considered as an expression of the steady-state inactivation of the mechanism responsible for providing the required voltage sensitivity to the contractile activation process. Such a relationship is also known to be affected in different ways by agents that modify depolarization–contraction coupling. In this section we describe experiments carried out to study the effect of Ca, Ba and La on voltage-clamp contractures, and on the steady-state inactivation curve. Figs. 6 and 7 compare the results obtained with several fibres under different experimental conditions. In each row the first contracture shows the effect of the particular experimental situation upon its time course, while the magnitude of the second contracture indicates the extent of repriming after a period of 30 s at a given membrane potential. The value of 30 s was chosen because in normal Ringer solution, and at 21 °C, it was sufficient for complete repriming at −100 mV (Caputo, Bolaños & Gonzalez, 1984).

The first response in the records of Fig. 6*A*, obtained with two different fibres in normal Ringer solution, demonstrate the characteristic time course of contractures

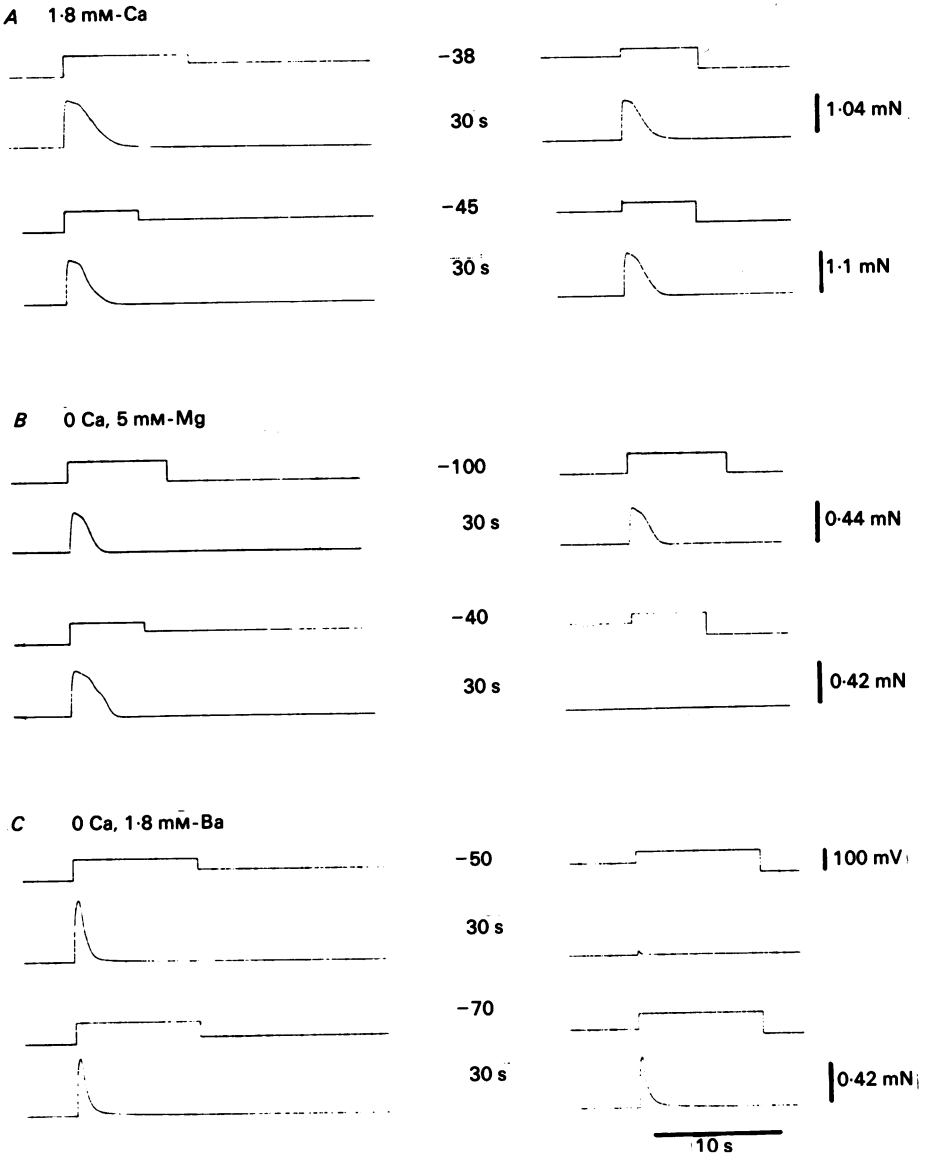

Fig. 6. Contractile responses and repriming capacity of several fibres under different experimental conditions. The responses were elicited with prolonged depolarizing pulses. The contractures on the left were obtained with fully reprimed fibres whose holding potential was −100 mV. After the spontaneous relaxation the fibre membranes were held at a given potential value, indicated in each case, for 30 s, before inducing another contracture with a pulse to 0 mV. *A* shows examples of repriming obtained with two different fibres in normal Ringer solution. *B* shows results obtained with two other fibres in the presence of a medium containing 0 Ca, 5 mM-Mg, showing a change in the first repriming capacity. *C* shows the results obtained with another pair of fibres exposed to a medium containing 0 Ca, 1·8 mM-Ba. Notice the marked change in the contracture time course and repriming capacity.

