Contractile Activation Phenomena in Voltage-Clamped Barnacle Muscle Fiber

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ABSTRACT Tension development in voltage-clamped barnacle muscle fibers occurs with depolarizing pulses so small as not to activate the potassium and calcium conductance systems. Peak tension and the tension time integral appear to be graded by both amplitude and duration of the depolarizing pulses. Subthreshold depolarizing conditioning pulses shorter than 500 ms potentiate the response to a given test pulse. This effect diminishes and reverts when the duration of the conditioning pulse is increasingly prolonged. The relationship between fiber membrane potential and tension developed in response to depolarizing pulses is described by an S-shaped curve. The tension saturates at a membrane potential of about +10 mV (inside positive). For a given pulse duration the saturation value remains constant even when the fiber interior reaches a value of +230 mV, which is well above what may be estimated to be the equilibrium potential of calcium ions ($E_{Ca} = +120$). In the presence of 5 mM external procaine, the shape of the tension-potential curve changes; the maximum value tension besides being diminished is not sustained by falls when the potential approaches the estimated value for E_{Ca}. These results suggest that under physiological conditions the contractile activator is probably released from an internal store, and that the calcium entering the fiber as inward current does not play a direct major role in contractile activation.

INTRODUCTION

Voltage clamp techniques have been increasingly used to study the phenomena involved in contractile activation in different muscular preparations (Hagiwara et al., 1968; Adrian et al., 1969; Heistracher and Hunt, 1969; Bezanilla et al., 1971). In principle, a detailed study of the eventual relationships between membrane potential, membrane currents, and tension development should be possible with this technique. It should also be possible to study contractile activation with a time resolution in the millisecond range. However, in practice, the complex membrane systems present in most muscle preparations introduce a factor that makes it difficult to achieve optimum clamping conditions because of the lack of spatial (longitudinal and radial) homogeneity.

Using a three microelectrode clamp technique and microscopic observation of signs of contractile activity, Adrian et al. (1969) described a strength duration relation for depolarizing pulses required to elicit a just detectable mechanical response in frog skeletal muscle fibers. They also found that under certain

conditions, two subthreshold pulses, when temporally added, could elicit a response.

These studies have recently been extended by Costantin (1974) who used high resolution microscopy to evaluate the contractile response of only a few superficial myofibrils.

Another approach has been followed by Heistracher and Hunt (1969) and by Bezanilla et al. (1971) using short fibers (1–1.5 mm long) dissected from snake intercostal muscle and from the lumbricalis muscle of the frog, respectively, and a two-microelectrode clamp system. With this preparation these researchers could study contractile activation measuring force development and the tension time integral in response to pulses of different configuration.

The giant muscle fiber of the barnacle can be voltage clamped with axially inserted electrodes in much the same way as the squid giant axons (Hagiwara et al., 1968). With these electrodes, longitudinal potential control is easily obtained; however, radial homogeneity seems more dubious because of the radially oriented systems of transverse tubules and membrane invaginations present in these fibers. Although the shortcomings inherent to this situation must be taken into account for a proper interpretation of the experimental data, the advantages obtained with this preparation render it suitable for a detailed study of the phenomena involved in contractile activation. Hagiwara et al. (1968) have studied the relationship between membrane potential and tension in voltage-clamped barnacle fibers. They have shown that for a depolarizing pulse of a given amplitude, the tension developed varied with the pulse duration. In the present work we have extended this information obtaining the relationship between the area under the tension curve and the pulse amplitude-duration product, either at constant amplitude or at constant duration.

A question of basic interest when dealing with contractile activation in barnacle fibers is the nature of the activator's immediate source. In fact, although these fibers have a conspicuous sarcoplasmic reticulum (Hoyle et al., 1973) with considerable calcium uptake capacity (Ashley et al., 1975), it has been proposed that the calcium that intervenes in contractile activation comes from the external medium (Atwater et al., 1974). This hypothesis is based mainly on the demonstration that calcium-dependent regenerative responses can be obtained in these fibers. Under voltage clamp conditions, inward currents carried by calcium ions may flow into the fiber in response to depolarizing pulses. The presence of these calcium-dependent regenerative responses and of the calcium currents can be best put into evidence in barnacle fibers injected with EGTA to reduce the internal ionic calcium concentration and with tetraethylammonium ions (TEA) to block the outward directed potassium currents (Keynes et al., 1973; Hagiwara et al., 1969, 1974). However, it is most probable that under these experimental conditions, important changes may be induced in the fiber properties, in particular those concerning contractile activation. Therefore, in the second portion of this work, we have attempted to clarify whether in normal nonperfused fiber the calcium eventually entering the fibers intervenes directly in contractile activation. Preliminary accounts of some of the experiments reported here have appeared elsewhere (Caputo and DiPolo, 1972, 1975).

METHODS

Preparation

The experiments reported in this work were performed in using single fibers from the barnacle *Balanus aquila*. The specimens were supplied by the Pacific Bio-Marine Laboratories, Inc. (Venice, Calif.). The mean fiber diameter ± SEM was 1070 ± 15 μm (n = 64). It is important to stress that in this work the fibers were not perfused nor internally dialyzed.

Experimental Procedure

The dissection of the fiber and its preparation were similar to those already described (DiPolo, 1972). Once isolated, the fiber was mounted in a chamber of the type shown schematically in Fig. 1. The cut end of the fiber was cannulated using a glass capillary of 450 μm in diameter. The tendon end of the fiber was tied to the shaft of an RCA 5734 mechano-electronic transducer (RCA Solid State, Somerville, N.J.) to record tension. Through the cannula a single compound electrode was introduced into the fiber. This penetration was carried out with a prism adjacent to the fiber to provide a side view of it. The electrode consisted of an internal potential sensing capillary of 100 μm in diameter filled with 0.5 M KCl and containing a floating platinum wire (50 μm). The current

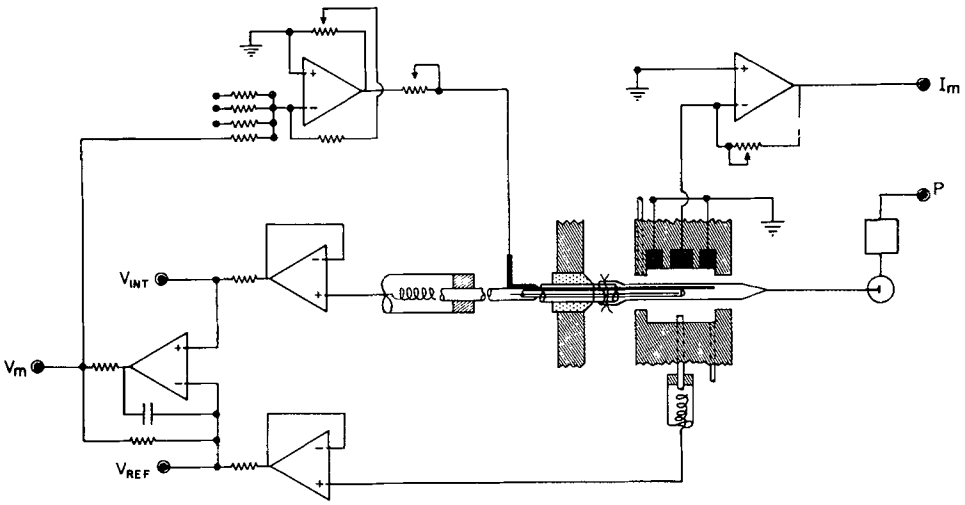

FIGURE 1. Diagram of the experimental arrangement. V_{INT} and V_{REF} represent the output of two unity gain followers connected to the internal and reference electrode, respectively. The output of these two amplifiers is connected to the input of a differential operational amplifier. The output of this amplifier is displayed on the screen of an oscilloscope and also compared at the summing junction (input) of the control amplifier with a DC holding potential and step command pulses. The output of the control amplifier supplies current to the membrane through a platinized platinum wire so that the difference of the sum of the holding and pulse potentials as compared to the fibre membrane potential is negligible. The current through the membrane (I_m) is measured by an operational amplifier connected as a current to voltage transducer. The tension developed by the fiber is recorded with an RCA 5734 mechano-electronic transducer. All operational amplifiers were model 148B (Analog Devices, Inc., Norwood, Mass.).

electrode consisted of a 100-μm platinum wire mounted onto a glass capillary and extended 0.65 cm beyond the tip of the capillary to provide a space clamp over the length of the fiber lying in the chamber. After the compound electrode had been inserted, the prism was replaced by three platinized platinum current electrodes (the lateral two for guards and the central one for collecting the current from the center region of the fiber under potential control) mounted on a single lucite block.

