Muscle Contraction during Hyperpolarizing Currents in the Crab

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ABSTRACT Isolated muscle fibers from the motor legs of the crab *Trichodactyulus dilocarcinus* were submitted to strong hyperpolarizing currents of varied intensities which produced tension during the current pulse. Threshold for tension was obtained with intensities of about $0.2 \times 10^{-6} A$, changing $E_m$ to ca. $-150 mV$ (starting from a resting potential of ca. $-80 mV$). At the closure of the anodic square pulse, a second phase of tension usually appeared superimposed upon the one obtained during hyperpolarization. The first phase of tension increased with the increase of Ca++ concentration in the bath. Sr++ produced the same type of mechanical output as Ca++. When added to the normal Ca++ concentration, Ba++ and Mn++ in low concentrations (up to 21.5 mM) also increased the tension of this phase, but at higher concentrations they blocked both phases while Mg++ did not alter the tension. Of all the divalent cations employed, only Sr++ is capable of developing tension as a substitute for Ca++ in the external media. Procaine administered in a dosage ($5 \times 10^{-4} W/V$) which would suppress the contracture due to caffeine (10 mM), did not modify the tension developed during the hyperpolarization.

The preceding data indicate that the Ca++ required for tension during hyperpolarization comes from sites which would differ from those usually postulated for tension due to depolarization in the muscle fibers of other crustaceans (American crayfish). Furthermore, the external source of Ca++ appears to be one mainly implicated in the induction of tension due to inward current pulses.

INTRODUCTION

The excitation-contraction coupling mechanism continues to be one of the most intriguing features of muscle physiology. Some doubt still remains regarding the role of membrane depolarization being the major and most relevant event in the entire process (7, 9, 10). This point of view is usually extended to many different types of muscle fibers disregarding the fact that the electrical process is not the same in all of them. In some cases "all-or-none" action potentials are generated while in other muscles graded responses are
responsible for a step-wise activation of the contractile machinery. Crayfish muscles have been most frequently employed in muscle physiology. In 1967, Reuben et al. (12) described a very unusual and apparently paradoxical manner in which tension is induced by hyperpolarizing currents in this muscle. These authors postulated that transfer of Ca from the external media to the myoplasm was the probable mechanism activating the contractile machinery.

Our experiments deal with another crustacean muscle fiber in which hyperpolarizing currents also evoke tension (15). The type and characteristics of this phenomenon are described and the probable mechanisms at work during contraction are discussed, particularly those connected with the role of divalent cations in the process.

MATERIAL AND METHODS

Muscle fibers from the flexor of carpopodite portion of the walking leg of the land freshwater crab (Trichodactilus dilocarcinus) were employed. After removing the exoskeleton, the muscle was mounted in a lucite chamber and a bundle of only a few fibers or a single one was carefully dissected. Usually these fibers were 150–250 μm in diameter and 5–7 mm long. The distal tendon was connected through a micro stainless steel clamp to a Grass strain gauge F. T. 03 (Grass Instrument Co., Quincy, Mass.). Fibers were impaled with glass micropipettes filled with 3 M KCl in order to record membrane potentials as well as to inject stimulating currents. Occasionally, 2 M K citrate instead of KCl was used to rule out the effects of chloride injection during strong hyperpolarization. In order to prepare an adequate Ringer solution, samples of hemolymph from six crabs were analyzed. The average values found were as follows: Na 198 meq/liter, K 6 meq/liter, Ca 13 meq/liter, Mg 6 meq/liter, osmolality 530 mosmol/Kg H2O, pH 7.1–7.3. According with these results, the following Ringer solution was prepared: NaCl 200 mM, KCl 5 mM, CaCl2 15 mM, MgCl2 5 mM, pH 7.1–7.2. NaCO3 was used as a buffer.

Signals were amplified by means of a Bioelectric amplifier (Bioelectric Instruments Div., General Microwave Corp., Farmingdale, N. Y.) and conventional electrophysiological techniques were employed. Stimuli were delivered by means of a Tektronix pulse and waveform generation units (Tektronix, Inc., Beaverton, Ore.). Fibers were stretched approximately 30% above their resting length. Tension was recorded isometrically by means of the Grass F. T. 03 strain gauge. All events were recorded in a Grass 5 DWC 4 channel polygraph and occasionally, a simultaneous display in a Tektronix 561 oscilloscope was used.