induced by membrane depolarization to 0 mV. The records also show that following relaxation from the first response, repolarization of the membrane to −38 mV for 30 s causes 86·4 % repriming, while at −45 mV repriming reaches 97·7 %. Fig. 8*A* shows the steady-state inactivation curve obtained with twenty-four different fibres

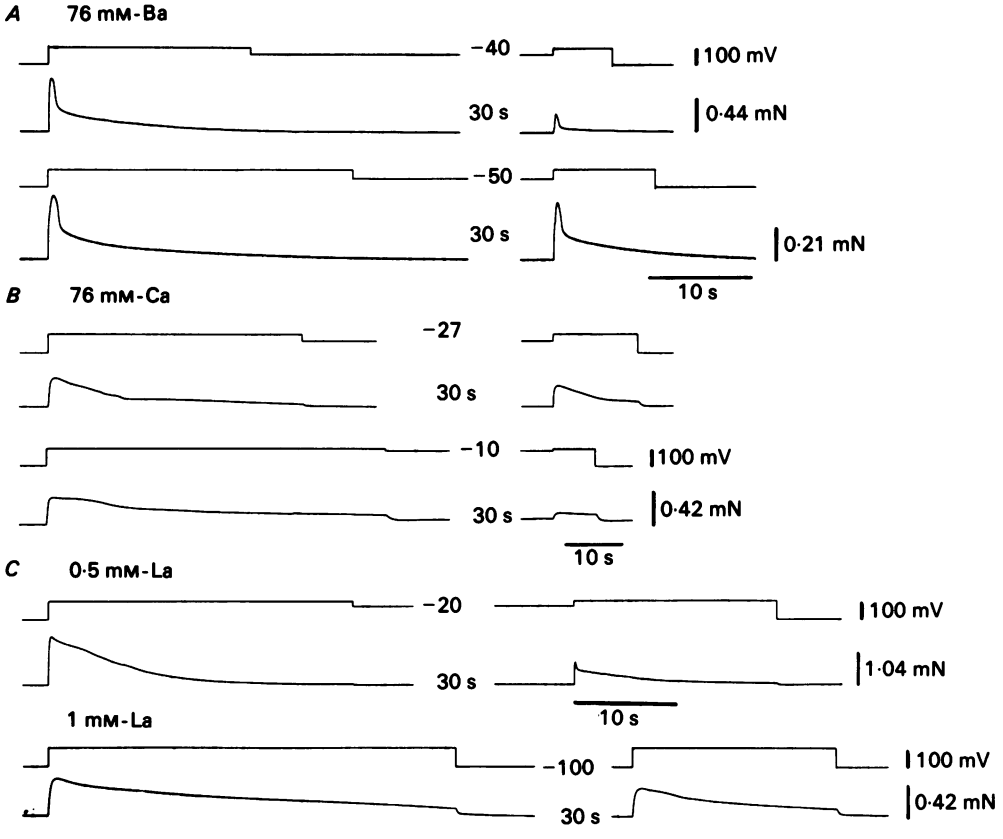

Fig. 7. Contractures and repriming capacity of several fibres under different conditions. The experiments were similar to those shown in Fig. 6. *A* shows the results obtained with two fibres in the presence of 76 mM-Ba, *B* shows the response of two fibres in the presence of 76 mM-Ca, and *C* shows the result obtained in the presence of La, 0·5 and 1 mM.

in normal Ringer solution. In this and the other similar graphs, the ordinate represents the fractional peak tension of the second response with respect to the first one, plotted against the value of the membrane potential during the 30 s repriming period. In the graph of Fig. 8*A*, the point at −100 mV represents the mean ± S.E. of mean obtained with twenty-four fibres, whose repriming was tested at −100 mV and at another potential value, with the exception of the fibre represented by the triangles, whose repriming was tested at −100, −50 and −30 mV.