The internal and the external voltage electrodes were both made of silver-silver chloride. The internal one was connected to the glass capillary directly. The external one was connected to the chamber via polyethylene tubing filled with 1% agar 0.5 M KCl. The voltage clamp circuit employed in this work is basically the same as that described by Fishman and Macey (1969), except that different operational amplifiers were used. Before clamping the fiber membrane to a holding potential of −70, inside negative and proceeding with the experiment, the portions of the fiber lying in the air gaps at both ends of the central chamber were allowed to dry. When this occurred, these portions became very stiff, thus contributing very little to the mechanical compliance of the system. This allowed recording the isometric tension from the portion of the fiber under potential control. The fibers were bathed in an external solution, the composition of which in mM/liter was as follows: Na^+ 442; TRIS 10; K^+ 12; Mg^{++} 53; Ca^{++} 11; Cl^- 592. Unless otherwise specified, the experiments were carried out at a temperature of 18–20°C.

Some experiments were carried out to test for longitudinal homogeneity of the membrane potential along the length of the fiber lying in the experimental chamber during voltage clamp pulses. For this purpose, a conventional glass microelectrode filled with 3 M KCl and connected via an Ag-AgCl electrode to an amplifier with high input impedance (NFI, Bioelectric Instruments, Hastings-on-Hudson, N. Y.) was transversally inserted at different points along the fiber. The gain of this amplifier was matched with that of the voltage control amplifier. Small hyperpolarizing and depolarizing pulses were applied to avoid fiber movement. It was observed that the change in membrane potential at the end of the preparation occurred somewhat more slowly than at the center although the steady state levels are the same. Other runs, carried out with the microelectrode inserted at different distances from the center, gave similar results.

RESULTS

Resting Membrane Potential

Before clamping the fibers and after the end portion of the fibers was allowed to dry as described in Methods, the fiber membrane potential was measured. The mean resting potential ± SEM was found to be 47 ± 1 mV (n = 64) immediately after the longitudinal composite electrode had been inserted in the fiber. The fibers were voltage clamped to a holding potential of −70 mV inasmuch this is the value of the resting potential in intact fibers measured with glass microelectrodes (DiPolo and Latorre, 1972). The lower membrane potential measured with the longitudinal electrode in this work is most probably due to the presence in these fibers of deep invaginations of the outer membranes which can be damaged by the introduction of the electrode in the interior of the fibers. In fact, in a few cases in which the fibers were not clamped immediately after measurement of the resting potential, it was observed that their resting potential increased with time by ≅5–10 mV. The difference of ≅20 mV between the normal resting potential and the holding potential in these fibers is rather large and requires passing steady currents of the order of 20 μA/cm^2 of

fiber surface membrane. However, it was felt necessary to do so in order to avoid possible effects of steady depolarization on contractile activation phenomena.

Tension Development in Response to Depolarizing Voltage Clamp Pulses

Fig. 2 shows membrane current records and the initial phase of tension developed in response to increasing depolarizing pulses of 130 ms duration.

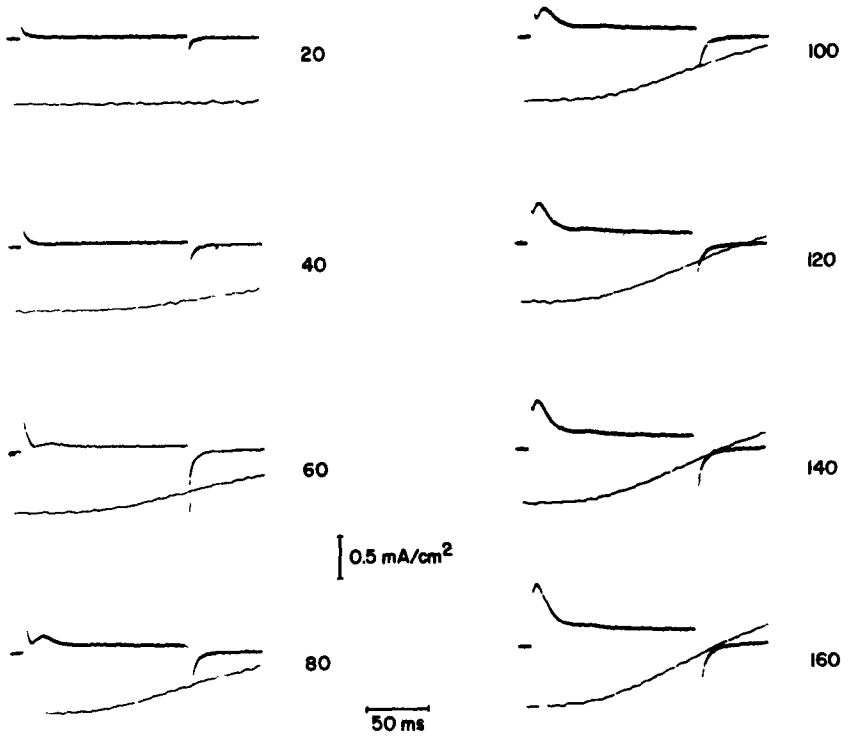

FIGURE 2. Records showing membrane currents (upper trace) and tension (lower trace) associated with depolarizing voltage clamp pulses of 130 ms duration and different amplitude. The amplitude of the depolarizing pulse appears on the right of each pair of traces. Inasmuch as in this and all fibers the holding potential was −70 mV, the membrane potential during the depolarizing pulses in this experiment ranged from −50 to +90 mV.

The current records are similar to those obtained by other authors (Hagiwara et al., 1969, 1974; Keynes et al., 1973). For pulses < 60 mV, the current trace shows a capacitive component at the beginning and end of the pulse and a steady, outwardly directed component. In addition, to these components an outward current bump appears after the capacitive current for pulses of 60 mV or greater. This bump can be identified with the first oscillation present in the current records of Keynes et al. (1973). In this case no inward current can be seen because, as shown by Keynes et al. (1973) and Hagiwara et al. (1974), these are made conspicuous only when the fiber is perfused with EGTA or when the potassium conductance system is abolished or reduced.

With regard to tension development, it is important to point out that, in the record obtained with a 40 mV pulse, tension development occurs without appreciable changes in the current record, indicating that no time-dependent currents are flowing through the membrane when the contractile mechanism is already activated. It is also to be noticed that with pulses of greater magnitude, the initial rate of tension development is the same, independently of the pulse amplitude. This finding contrasts markedly with the behavior of barnacle fibers perfused internally with TEA and bathed in 100 mM Ca used by Atwater et al. (1974).

Fig. 3 shows two sets of tension records, one obtained with pulses of constant duration (50 ms) and of increasing amplitude, the other with pulses of constant amplitude (80 mV) and increasing durations.