RESULTS

Muscle fibers submitted to strong hyperpolarizing currents showed an increase in tension when a certain threshold was reached (more than −150 mV) (Fig. 1). The tension development was slow and a latency was always observed. In most cases, a secondary increase was observed when the hyperpolarizing pulses were discontinued. This mechanical event was coincident with an anode break response observed in the membrane potential. A syn-
chronized shortening of the sarcomeres appears within a constant length during the application of the hyperpolarizing pulses. This is in marked contrast to the asynchronous contractile phenomenon occurring after the hyperpolarization. In this latter case, sarcomere shortening was not limited to regions close to the current electrode and propagating contractile waves were frequently observed.

In Fig. 2, a record of contractions elicited by successive depolarizing and hyperpolarizing pulses can be seen. The values of mechanical output (80 and 100 mg) maintained without change during the entire series indicate the reproducibility of the phenomenon.

A characteristic latency period between the onset of the inward current pulse and the beginning of the mechanical response is also evident. It is noteworthy that tension does not decline immediately after the end of this pulse. It should be finally pointed out that tension evoked by depolarizing and hyperpolarizing pulses depicts different rates of rise. Tension during hyperpolarization seems to have an electrical threshold. It was always observed that mechanical response appeared when \( E_m \) attained approximately \(-150\) mV and that its amplitude increased with both pulse duration and current intensity (Fig. 3 A-B). It is important to point out that the delay between the onset of the hyperpolarizing pulse and the beginning of tension was always present and ranged between 100 and 500 ms.

In all cases, the maximal tensional values obtained under these conditions at the end of a 3-s hyperpolarization of \(-190\) mV reached 0.5 kg/cm² of muscle fiber. As the \( R_{\text{eff}} \) varies from fiber to fiber, currents ranging from 0.15 to \(2.5 \times 10^{-9}\) A were delivered in order to attain the values of hyperpolarization mentioned above.
Action of Ca

Ca has been shown to be the principal cation directly involved in the excitation-contraction coupling mechanism elicited by depolarization in muscle fibers of different species (14). Reuben et al. (12) suggested that Ca could also be involved in the development of tension during hyperpolarization.

In order to test the possibility that external Ca could also be essential for this contractile phenomenon, we studied the mechanical output in muscle fibers bathed in solutions containing different Ca concentrations. Six experiments were done, keeping the parameters of the stimulating current pulses constant throughout. In all cases, the study started no longer than 3 min after changing the bathing medium. Fig. 4 A illustrates a typical experiment performed under these conditions. Fig. 5 also related to this series, shows that peak tension increases several times as a result of rising external Ca following
Figure 4. Three different cells, A, B, and C. A: (a) standard Ringer, (b) +21.5 mM CaCl₂, (c) +65 mM CaCl₂. B: (a) standard Ringer, (b) +21.5 mM MgCl₂, (c) +65 mM MgCl₂. C: (a) standard Ringer, (b) +21.5 mM MnCl₂, (c) +65 mM MnCl₂. In each set, the hyperpolarization value was kept constant and the osmolarity unchanged by NaCl removal. In A, the tension increases with the increase of Ca in the external media; in B, Mg addition does not modify substantially the mechanical output during hyperpolarization. In C, the Mn increases the tension at low concentrations but decreases the control value with the higher concentration. For A, B, and C from top to bottom: tension, voltage, and current intensity.

Figure 5. Plot of the different tensions obtained with different concentrations of divalent cations (X) added to the normal Ca concentration, the osmolarity being constant by NaCl removal. The Ca (●) addition increases the tension up to 600% of the control. Similar results were obtained with Sr (■). The Mg addition (○) does not produce important changes, while Mn (□) and Ba (△) increase the tension at low concentration but progressively diminish it at higher ones.

A nearly linear relationship. Whereas the rate of rise of tension increased with increasing external Ca, the delay remained constant.

Action of Other Divalent Cations

In view of the above-mentioned results obtained with different Ca concentrations, other divalent cations which have been demonstrated to act on the contractile mechanism were tested. At first, we analyzed the influence of Mn⁺⁺, Ba⁺⁺, and Sr⁺⁺ on the development of tension when they were added in various concentrations to the external media, while maintaining Ca constant
at 15 mM. The general results of these experiments are shown in Fig. 5, where correlation between tension and divalent cations is shown (Ca++, or Ca+++ and C++, C+++ being divalent cations other than Ca++). In the series in which Sr was added we found that the tension was quite similar or slightly higher than in experiments in which Ca was the only divalent cation present.

Mn and Ba behave differently. Both cations facilitate the tension up to a certain value (21.5 mM). At higher concentrations, a reduction of the tension was observed, to such an extent that at 65 mM the mechanical output fell to less than control. No facilitation effect was seen in fibers incubated longer than 10 min in low Mn concentrations. Ba has not shown depressing effects at low concentrations even when the preparation was incubated for 20 min. On the other hand, the series of different concentrations of Mg studied show no effect on the tension output when it was added to normal Ca Ringer (Fig. 4 B, Fig. 5).