Part *B* of Fig. 6 shows two records obtained with two different fibres exposed for at least 5 min to a medium containing 0 Ca, 1 mM-EGTA and 5 mM-Mg. In agreement with previous observations, it appears that the plateau phase is shortened, while the

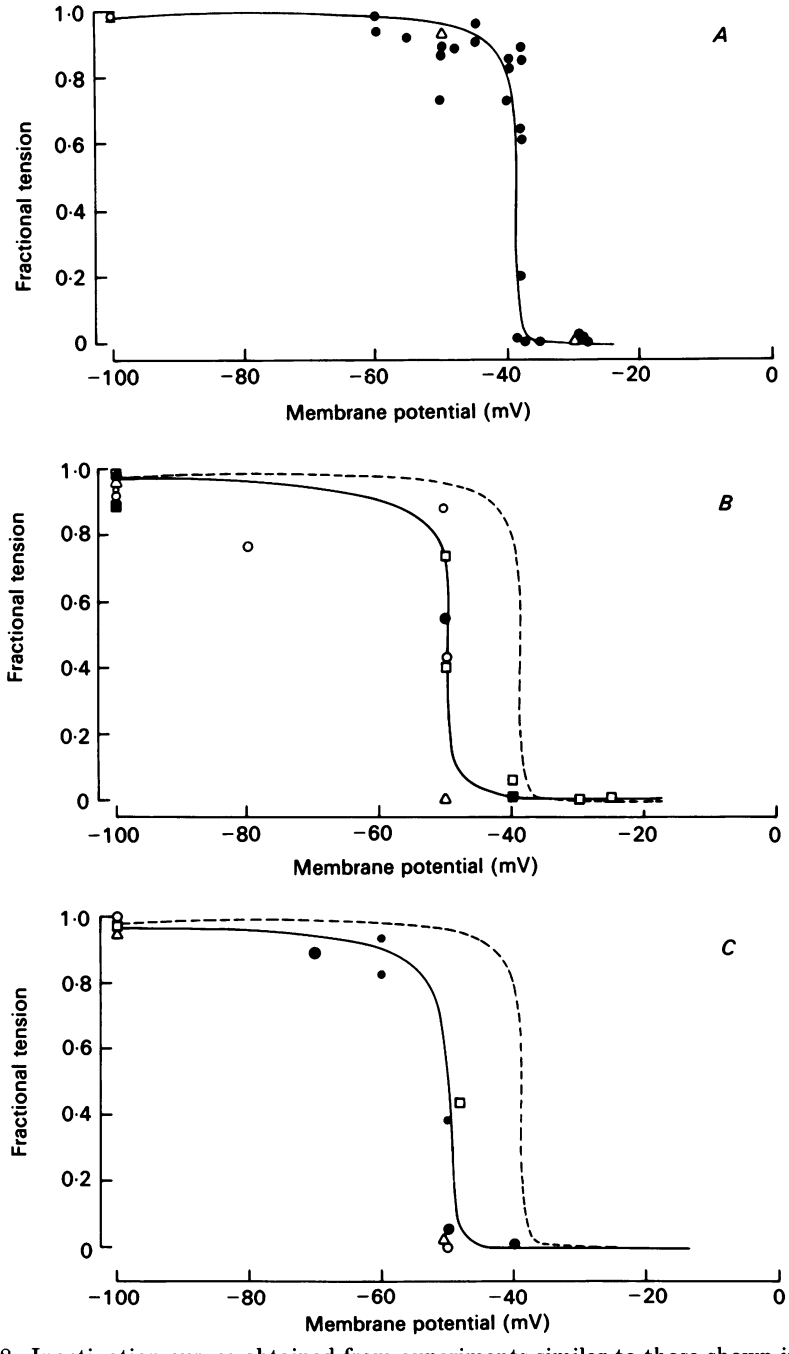

Fig. 8. Inactivation curves obtained from experiments similar to those shown in Fig. 6. The peak tension of the second response, is expressed as fractional value of the peak of the first response and plotted against the membrane potential during the repriming period. The results were obtained with different groups of fibres in the presence of 1·8 mM-Ca, graph A; in the presence of 0 Ca, 5 mM-Mg, graph *B* and finally in the presence of 0 Ca, 1·8 mM-Ba, graph *C*. In all cases the repriming curves have been fitted by eye to the experimental points. The repriming curve shown in *A*, has been reproduced as a dotted curve in the other graph for comparative purposes.

relaxation phase is only affected to a small extent, due to the presence of 5 mM-Mg. Repriming in this medium is also affected since at −100 mV, and −40 mV, 91·3 and 88 % recoveries are obtained respectively. This effect is better illustrated in Fig. 8*B*, which shows the steady-state inactivation curve with five fibres exposed to this solution. In the graph each symbol corresponds to a different fibre. The dotted curve included for comparative purposes in this and in the following similar graphs corresponds to that shown in Fig. 8*A*, obtained in normal Ringer solution. The graph shows that in the absence of external Ca, the steady-state inactivation curve is shifted by about 11 mV toward more negative membrane potential values.