For pulses of constant duration, it can be seen that with pulses > 70 mV, the initial rate of tension development is the same. Although the peak tension

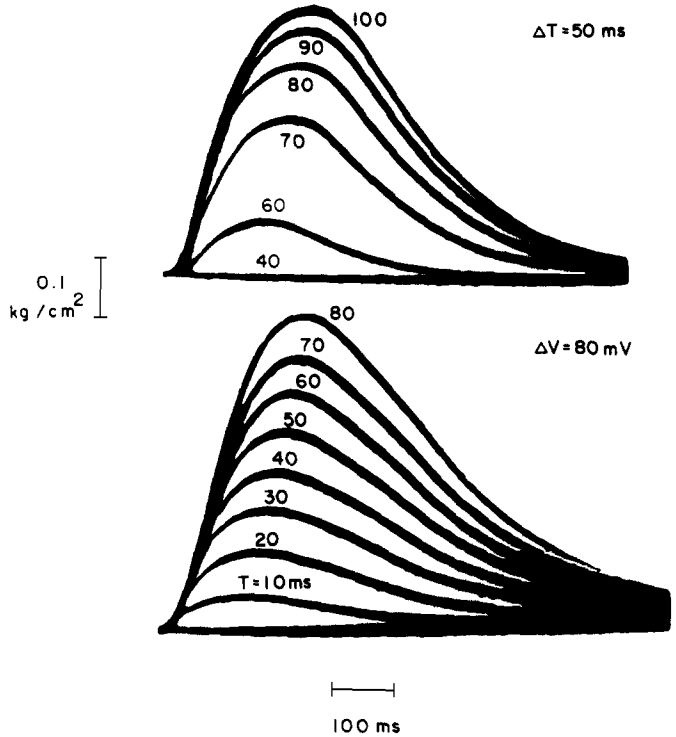

FIGURE 3. Sets of tension records obtained with pulses of varying amplitude and constant duration (upper set) and of constant amplitude and varying duration (slightly retouched photograph). Note that, in both sets of curves, not only the peak tension but also the area under the tension curves increases with the variable parameter of the depolarizing pulse. Note also that, for the case of the pulses of constant duration, the peak tension and the area under the tension curve tend to saturate with increasing depolarizations. This is better shown in Fig. 5.

increases with the pulse amplitude, it also increases with pulse duration for pulses of constant amplitude. A noteworthy feature of this experiment is that not only peak tension, but also the area under the tension curve, i.e., the tension time integral, increases with both amplitude and duration of the depolarizing pulse.

The increase in the tension time integral probably reflects an increase in the amount of contractile activator made available to the contractile protein. If this were so, the use of the area under the tension curve could be used as an index of the contractile activation process. Fig. 4 A shows a plot of the tension time integral ($\int P dt$) vs. the stimulus amplitude for the case of stimuli of three different durations. Inasmuch as both pulse amplitude and duration affect the contractile response, it appears that neither variable by itself provides a complete index of the electrical stimulus; it therefore seems convenient to use the product of the amplitude and duration as a measurement of the electrical stimulus. Fig. 4 B, therefore, shows how the tension time integral varies as a function of the stimulus amplitude duration product for the same case of Figure 4 A. It may be observed that, for each pulse duration, the tension time integral is zero until a threshold value of the stimulus amplitude (Fig. 4 A) and of the stimulus amplitude duration product (Fig. 4 B) is reached. Beyond these values, the tension time integral increases following a sigmoidal curve. Both the threshold stimulus amplitude duration product and the maximum value of the tension time integral increase with increasing pulse duration. The decreased stimulus amplitude threshold and increased stimulus amplitude duration product threshold with increasing pulse duration can be considered as an expression of the strength duration curve for mechanical activation (Adrian et al., 1969). Other examples of this feature are shown in Figs. 9 and 10.

The increase of the maximum value of the tension time integral with pulse duration seems to be peculiar to barnacle fibers and may indicate that, for a given pulse amplitude, the amount of contractile activator made available is a function of the pulse duration, for pulse durations shorter than that beyond which spontaneous relaxation occurs.

This is better shown in Fig. 5 which demonstrates the dependence of tension time integral on the stimulus amplitude duration product for two fixed pulse amplitudes while the duration was varied.

It is clear from this figure that there is a linear dependency between the tension time integral and the pulse amplitude duration product for pulses of relatively short duration as those used in this experiment (< 100 ms). The slopes of the lines being 0.033 and 0.018 $K \cdot g/cm^2 \cdot mV$ for the 80 and 60 mV pulse, respectively.

Effect of Conditioning Prepulses

It is known that in frog skeletal muscle steady-state depolarization induces inactivation of the fiber contractile capacity (Hodgkin and Horowicz, 1960), whereas short depolarizing conditioning pulses enhance the tension in response to a test pulse in voltage-clamped frog short muscle fiber (Bezanilla et al., 1971) and frog ventricle (Morad and Orkand, 1971). These results support the finding

of Adrian et al. (1969) that two subthreshold pulses produce a contractile response when applied sequentially. Hagiwara et al. (1968) reported that tension development in barnacle fibers depends on the absolute value of the membrane

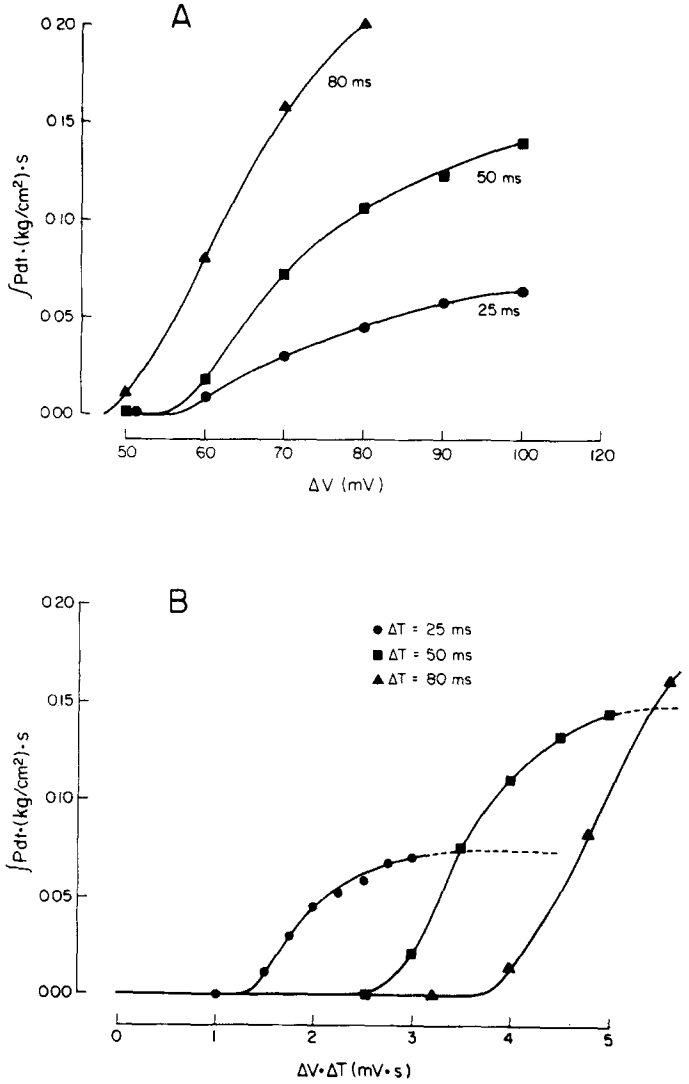

FIGURE 4. Curves relating the time integral of tension to (A) stimulus amplitude and (B) stimulus amplitude duration product for the cases of stimuli of three fixed durations and of varying amplitude. Note the tendency of the tension time integral to saturate with the pulse amplitude and that the saturation values increase with the pulse duration.

potential and is independent of the base line potential. More recently, Atwater et al. (1974) have failed to observe any signs of summation of two barely subthreshold pulses sequentially applied. In contrast to these findings, the experiments to be described next, show that in nonperfused barnacle muscle

fibers, conditioning pulses do have an effect on the contractile response to a test pulse and that this effect depends on the pulse configuration.