Since these experiments were performed with the addition of different cations to a normal Ca concentration in the external media, it was of interest to know whether these cations could replace Ca. In the following experiment, different Ringer solutions were prepared in which Ca was progressively substituted up to complete replacement, keeping the total divalent cation concentration constant (15 mM).

As can be seen in Figs. 6 A and 7, only Sr is capable of maintaining the tension whereas the other divalent cations fail to do so. In other words, mechanical output during hyperpolarization is completely abolished unless Ca or Sr ions are present in the external media (Figs. 6 B and 7). In marked contrast to the results observed when divalent cations were added to a normal concentration of Ca, no difference appeared in this experimental series between Mn and Ba and Mg, meaning that no tension can be obtained in zero Ca.

**Action of Procaine**

The results shown above suggest that the external Ca was directly involved in the induction of tension during hyperpolarization. Attention was then focused on the possible contribution of Ca from internal organelles in the hyperpolarizing activation process. Since procaine has been reported to have a selective action on this subcellular structure by blocking the release of Ca (3), a group of fibers were submitted to the action of the local anesthetic at a dose of $5 \times 10^{-4}$ W/V. Under these conditions, hyperpolarizing pulses elicited the same tension observed in the previous control, even after 30 min of exposure to procaine.

Failure to abolish tension during hyperpolarization by exposing the muscle to procaine suggests that there is a major difference in the excitation-contrac-
Action of Ca on Tension Produced by Depolarizing Currents

In view of the difference found in the excitation-contraction coupling mechanism underlying the two excitation conditions described above, it was of interest to analyze the changes in tension induced by depolarization when the Ca concentration of the external bath is increased. Three fibers were soaked in different Ca concentrations and tension was elicited by pulses of 120-ms duration and of the same depolarization level. As shown in Fig. 8, when the...
concentration of Ca increased, the tension developed decreased, so that at the highest concentration the tension was practically abolished. When the $I/V$ relationship was studied, using 15 and 42.5 mM Ca in the external media, it was clearly observed that the two curves superimposed in the hyperpolarizing quadrant (Fig. 9 A). These results indicate that the added Ca does not change the electrical response of the membrane to hyperpolarization. The same conclusion is valid for Mg (Fig. 9 B). The analysis of the depolarizing quadrant shows that the onset of delay rectification was affected, as it was observed in frog muscle (4) by the presence of higher concentrations of Ca or Mg. It should be noted that despite these results observed either under Ca or Mg in the $I/V$ plot, tension elicited by inward currents is clearly increased when Ca is raised (from 15 to 42.5 mM) but it is not affected when Mg is added replacing Ca (Fig. 5).

**DISCUSSION**

The development of tension during the injection of hyperpolarizing current has been reported previously (12) in other preparations. Although Orkand (11) did not observe tension when he applied inward currents in experiments
performed in the contractor epimeralis of the crayfish, Reuben et al. (12) working on another muscle of the same crustacean did record tension.

The tension we have observed depicts characteristics similar to those mentioned by the latter authors. In our experiments, tension begins to rise when the fiber has been hyperpolarized to \(-150\) mV for more than 100 ms. It is important to point out that in order to reach these values, the current employed varies between 0.15 and \(2.5 \times 10^{-6}\) A in different fibers. This wide range can be explained by the scattering of the effective resistance of the fibers (5–80 kΩ). The space constant (\(\lambda\)) derived from the preceding data, using the cable equation (8) and assuming an \(R_s\) of 310Ω cm for our fibers of 150–250 μm, ranged between 0.2 and 1 mm. These limits roughly coincide with the sarcomere shortening observed during hyperpolarizing pulses.

As can be observed in Fig. 3, the tension increases as a function of both the hyperpolarizing current intensity and duration. In this respect, tension evoked by hyperpolarization does not differ from that observed during the injection of depolarizing currents.

The second phase of tension that develops immediately upon the cessation of the hyperpolarizing current (Figs. 1, 3) is very irregular with regard to its shape, amplitude, and duration, suggesting that a process different from that underlying the phenomena described above occurs as far as excitation-contraction coupling is concerned. Tension during hyperpolarization or depolarization is produced by a very orderly sarcomere shortening, while the opposite is true for the contraction at the break of the inward current. These
two types of contractile activity also appear under different experimental con-
ditions when Ca is iontophoretically injected into the myoplasm of crayfish
muscle fibers (1).