Finally, Fig. 6*C* shows the results obtained with two fibres bathed in a medium containing 0 Ca and 1·8 mM-Ba. The first response in each row shows more marked changes in the time course, since beside the abolition of the plateau phase, the spontaneous relaxation occurs more rapidly than in the previous records, due to the absence of Mg. These results indicate that at a concentration of 1·8 mM, Ba is not an effective substitute for Ca in restoring the contracture time course. Repriming is also affected under this condition, since at −50 and −70 mV, repriming values of 6·1 and 88·3 % respectively are achieved for the two fibres. Fig. 8*C* summarizes the results obtained with five fibres under the same conditions as shown in Fig. 6*C*.

The results demonstrate that in the presence of 1·8 mM-Ba and in the absence of external Ca, the steady-state inactivation curve is shifted to nearly the same extent as in the Ca-free medium.

Fig. 7 shows the effect of high (76 mM) Ba (*A*) and Ca (*B*) and of La, 0·5 and 1 mM (*C*) on the contracture time course and contractile repriming.

The records of Fig. 7*A* were obtained in the presence of 76 mM-Ba in the absence of Ca, and appear to lack the plateau phase. However, it is noteworthy that following a rapid, partial relaxation phase, a second slowly declining relaxation component appears. The reprimed responses show the same characteristic features. For the case of the two fibres used in these runs, at −40 and −50 mV repriming values were 35·9 and 90·1 % respectively.

Fig. 9*A* shows the effect of 76 mM-Ba on the steady-state inactivation curve of five fibres, each represented by a different symbol. In this case it is evident that the inactivation curve is much the same as that obtained in normal Ringer solution.

Fig. 7*B* shows the results obtained with two fibres in the presence of 76 mM-Ca while the records of Fig. 7*C* were obtained in the presence of 0·5 or 1 mM-La, 0 Ca. In the presence of high Ca the plateau phase is appreciably prolonged, and the spontaneous relaxation is slowed. The repriming capacity appears to be modified since at −27 and −10 mV, repriming of values 7·2 and 22·4 % are obtained respectively.

In the presence of La (0·5 or 1 mM) the response time course appears to be prolonged due to an increase of the plateau duration and to slowing of the relaxation phase, confirming the results obtained with potassium contractures by Andersson & Edman (1974*b*).

In the presence of La, repriming appears to be affected in a similar way as in the presence of 76 mM-Ca. For the two fibres repriming values of 46 and 75 % were obtained at −20 and −100 mV respectively. These effects are better shown in Fig. 9*B* and *C* which shows the inactivation curve obtained with several other fibres in the presence of 76 mM-Ca (*B*) or 2 mM-La (*C*). It is clear that under these conditions