The upper two records of Fig. 6 show the effect of a short depolarizing

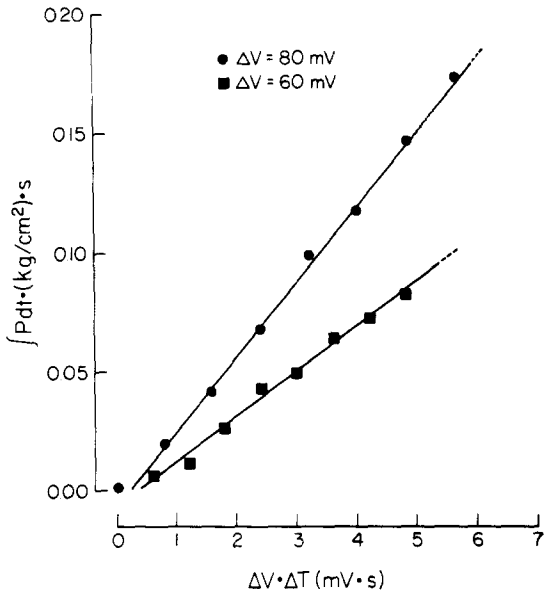

FIGURE 5. Curves relating the time integral of tension to the stimulus amplitude duration product for the case of stimuli of two fixed amplitude and varying duration, (between 10 and 80 ms). Note that for the same range of amplitude duration product as in Fig. 9, no saturation of the tension time integral is evident.

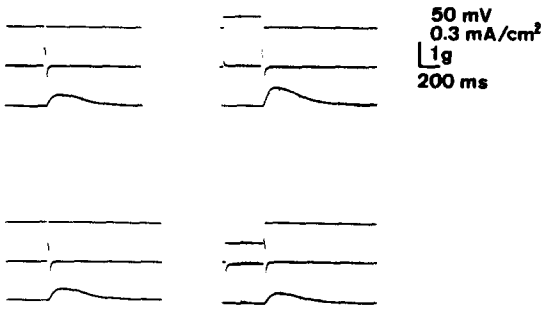

FIGURE 6. Effect of conditioning depolarizing and hyperpolarizing pulses on the contractile response to a test pulse. Note the increase induced by the subthreshold depolarizing conditioning pulse and the relatively small decrease caused by the hyperpolarizing conditioning pulse.

conditioning pulse of 350 ms duration and 22 mV amplitude on the tension produced in response to a 20-ms 80 mV test pulse. The first record shows the response to the test pulse alone; the second record shows that preceding the test pulse with a depolarizing conditioning pulse causes potentiation of the response.

The possibility that this effect might result from increased radial recruitment of additional myofibrils because of incomplete radial penetration of the test pulse alone should be considered. The lower two records show the effect of an hyperpolarizing conditioning pulse of 350 ms and 40 mV amplitude. It is clear that although a diminution of the response is observed, this is smaller than the increase caused by the conditioning depolarizing pulse even though the amplitude of the hyperpolarizing pulse is greater. This would not be expected if the effect of the conditioning pulse were limited to increase or decrease the membrane potential before the test pulse. Another reason that weakens the above mentioned possibility is the observation that the effect of the prepulse depends on its duration. This is shown in Fig. 7. In it the ordinate shows the tension obtained when the test pulse was preceded by the conditioning pulse relative to the tension in response to the test pulse alone. The abscissa represents the conditioning pulse duration. The insert shows schematically the experimental procedure. The conditioning pulse duration was varied whereas the test pulse duration had a fixed duration of 20 ms. It can be observed that for this case the maximum potentiating effect is obtained with a prepulse of about 350 ms. For conditioning pulses shorter than this value, their effect on the response increases monotonically with their duration. Therefore, a marked difference is

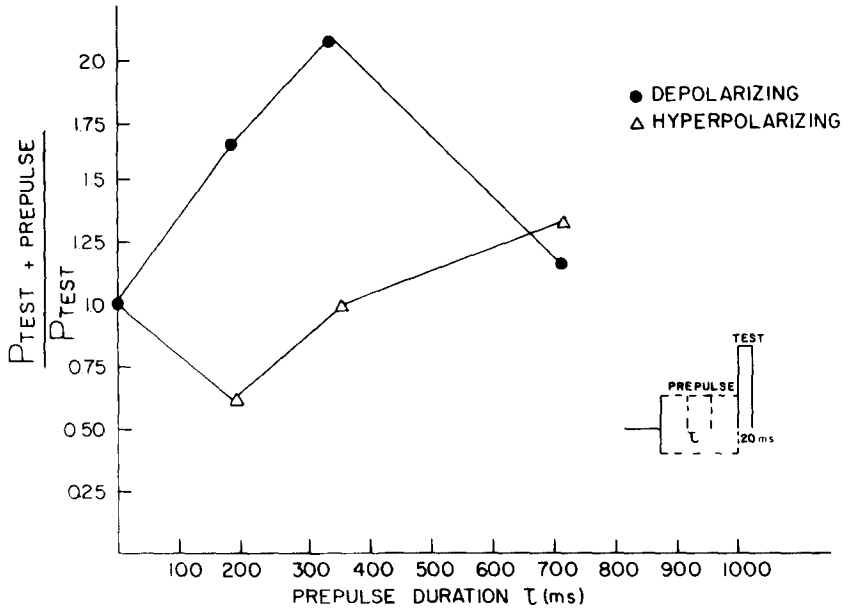

FIGURE 7. Curves showing the effect of the duration of conditioning depolarizing and hyperpolarizing pulse on the tension induced by a fixed test pulse. The ordinate shows the fractional increase or decrease of the peak tension caused by the conditioning pulse. The abscissa represents the duration of the conditioning pulse. The test pulse had an amplitude of 60 mV and a duration of 20 ms. The amplitude of the subthreshold conditioning depolarizing was 25 mV whereas that of the hyperpolarizing conditioning pulse was −40 mV.

observed between the effect of prepulses of 200 and 300 ms. This difference cannot be due to increased myofibrillar recruitment derived from poor radial control. When the duration of the prepulse is prolonged, the effect decays and is abolished when the prepulse is 700 ms long. In other experiments using similar pulse configurations, it was found that when the conditioning and the test pulses were separated by increasing time intervals, the potentiating effect of the conditioning pulse decayed with a half time of ≅100 ms and disappeared completely when the separation between pulses was of 500 ms.

Two conclusions can be derived from these experiments. First, as already mentioned, they render improbable the view that the effect of the prepulse might be primarily due to increased radial penetration of the depolarizing signal. Second, they indicate that, whatever the mechanism underlying the potentiating effect described, there seems to be a process that is time dependent.

It is interesting to note that these results show some agreement with those obtained by Adrian et al. (1969) and Costantin (1974) in frog skeletal muscle.

These results can be interpreted assuming that subthreshold pulses either make available subthreshold amount of contractile activator or facilitate the effect of test pulses. In both cases one would expect that the effect of conditioning pulses should diminish when the amount of activator released by the test pulse alone is made greater. This was found to be the case when larger test pulses were applied.

It has been shown above that the effect of conditioning subthreshold pulses depends on their duration. This suggests the presence of an inactivation process analogous to the one described for skeletal muscle (Hodgkin and Horowicz, 1960; Frankenhaeuser and Lannergren, 1969).