The possibility that the type, intensity, and duration of the current used in
our experiments might create anomalous situations in the myoplasm or in the
membrane and could impair the development of tension by depolarization
was discarded by the experiments described in Fig. 2. In this case, unaltered
mechanical output was repeatedly obtained by successive inward and out-
ward current pulses; a condition where the process leading to the contraction
in both cases occurs in the same cell in the same place. In addition, this ex-
periment also discards any possible deleterious action caused by the strong
current injected into the fiber.

Regarding the activator of the contractile machinery, it should be pointed
out that in our experiments all the results indicate that the tension obtained
during hyperpolarization is highly dependent on extracellular Ca. There is
abundant data in the muscle literature (14) referring to Ca as the basic ion
playing the fundamental role as the activator of the contractile system.

However, discussion continues as to the principal source of Ca in the process
triggered by different types of stimuli (K, outward current, caffeine). Zacha-
rova and Zachar in crayfish (18) and Gainer (6) in the lobster reported that
K contracture is dependent on the concentration of Ca present in the ex-
ternal media and that the function follows an S-shaped curve, approaching
saturation when Ca reaches 30 mM/liter. However, in our experiments the
relationship between tension and Ca concentration is practically linear and
even in concentrations as high as 100 mM/liter, we have observed that ten-
sion still rises reaching six times the control level. The flux studies performed
on lobsters (6) strongly indicate that K contracture is due to the influx of Ca
from the outside to the inside of the cell. The tension elicited by hyperpolariza-
tion in conditions where the electrochemical gradient for Ca is highly in-
creased, supports the idea that a direct relationship would exist between Ca
influx to the myoplasm and mechanical output. The fact that tension was ob-
tained after a long delay (100 ms) hardly supports the idea that Ca from a
source other than an external one could participate in the process as can be
observed during depolarization (14).

Reuben et al. (12) reported potentiation of the tension elicited by hyper-
polarization in fibers bathed in zero K saline, a condition which enhances the
influx of Ca. It was therefore postulated that such potentiation could be due to
the additional entry of extracellular Ca during the inward current pulses.

Ca from the internal organelles could also participate in the contraction
during hyperpolarization. Procaine is known to stop the release of Ca from
the sarcoplasmic reticulum in the frog (16, 17). In this preparation, procaine
has been shown (Uchitel and García, unpublished data) to block contracture
due to caffeine which acts through the release of Ca from the sarcoplasmic reticulum (16, 17). Therefore, the absence of effect of procaine on the tension during hyperpolarizing pulses observed in our experiments indicates that the release of Ca from the sarcoplasmic reticulum is unlikely and strengthens the hypothesis of an external Ca source.

Further support for the external Ca hypothesis is given by the results observed with Sr since both Frank in the frog muscle fibers (5) and Caldwell and Walster in crustacean fibers (2) reported that this cation has the highest capability for replacing Ca in the development of the contractile phenomena. This was also observed in our experiments where the addition of Sr to a normal Ca concentration increases tension in a way similar to that which occurs when Ca is added (Fig. 5). This property of Sr as a Ca substitute is more clearly shown when Ca is progressively replaced at normal divalent cation concentrations (Figs. 6 A and 7). At zero Ca, Sr is the only divalent cation that fully maintains the control tension values and whose external concentration holds a direct relationship with the tension, suggesting that the extracellular Sr is available for the contraction during the hyperpolarization.

As previously described, Ba and Mn as well as Mg do not substitute for Ca (Figs. 6 B and 7) to maintain tension. Caldwell and Walster (2) demonstrated in crab muscle fibers that intracellularly injected Mg does not produce tension. We have also shown that at normal Ca concentration, the addition of Mg is irrelevant to produce a change in the initial tension. These data led us to consider in greater detail the double effect observed for Ba and Mn. Neither of these cations substitute for Ca, but at normal Ca concentration (15 mM), the tension increased when they were added at concentrations up to 21.5 mM. At concentrations above this level, the tensional output is progressively depressed and reached the control level when the concentration approached 65 mM.

One alternative to be considered to explain this double effect of Ba and Mn is that Ba might activate the contractile system in a way similar to Ca and Sr according to the observation made after intracellular injections by Caldwell and Walster (2). Reuben et al. (unpublished data) showed that Mn injected or applied to skinned fibers also produces tension. Thus, the inability of Ba and Mn to substitute for Ca in the external medium (Figs. 6 and 7) most probably indicates that they cannot penetrate the muscle fiber. This hypothesis is also consistent with the well-known Ca-conductance blocking action of Mn (13).

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BIBLIOGRAPHY