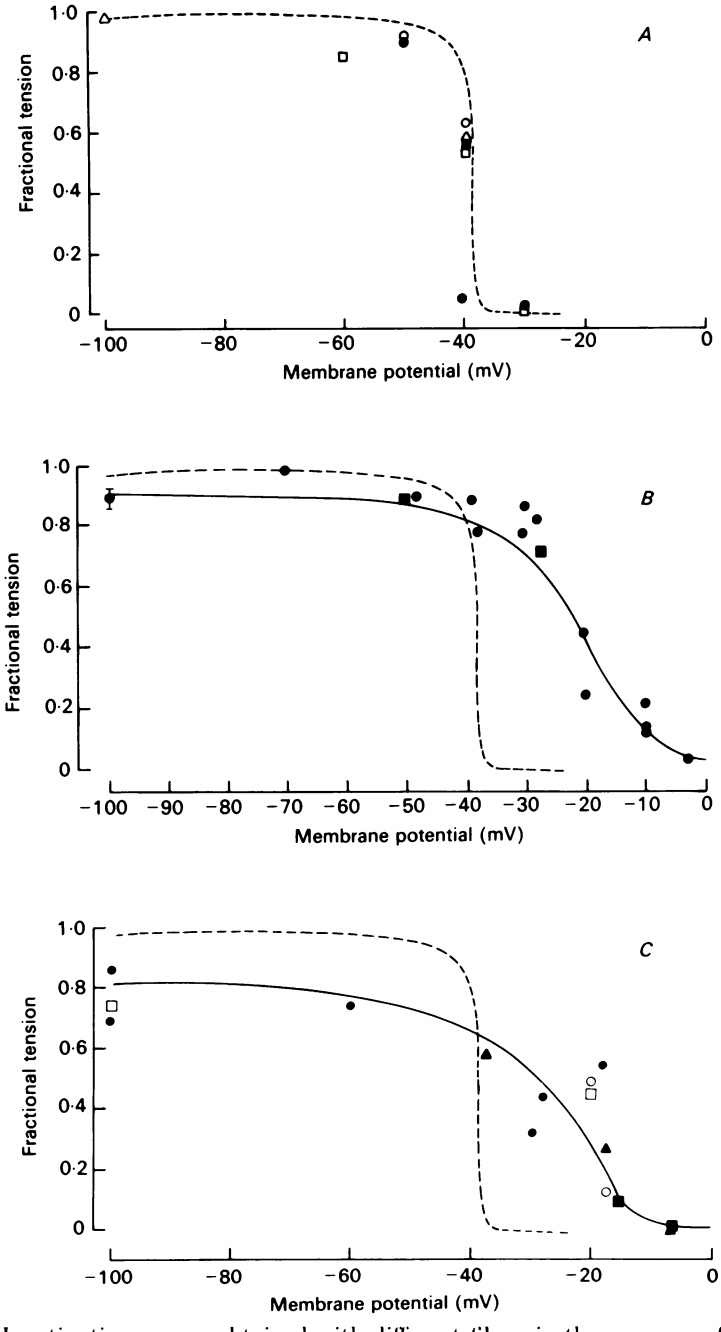

Fig. 9. Inactivation curves obtained with different fibres in the presence of 76 mM-Ba, graph *A*, 76 mM-Ca, graph *B*, and 2 mM-La, graph *C*. As in Fig. 8 the dotted curve represents the results obtained in the presence of normal Ringer solution, and shown in graph *A* of Fig. 8.

the inactivation curve is shifted toward more positive potentials. For the case of La, repriming at −100 mV appears also to be depressed.

The second slow component of the relaxation phase observed in the presence of 76 mM-Ba, as well as the decreased relaxation rate observed with short pulses, could be due to the entry of Ba during depolarization or following membrane repolarization (tail currents).

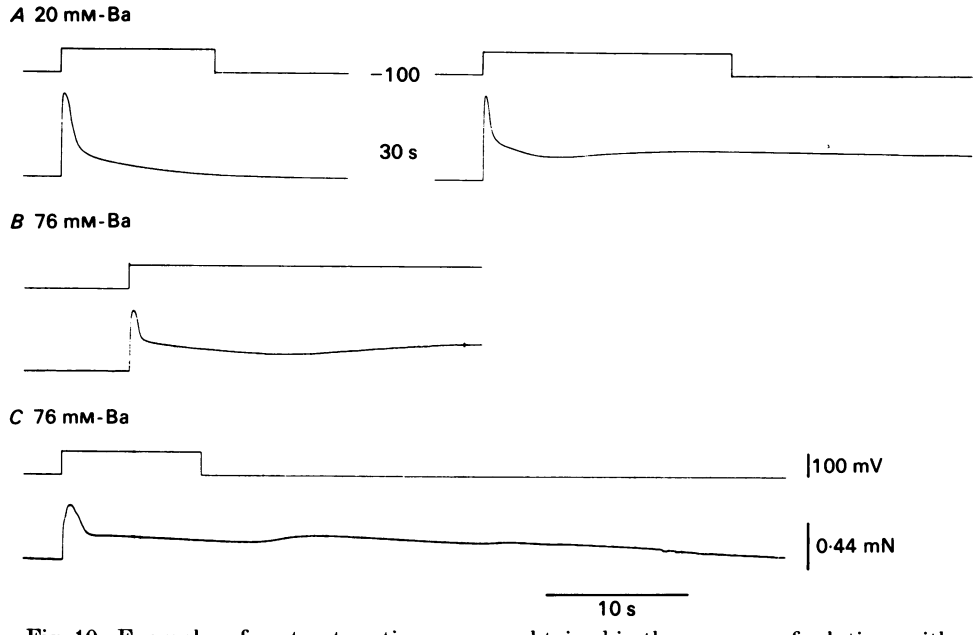

Fig. 10. Examples of contracture time courses obtained in the presence of solutions with high Ba. *A* shows a contracture obtained in the presence of 20 mM-Ba, with the characteristic slow relaxation phase starting after a fast one in the absence of the plateau phase; after a repriming period of 30 s at −100 mV a second contracture was elicited which shows an increase in the second slow relaxation phase and a secondary tension development phase. *B* shows a contracture with the same characteristics as those of the second contracture of *A*. Finally *C* shows a third response, obtained in a different fibre, whose slow relaxation phase continues after membrane repolarization and also shows a secondary tension development.

In several experiments, it was found that the slow relaxation could even be reversed by the development of a secondary tension component. Examples of this behaviour are given in Fig. 10. In some cases, but not always, microscopic observation of the fibre during this secondary component of tension development, indicated a contraction clot near the current-passing electrode. Although damage of the fibre was clear in these cases, the observation is still indicative that massive entry of Ba in damaged, and possibly in undamaged fibres, can give raise to tension development.