Other experiments were carried out to test whether a transient potentiation phenomena could also be observed for the cases of short test pulses superimposed upon long suprathreshold pulses. Fig. 8 shows one such experiment. The first record shows the tension in response to a pulse of 2 s duration and 20 mV amplitude. The second record shows the tension developed in response to 20-ms 50 mV pulse. The other records show the responses developed when the short pulse was superimposed at various times upon the long pulse. It can be seen that, when the short pulse is superimposed, the fiber develops an extra tension whose time-course is roughly equivalent to that developed in response to the short pulse alone. The amplitude of this extra response is greater when the short pulse occurs early during the long pulse, and diminishes progressively when the short pulse occurs at later stages. This effect then appears to be similar to that observed for the case of conditioning subthreshold pulses, indicating that probably the underlying mechanism is the same. For the case of this fiber, it can also be observed that the tension in response to the long pulse is not maintained at its maximum value, but decreases slowly with time, thus showing a behavior similar to that found in frog skeletal muscle fibers.

Source of Contractile Activator

From the work of Hagiwara et al. (1969, 1974), Keynes et al. (1973), and Atwater et al. (1974), it is clear that calcium currents, obtained under determined

experimental conditions can be described by $I_{Ca} = G_{Ca} (E_{Ca} - E_m)$, where I_{Ca} is the calcium current, G_{Ca} the calcium conductance, E_m is the membrane potential, and E_{Ca} is the calcium ion equilibrium potential obtained by the Nernst relationship.

$$E_{Ca} = \frac{RT}{2F} \ln [Ca]_o/[Ca]_i.$$

As expected from this formulation, Keynes et al. (1973) found that I_{Ca} increased

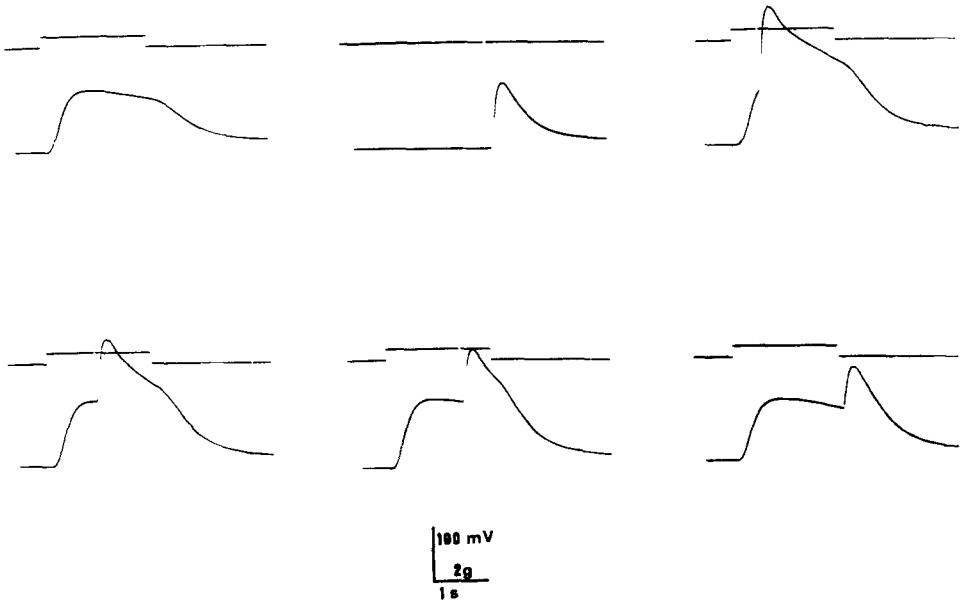

FIGURE 8. Effect of superimposing a short pulse (20 ms, 50 mV) over a long suprathreshold pulse (2 s, 20 mV) at different times. The fiber was clamped at a holding potential of −65 mV. The first two records in the upper row show the contraction developed in response to the two pulses alone. Note that when the short pulse is superimposed upon the long one, the fiber membrane potential during the short pulse has the same value as when the short pulse is applied alone (−15 mV).

with E_m until it reached a maximum value at about + 50 mV and then decreased when the value of E_m approached the value of E_{Ca}.

These considerations suggest a further test for the hypothesis according to which development of tension in barnacle fibers is associated with the inward flow of Ca currents (Atwater et al. (1974). In fact, one would expect tension to fall when E_m approaches the value of E_{Ca} in a way similar to that of I_{Ca}. Fig. 9 shows that in nonperfused barnacle fibers bathed in artificial sea water containing 25 mM Ca, the peak tension obtained with pulses of different duration reaches a maximum at a given voltage and maintains this maximum value even when the pulse amplitude is increased so as to exceed what can be considered reasonable values for E_{Ca}.

In the experiments carried out with solutions containing 25 mM Ca, assuming internal free calcium concentrations between 10^{-8} and 10^{-7}, the E_{Ca} values should lie between +186 and +157 mV at 20°C. It is clear from the results shown in Fig. 9, that tension does not behave as one would expect if it were dependent on I_{Ca} only. These results suggest that in normal fibers the calcium involved in contractile activation comes from an intracellular source. This type of experiment can be criticized on the basis of the complex intracellular membrane system of cleft and transverse tubules present in these fibers. The complex geometry of the membrane system of these fibers has been diagrammatically represented by electrical equivalent circuits by Keynes et al. (1973) and by

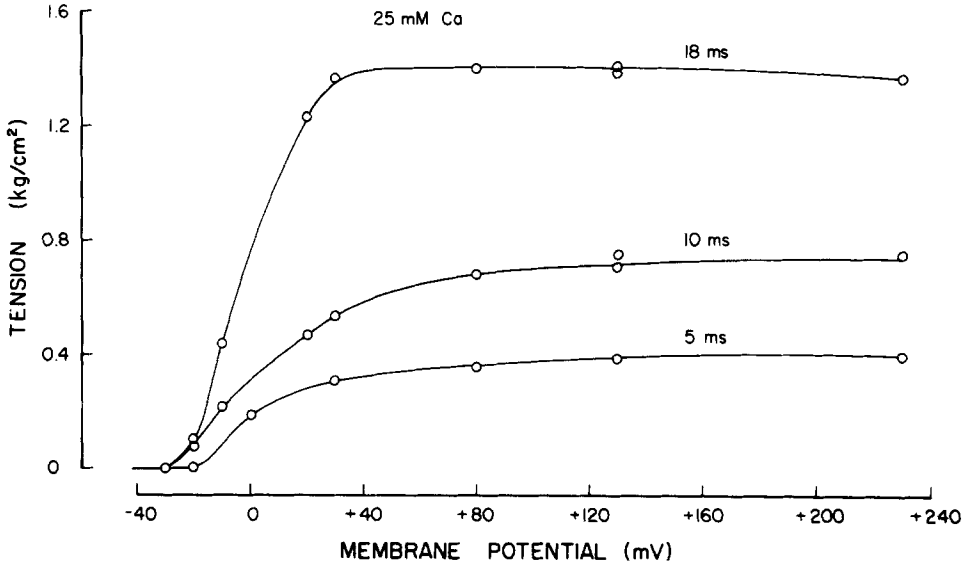

FIGURE 9. Tension voltage relationships obtained in one fiber bathed in artificial sea water containing 25 mM Ca, with pulse of different durations. Note that different tension saturation values are obtained with each pulse duration.

Padrón (1973). The presence of radially oriented series resistance in these circuits renders possible the view that, because of the voltage drop across these resistances, the potential across the tubule and cleft membrane may not necessarily follow the command potential imposed on the surface of membrane, especially under conditions of regenerative currents at the level of the tubular and cleft membranes. One way to reduce the magnitude of this experimental problem is to work under conditions in which the potential dependent ionic currents are reduced. The magnitude of the Ca currents should have been reduced in the experiments shown in Fig. 10, in which the external [Ca] was lowered to 1 mM. In this case the E_{Ca} values would lie between +145 and +116 mV. It is important to note that the maximum tension obtained with pulses of the same duration is much reduced in the solution containing 1 mM Ca. A few experiments were carried out to study the time-course of tension decline when the low calcium solution was substituted for the normal one, using pulses of

fixed amplitude and duration. It was found that the half times of tension decay were 370, 190, and 300 s for three fibers with diameters of 780, 620, and 800 μm, respectively. These decays are consistent with what would be expected considering diffusion delays out of cylinders of the fiber diameters. Interestingly, the tension recovery on replacing the normal calcium concentration proceeded with a much faster time-course. These results show that the fiber tension is clearly dependent on external calcium, although it appears that the calcium that

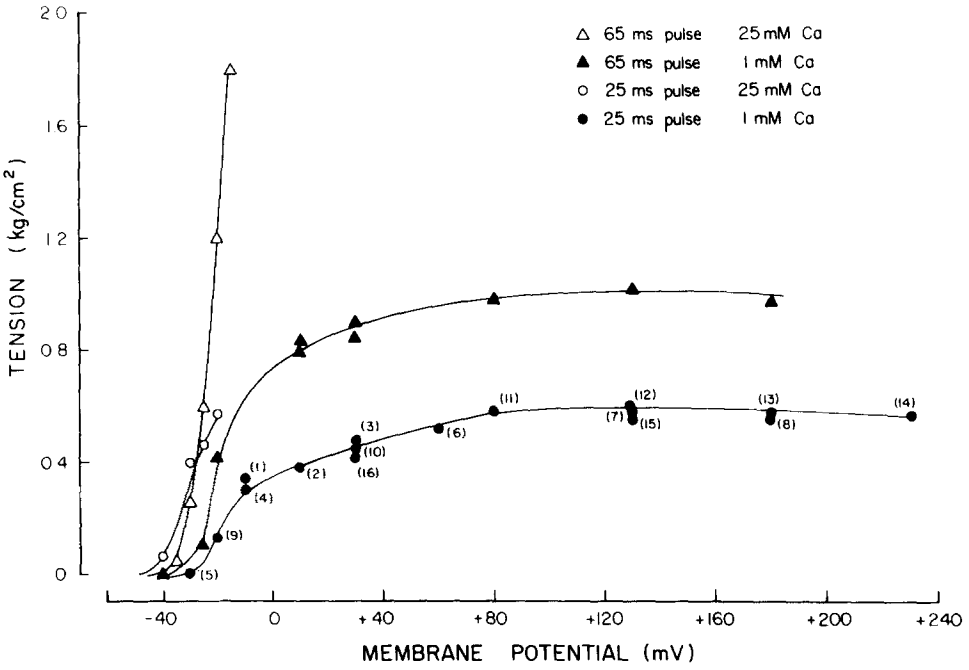

FIGURE 10. Effect of lowering the calcium concentration in the artificial sea water in the tension voltage relationship obtained with pulses of different durations in the same fiber. For the case of the curve obtained with pulses of 25 ms in 1 mM Ca solution, the numbers beside each point indicate the order in which these were obtained.

might be entering the fiber down its electrochemical gradient does not act as the major contractile activator. This would suggest a role of external calcium in supporting some step of the excitation-contraction coupling process, or in maintaining a rather labile intracellular calcium store.

To reduce the magnitude of potassium currents, experiments were also carried out in presence of externally applied TEA, at low external calcium. Fig. 11 shows that substituting 20% of external sodium by TEA effectively reduces the outwardly directed currents and that in the case of the 60 and 70 mV pulses the net ionic current is inwardly directed. Under these conditions, however, the tension membrane potential relationship is not significantly altered, as shown in Fig. 12. Different results were however obtained in few experiments in which

fibers from Chilean barnacle *Megabalanus psittacus* were used and in which all the external sodium was substituted by TEA. In this case the tension reached a maximum at a given membrane potential and then decayed when the membrane

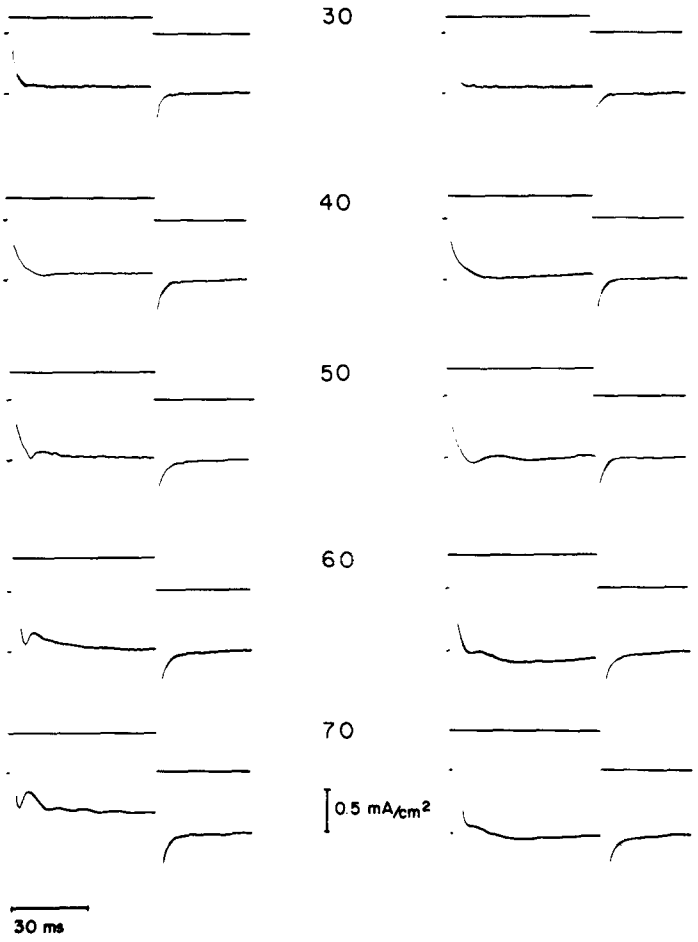

FIGURE 11. Membrane currents obtained in response to depolarizing pulse of different magnitude in the absence and presence of TEA (90 mM). The fibers were in artificial sea water containing 1 mM Ca.

potential was more positive than 150 mV. Similar results were obtained with fibers exposed to procaine.

Hagiwara and Nakajima (1966) have reported that in barnacle fibers, procaine does not suppress the Ca conductance system, but could affect the potassium conductance one. On the other hand, it is known that local anesthetics affect the release of calcium from the sarcoplasmic reticulum of frog fibers, either in

response to caffeine or in response to membrane depolarization (Feinstein, 1963; Luttgau and Oetliker, 1968; Caputo, 1976). Fig. 13 shows some current records that confirm the ideas of Hagiwara and Nakajima (1966). The set of records on the left side was obtained in a medium containing 10 mM Ca. The records on the right were obtained after adding to the medium 10 mM procaine. It is evident that the outward directed current is reduced in the presence of this drug. Interestingly, the tension potential relationship obtained in the presence of procaine is completely different from those presented so far. This is shown in Fig. 14.