The physiological relevance of this observation is not clear, since entry of Ca in undamaged fibre did not have the same effect, since we never observed a secondary tension development in fibres not obviously damaged. The observation, however, is interesting since it confirms the early observations of Heilbrunn & Wiercinski (1947)

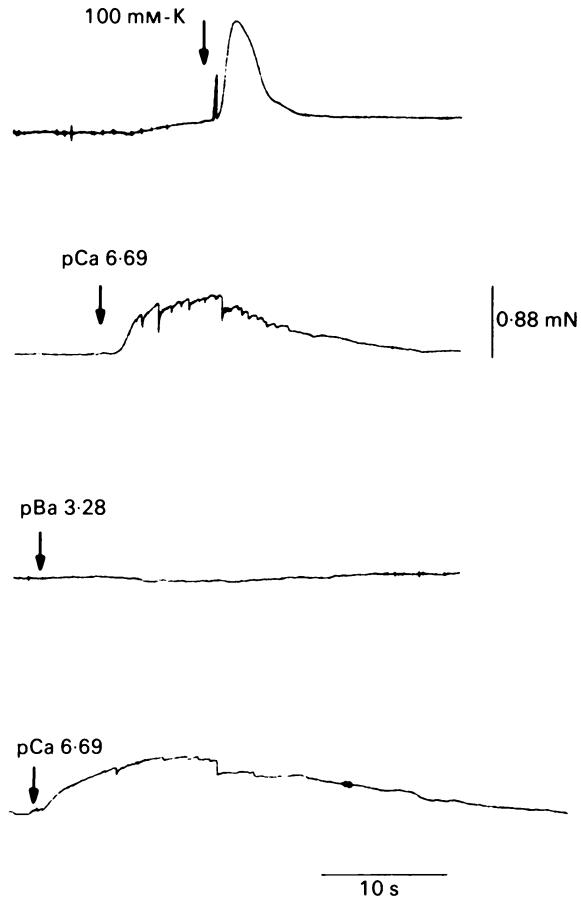

Fig. 11. Contractile activation in a chemically skinned fibre. The upper trace shows a contracture induced by 100 mM-K prior to exposing the fibre to the detergent solution. After removal of the membrane was complete exposure to a solution of pCa 6·69 induced the response shown in the second trace. The third trace shows that exposure to a solution with pBa 3·28 was without effect. Finally, the bottom trace shows again a response to pCa 6·69.

and of Caldwell & Walster (1963), that Ba injection may induce contraction in muscle fibres. It also appears to be consistent with the idea of Potreau & Raymond (1980*a*) of a Ba-induced Ca release.

Effect of Ba on skinned muscle fibres

In view of the reports that both in frog (Heilbrunn & Wiercinski, 1947) and crab (Caldwell & Walster, 1963)) striated muscle fibres intracellular injection of Ba ions induces contractile activation, a few experiments were carried out to test whether Ba ions could induce contraction in chemically skinned fibres by direct interaction with the contractile machinery. Fig. 11 shows one such experiment. The upper record shows a contracture induced by a high K solution prior to the treatment of the fibre with the detergent solution. The second record shows tension development of the

chemically skinned fibre in response to raised Ca concentration, pCa 6·69. In agreement with the work of Julian (1971) it was found that at this pCa tension was developed, which in this fibre amounted to 65% of the maximal contracture peak tension. The third record shows that a much higher Ba concentration, pBa 3·28, was ineffective in inducing a contraction. Finally the bottom record shows a final response to pCa 6·69. From this and other similar results, it can be concluded that Ba ions do not activate directly the contractile mechanism.

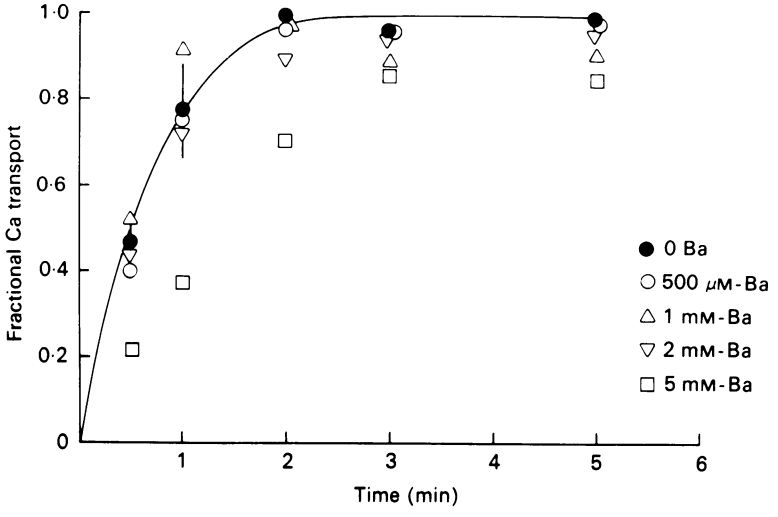

Fig. 12. Effect of Ba on the fractional Ca transport. The filled symbols show Ca uptake in the absence of Ba expressed as a percentage of control in the presence of 0·1 mM-$CaCl_2$ and plotted against time. Each point represents the mean ± S.E. of mean obtained in four determinations. The empty symbols represent the results obtained in the presence of different Ba concentrations. Temperature 21 °C.