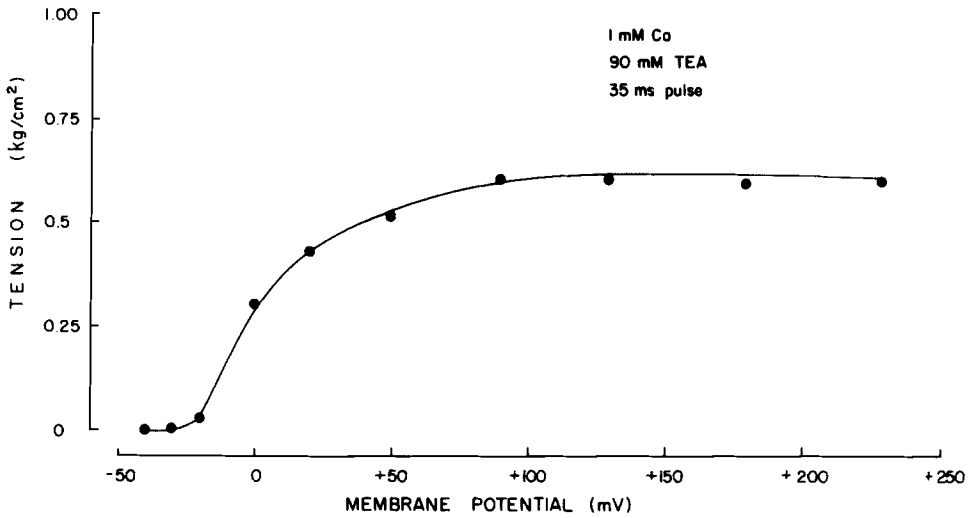

FIGURE 12. Tension voltage relationship obtained in one fiber bathed in artificial sea water containing 1 mM Ca and 70 mM TEA (substituted for sodium).

The tension voltage relationship shown here was obtained with a fiber bathed in a medium containing 11 mM Ca and 5 mM procaine. In this case, the contractile threshold was shifted to less negative potentials, and maximal tension was much reduced. More interestingly, it can be seen that the tension-potential curve obtained in the presence of procaine attains a maximum value at a potential of $\cong$ +50 mV and then decays to practically zero value at potentials more positive than +130 mV. This behavior agrees well with the behavior of calcium currents described by Keynes et al. (1973). Our preliminary interpretation of this result is that in the presence of procaine, the release of calcium from the sarcoplasmic reticulum is practically blocked and the calcium entering the fiber as inward calcium current may serve, under these circumstances, as contractile activator. It is not known whether the same amount of calcium enters the fiber in the presence and in the absence of procaine. The maximum tension is much reduced in the presence of procaine. However, if calcium entered the fiber in the absence of procaine, one would expect a small but observable decrease in the peak tension when the fiber membrane is more positive than +50 mV.

The conclusion that seems fair to derive from these experiments is that, if under normal conditions calcium ions enter the fiber down their electrochemical gradient, they do not contribute directly to contractile activation. The activator calcium probably comes from an intracellular store, which should be the sarcoplasmic reticulum which is known to be well developed in these fibers.

Additional evidence that intracellular activator stores may supply calcium for contractile activation was obtained inducing caffeine contractures in fibers

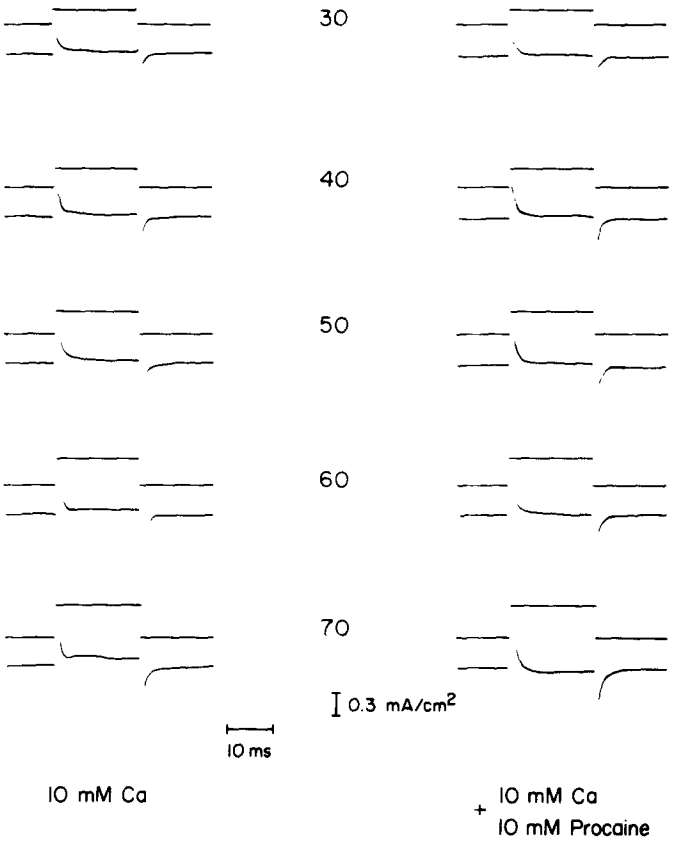

FIGURE 13. Membrane currents obtained in response to depolarizing pulse in the absence and presence of 10 mM procaine.

bathed for > 20 min in ASW prepared without calcium and with 2 mM EGTA. The results of one such experiment are shown in Fig. 15.

The peak tension of the contracture obtained in the virtual absence of external calcium (10^{-9}) was reduced to about half of the value obtained in the presence of a normal calcium concentration. When the drug was washed out and then reapplied after a rest period, the tension of this second response, although reduced, was still considerable. These experiments, show that intracellular calcium may indeed participate in the process of contractile activation, supporting the conclusions derived from voltage clamp experiments, and in agreement with the results of Lin and Bittar (1975).

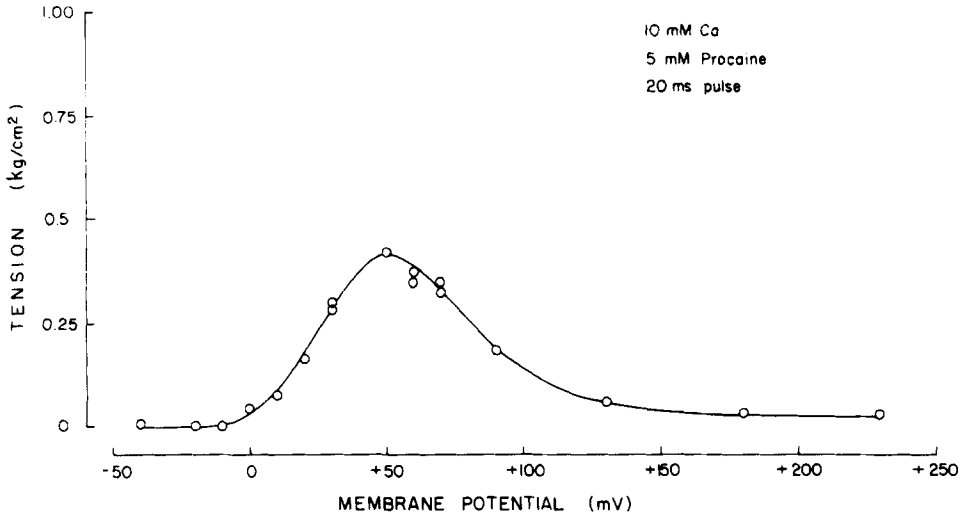

FIGURE 14. Tension voltage relationship obtained with one fiber bathed in artificial sea water containing 10 mM Ca^{++} and 5 mM procaine. Note the striking difference with the tension voltage curves shown in Fig. 9, 10, and 12.