Effect of Ba on the Ca uptake by fragmented sarcoplasmic reticulum

Since we observed that in the presence of high external Ba the relaxation phase of short and prolonged contractile responses is markedly slowed, it was thought important to test whether Ba ions could interfere with the Ca uptake function of the sarcoplasmic reticulum. Fig. 12 indicates that even in the presence of 2 mM-Ba, the uptake capacity of isolated sarcoplasmic reticulum vesicles is basically unchanged, while in the presence of 5 mM-Ba a small decrease in the uptake rate is observed. In the graph, the Ca transport expressed as a percentage of the values obtained in the presence of 0·1 mM-external Ca, is plotted against time.

DISCUSSION

Contractile activation of frog skeletal muscle fibres in response to a variety of stimuli may occur in the virtual absence of extracellular Ca (Armstrong, Bezanilla & Horowicz, 1972; Lüttgau & Spiecker, 1979; Caputo, 1981). In agreement with this, in the present work we have found that contractile responses to short ($\ll 1$ s)

depolarizing pulses are practically the same irrespective of the presence or absence of Ca in the external medium.

Furthermore, it has been shown that a low concentration (1·8 mM) of Ba in the absence of external Ca does not have any effect on these responses. In the absence of external Ca, and irrespective of whether 1·8 mM-Ba is used to substitute for it, the relationship between fractional tension and membrane potential is barely affected.

The steady-state inactivation curve obtained with repriming experiments, is shifted by about 10 mV toward less negative potentials in the absence of external Ca, in qualitative agreement with the results of Lüttgau & Spiecker (1979), and a similar shift is observed when 1·8 mM-Ba substitutes for Ca. At high concentrations (76 mM), Ca and Ba cause a marked shift of about 30 mV toward less negative potentials in the tension–voltage relationship, also causing a change in the slope of the curve which describes the relationship. La at lower concentrations (2 mM), appears to cause a similar shift, without an apparent effect on the curve slope. Interestingly, while La (2 mM) and Ca (76 mM) also affect the steady-state inactivation curve, causing a shift toward less negative potential and a decrease in the curve steepness, Ba at 76 mM, only appears to reverse the shift observed in Ca-free media.

Considering the shift in the tension–voltage relationship caused by high Ca or La, the present results confirm the previous work of Andersson & Edman (1974*a*,*b*) and of Dörrscheidt-Käfer (1976, 1981). Ba at 76 mM, appears to induce the same effect as Ca although less efficiently. The effect of high Ca has been explained assuming that this ion could affect the surface potential of the transverse tubular membrane (possibly near the T-sarcoplasmic reticulum junction) by either binding to or by screening off, fixed charges (Dörrscheidt-Käfer, 1976). Since, however, La affects the contractile threshold more efficiently than Ca, a further interaction of La was postulated in terms of adsorption to neutral but amphoteric sites, resulting in additional positive charges (Dörrscheidt-Käfer, 1981).

The relationship between tension and voltage appears to be described by the same equation as the charge–voltage one (Chandler, Rakowsky & Schneider 1976; Shlevin, 1979). Tension however is more steeply related to voltage than charge movement. This might indicate that a component of charge movement, more steeply dependent on membrane potential, is involved in contractile activation.

The shift of the contractile threshold observed in 76 mM-Ca is larger than the shift in the threshold potential for charge movement measured by Shlevin (1979) in 50 or 100 mM-Ca. However, the shift in the V value for the two phenomena, appears to be similar (41 mV shift for contraction, and 50 mV shift for charge movement). A good agreement is also found if one compares the shift for contractile threshold and that for activation of $\frac{1}{3}$ of the charge, which according to Horowicz & Schneider (1981) is the necessary amount of charge to be removed for attaining contractile threshold (which amounts to 38 mV). Interestingly, the tension–voltage relationship, in the presence of high Ca or Ba, but not in the presence of La, shows a decreased steepness evidenced by an increase of the parameter K of eqn. (1), from 4 to 6·5. A steepness change in the same direction was also found by Shlevin (1979) who studied the effect of high Ca solution on intramembrane charge movement. The similarity of the shift and the steepness change is perhaps indicative of the relationship existing between the two phenomena. The steepness of the tension–voltage relationship is not affected

by La, but only by Ba and Ca. Perhaps this difference is either due to the different permeation of these ions, or it is a result of the additional interaction of La with the surface membrane postulated by Dörrscheidt-Käfer (1981).