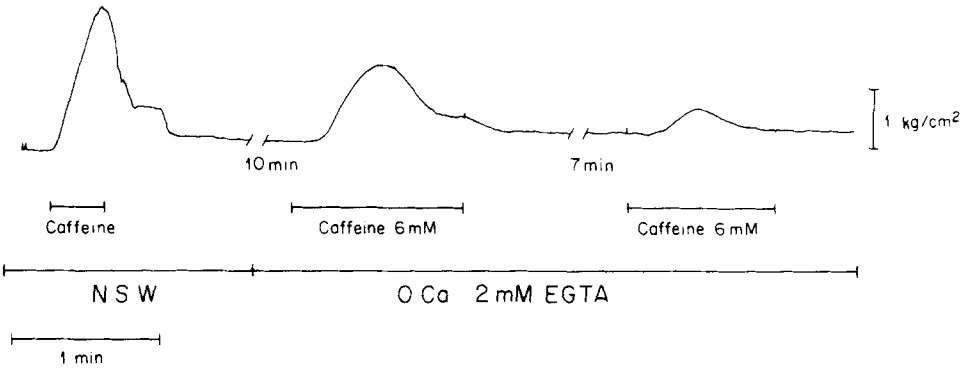

FIGURE 15. Caffeine contractures obtained in the presence of calcium and after 10 and 17 min after deprivation of this ion from the external solution.

DISCUSSION

In agreement with the work of Hagiwara et al. (1968) and of Ashley and Ridgway (1970), the experiments reported here show that the contractile response of voltage-clamped barnacle fibers can be graded by varying either the amplitude or the duration of depolarizing pulses. The evidence presently available does not allow discarding completely the possibility that increasing depolarization causes a larger fraction of the fiber cross-sectional area to be activated, thus partially explaining the gradation of the contractile response. However, Costantin and Taylor (1973) working with voltage-clamped frog muscle fiber and high speed cinematography have shown that most of the gradation phenomena in these fibers can be accounted for by gradation of

activation of the level of the myofibrils themselves. This is probably also the case for barnacle muscle fibers. The dependency of the contractile response with the pulse duration observed in these fibers is particularly interesting. In fact, contrary to what appears to be the case for frog muscle fibers (Bezanilla *et al.*, 1971), the peak tension that can be obtained with large depolarizations depends on the pulse duration as first shown by Hagiwara et al. (1968) and confirmed here. These findings support the view that most of the gradation of the contractile response can be explained assuming that it occurs at the level of the individual myofibrils. In fact, it is conceivable that increasing the duration of the pulse for a given amplitude causes more activator to be available to the contractile machinery instead of causing a deeper penetration of the depolarization. This interpretation agrees with the already mentioned findings of Ashley and Ridgway (1970) who showed that the integral of the light transient in barnacle fibers injected with aequorin increased with both pulse amplitude and pulse duration. In the present work, we have observed tension saturation with amplitudes of ≅100 mV at different pulse durations, whereas saturation of tension was not observed with durations up to 100 ms at different pulse amplitudes. One would, of course, expect to observe saturation with pulses much longer than the values used here, because, as has been shown in this work and will be discussed later, an inactivation type of phenomena is present in these fibers that is reflected in the occurrence of spontaneous relaxation during very long pulses (> 5 s). The results obtained with barnacle fibers can be reconciled with those obtained with vertebrate muscle, assuming that the inactivation process in barnacle occurs much more slowly. This appears to be the case inasmuch as a contraction in response to increase external potassium or to a long-lasting depolarizing pulse relaxes completely in ≅5 or 6 s at 20°C in frog fibers and much more slowly in barnacle. This characteristic of barnacle fibers is probably associated with their function of tonic fibers.

With respect to the effect of conditioning pulses, Atwater et al. (1974) have reported that two subthreshold pulses sequentially applied were not sufficient to activate contraction in their preparation. The discrepancy with these results presented here could be explained by considering the different experimental conditions. In our case, nonperfused fibers were used, whereas in the case of the experiments of Atwater et al. (1974) fibers perfused with TEA were used. Apart from any effect of TEA itself, it is most probable that perfusion may induce important changes in the fiber behavior. Supporting this view, Atwater et al. (1974) state that perfused fibers slowly lose their ability to develop tension. This fact was confirmed by us in a few unpublished experiments. In our case, the potentiation of contractile response to a test pulse caused by a depolarizing conditioning prepulse can probably be explained best by assuming that facilitation of calcium release is caused by the subthreshold depolarization. This view finds support in the results obtained in the experiments in which the test pulse was superimposed upon suprathreshold depolarizing pulses. However, even for this case, the possibility that the effect might be due to a deeper penetration of the test pulse and extra recruitment of more central myofribrils should be considered. The fact that the effect reverses when long prepulses are applied is

not necessarily a valid argument against this possibility, because it could be due to an idependent inactivation process. Better arguments against this possibility are (*a*) the findings that the potentiation effect at the beginning increases rather slowly with the prepulse duration (Fig. 7 shows that maximum effect is obtained with a prepulse of 350 ms and (*b*) that the effect also decays very slowly when the threshold conditioning pulse and the test pulse are separated. With longer conditioning prepulses, the effect diminishes and then reverses itself causing inactivation of the contractile response. It is not known whether two separate processes are involved in this behavior or whether only one process is responsible for it. What is clear is that a steady-state inactivation process similar to that found in frog fibers (Hodgkin and Horowicz, 1960) also appears in barnacle fibers.

Atwater et al. (1974) suggested that development of tension is linked with the flow of inwardly directed current, which they showed to be carried by calcium. In their work, calcium currents were made evident by eliminating the outwardly directed potassium currents using tetraethylammonium ions. This precaution was deemed necessary because, as shown by Keynes et al. (1973) and confirmed by Hagiwara et al. (1974), with both the calcium and the potassium systems activated, oscillation occurs in the membrane current. In our work, however, in the absence of TEA, tension development has been observed without observing oscillations in the current records. This probably means that in untreated fibers tension can be developed without the occurrence of calcium currents, suggesting that an intracellular release of calcium activates contraction.

Other evidence indicating that the calcium that enters into the fibers down its electrochemical gradient during depolarizing pulses does not play a major direct role in the contractile activation process, derives from the tension-potential diagrams shown in Figs. 9, 10, and 12. In fact, if the opposite were true, one would expect tension to reach a maximum and then decline to a zero value with increasing depolarizing pulses, in much the same way as the calcium inward current does. Instead, under different experimental conditions it was found that the maximum level of tension was maintained over a large potential range. Only in the presence of procaine was the opposite effect observed.

Although further work is needed to clarify this point, the experimental data presented here can be explained assuming that the whole fiber cross section is mechanically activated and that the level of activation is unchanged over a wide potential range, making plausible the explanation that the calcium entering the fiber as ionic current during voltage clamp depolarizing pulses does not play a major direct role in contractile activation.

There are examples in the literature that this is the case for other muscular preparations that exhibit calcium currents. Thus, Beeler and Reuter (1970) have proposed the existence of intracellular sites where calcium ions entering into the cell during depolarizing pulses are accumulated and from which they can be released, by an unknown mechanism dependent on the membrane depolarization, to serve as contractile activator.

In view of the fact that in barnacle fibers there exists a conspicuous sarcoplasmic reticulum (Hoyle et al., 1973), it is quite possible that the calcium

that intervenes in contractile activation is released from there. Strong support for this conclusion is given by the work fo Ashley et al. (1975) who, when using isolated bundles of myofibrils for barnacle muscle and the photoprotein aequorin, obtained evidence that the calcium that activates contraction must come from intracellular stores such as exist in the longitudinal elements of the sarcoplasmic reticulum. The maintenance of caffeine contracture in the absence of external calcium confirms this view. However, to explain the decrease in tension in media with low Ca, it must be assumed either that the sarcoplasmic reticulum equilibrates rapidly with the external calcium, or that the release mechanism is sensitive to external calcium concentration.

Recent work by Desmedt and Hainaut (1977) is also conclusive in showing that during contractile activation in barnacle fibers, there is a calcium release from the sarcoplasmic reticulum, which can be inhibited by dantrolene.

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