A noticeable effect of external Ca deprivation is the reduction of the time course of contractures induced by raised external K (Lüttgau, 1963; Caputo & Gimenez, 1967; Lüttgau & Spiecker, 1979; Cota & Stefani, 1981), or by prolonged voltage-clamp depolarization, (this work). Different divalent and trivalent cations, such as Sr, Co, Ni, and La, at low concentrations, have been shown to substitute for Ca in restoring the contracture time course (Edwards, Lorković & Weber, 1966; Lorković, 1967; Andersson & Edman, 1974*b*; Caputo, 1981; Lorković & Rüdel, 1983). Of these, the action of Ni and La are noteworthy, since Ni does not permeate through the Ca channels and has been reported to block them, (Almers & Palade, 1981), while La has been shown not to permeate muscle fibre membranes (Fink, Grocki & Lüttgau, 1980) and causes a marked prolongation of the contracture response. On the other hand, Ba ions, which may flow into the fibre at low concentrations (1·8 mM), are not effective in restoring the contracture time course, and high concentrations (76 mM), are unable to restore the response plateau, although they cause a slow secondary relaxation phase. These considerations indicate that membrane permeation of di- and trivalent cation, is neither a necessary nor a sufficient condition for obtaining a contracture response with normal time course. Therefore it is possible to postulate that the time course of these responses, and most probably the time course of Ca release, is regulated by di- or trivalent cations at a site localized on the transverse tubular membrane.

An interesting effect of Ba at high concentration is the reduction in the relaxation rate after short depolarizing pulses. This effect is peculiar to Ba, and most probably due to the entry of this cation into the fibre, during the depolarizing pulse and immediately after (tail currents).

When used at high concentration, Ca and Ba are effective in prolonging the contracture time course, and La at 0·5–2 mM also has a similar effect. Thus, in the presence of high Ca or La a marked prolongation of the plateau phase is observed, with a slowing of the spontaneous relaxation phase. In the presence of high Ba the plateau is absent, as is the case for the Ca-free contracture, however, the spontaneous relaxation phase shows a late slowly relaxing component, sometimes during which a secondary tension phase develops. This effect is probably related to the decrease in the relaxation rate observed after short pulses, and due to the massive entry of Ba into the fibre. Since the skinned fibre experiments indicate that the contractile machinery is not directly activated by Ba, the effect could be due either to decreased uptake of Ca by the sarcoplasmic reticulum in the presence of increased Ba concentration, or to a secondary release of Ca from the sarcoplasmic reticulum in the presence of Ba. The first possibility is not supported by the results of the experiments with isolated sarcoplasmic reticulum vesicles, which demonstrate that Ca uptake rate is decreased only when Ba concentration is 5 mM. For the case of our fibres, with a volume of $4{\cdot}2 \times 10^{-6}$ cm^{-3}, to achieve an intracellular concentration of 5 mM an entry of $21{\cdot}2 \times 10^{-12}$ mol of Ba would be necessary. This amount would be equivalent to a transfer of $4{\cdot}1 \times 10^{-7}$ C of charge, which is at least one order of magnitude higher than that calculated by Potreau & Raymond (1980*a*) for a pulse of 100 mV and 600 ms,

in the presence of 76 mM-external Ba. With regard to the second possibility, in the work of Potreau & Raymond (1980*a*) the idea has been held that Ca and Ba currents are involved in contractile activation. Since Ba does not interact with the contractile proteins, the idea of a Ba-induced Ca release, is not easily discarded. Such an idea is also based on the old observation that Ba injection in muscle fibres, causes contractile activation (Heilbrunn & Wiercinski, 1947). It is also possible that Ba can displace Ca from myoplasmic buffering systems such as parvalbumins or other Ca-binding proteins. In the present work we have presented results which are compatible with these ideas, since under certain conditions, a tension development could be observed even when the fibre was repolarized, indicating that massive Ba entry could cause tension development. This effect however appears to be specific for Ba. The presence of a Ba-induced Ca release, is perhaps of no physiological interest, since it is known that a Ca-induced Ca release can occur under particular experimental situations, which, however, are considered not to play a significant role under physiological conditions (Endo, 1977).

It has recently been shown (Eisenberg *et al.* 1983; Frank, 1984) that the Ca-current blocker D-600 may have an inhibitory effect on contracture response. However, it is important to stress that the simultaneous presence or the simultaneous abolition of Ca currents and contractile responses does not constitute an unequivocal demonstration of a causal relationship between the two phenomena.

With respect to this point experiments with pharmacological agents which differentially affect depolarization contraction coupling and inward Ca currents, indicate that the site of Ca release from the sarcoplasmic reticulum is pharmacologically different from the Ca channel (McCleskey & Almers, 1981; Gonzalez-Serratos, Valle-Aguilera, Lathrop & García, 1982).

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