

The Role of Calcium in Excitation- Contraction Coupling of Lobster Muscle

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ABSTRACT Potassium contractures were induced in lobster muscle bundles under conditions which produced varying KCl fluxes into the fibers. The presence or absence of chloride fluxes during depolarization by high concentrations of potassium had no effect on the tensions developed. The curve relating tension to the membrane potential had a typical sigmoid shape with an apparent "threshold" for tension at -60 mv. Soaking the muscles in low (0.1 mM) calcium salines for 30 min completely eliminated the potassium contractures but the caffeine contractures were only slightly reduced under these conditions. The potassium contracture could be completely restored in less than 2 min by return of the calcium ions to the saline. Evidence is presented for independent, superficial, and deep calcium sites; the superficial sites appear to be involved in the coupling mechanisms associated with potassium contractures. These sites are highly selective for Ca^{++} , and attempts to substitute either Cd^{++} , Co^{++} , Mg^{++} , Ba^{++} , or Sr^{++} for Ca^{++} were unsuccessful. However, K^+ appeared to compete with Ca^{++} for these sites, and the evoked tension could be reduced by prestimulation of the muscle fibers with high K^+ salines. The results of studies on the influx of ^{45}Ca during potassium contractures were compatible with the view of muscle activation by the entry of extracellular calcium.

INTRODUCTION

Recent studies on the mechanisms of excitation-contraction coupling in muscle have focused on two fundamental issues. The first is whether the activation mechanism for tension is coupled to the absolute membrane potential (Hodgkin and Horowicz, 1960 *a, b*; Orkand, 1962; Zachar and Zacharova, 1966) or to the quantity of membrane current (Reuben et al., 1967). The second issue is related to the origins of the calcium released by excitation. One hypothesis proposes that there is a translocation of intracellular calcium (Sandow, 1965), while the other holds that there is an influx of extracellular calcium into the muscle fiber (Bianchi and Shanes, 1959; Edwards and Lorkovic, 1967). This study attempts to characterize the excitation-contraction coupling process in lobster muscle within the framework of the above issues.

MATERIALS AND METHODS

Flexor muscles of the carpopodite from the walking legs of the Maine lobster, *Homarus americanus*, were used exclusively in this study. The lobsters were obtained from the Gloucester Lobster Co., Washington, D. C., and were maintained in a lobster aquarium (Ruet Co., N. Y.) containing artificial sea water (Neptune Salts) at a temperature of 4°C. Each lobster was equilibrated in this environment for at least 1 week before use in the experiments.

The standard lobster saline contained 10 mM KCl, 455 mM NaCl, 24 mM CaCl₂, 8 mM MgCl₂, 6 mM H₃BO₃, and was adjusted to a pH of 7.4 ± 0.1 . Propionate was used as an impermeant anion substitute for chloride. The various changes in ionic composition of the salines will be described in the text in connection with specific experiments. In order to minimize the variability of the muscles from different lobsters, each fully dissected muscle bundle was first equilibrated in the standard saline at 6°C for 14–15 hr, and then brought to room temperature (21–23°C) before the initiation of the experiments.

In order to make the muscle preparation, the exoskeleton of the meropodite of the lobster walking leg was routinely removed, leaving only the portion of the exoskeleton to which the muscle was attached. The muscle preparation was then placed in standard saline, and the extensor muscle (of the carpopodite) was completely dissected away. Thus a large band of flexor muscle (of the carpopodite), which was attached to a thin exoskeleton at one end and to the central tendon of the meropodite at the other end, was exposed. The flexor muscle was then carefully dissected under saline until a clean preparation containing a small bundle of muscle fibers (usually 5–10 fibers) extending from the exoskeleton to the central tendon was obtained. These muscle bundles were soaked overnight in fresh standard saline as described above, and were examined immediately before the experiments for any damage to the fibers. Only muscle fibers which survived the dissection procedures without damage were used in these experiments.

In the experiments, the muscle preparations were rigidly mounted under saline in paraffin chambers by pushing stainless steel pins through the remaining exoskeletons deep into the paraffin floors of the chambers. Muscle tension was measured by a Grass Ft-03 force displacement transducer (Grass Instrument Co., Quincy, Mass.) and was displayed on a Tektronix 502A oscilloscope (Tektronix, Inc., Beaverton, Ore.). The central tendon (close to the insertion of the muscle) was connected by a small stainless steel clamp to the transducer, and the excess tendon was cut away. In each individual experiment the position of the transducer was adjusted relative to the muscle bundle so that the resultant geometry of recording would produce the maximum sensitivity of measurement for that muscle bundle. In order to compare different muscle bundles with different intrinsic geometries, a standard test procedure was adopted which will be described in the results section. Each muscle bundle was then stretched until a resting tension of 2 g was produced. No attempt was made to determine the physiological "resting length" for each muscle bundle. In preliminary experiments it had been determined that maximum potassium contractures were obtained with about 2 g of resting tension on the muscle bundles. Furthermore, any variation in the

“resting lengths” between the muscle preparations was compensated for by the standard test procedure which was used. Experiments were not begun until the base-line tension (2 g) remained stable for at least 15 min in standard lobster saline. Tension responses to various stimulation salines were recorded photographically and measurements were made from enlarged records of the film.

The electrophysiological experiments and the calcium influx experiments were done on muscle bundle preparations which were identical to those described above. In one study (Fig. 8), electrophysiological measurements were made on single isolated fibers in which the muscle bundle preparations had been further dissected until only one fiber was left intact. Membrane potentials were measured and intracellular currents were applied using conventional glass microelectrodes filled with 3 M KCl. Intracellular potentials were amplified by a high input impedance, negative capacity pre-amplifier and displayed on a 502A Tektronix oscilloscope. Only microelectrodes with tip potentials of 5 mv or less were used for the membrane potential measurements.

The calcium influx in the muscle fibers of the lobster was determined from the rate of entry of ^{45}Ca . The approach used was based upon similar experiments by Bianchi and Shanes (1959) on frog sartorius muscle. All the radioactive salines contained the same specific activity of ^{45}Ca (i.e. $18.3 \mu\text{c}$ per mM Ca^{++}). These were prepared by adding the ^{45}Ca to the salines from a stock of neutralized, high specific activity CaCl_2 (lot no. 9221) obtained from the New England Nuclear Corp., Boston, Mass. Muscle bundles were prepared and presoaked as described above for the tension experiments. After selecting the undamaged preparations, these muscles were then routinely soaked for 15 min in standard (non-radioactive) lobster salines. When the muscles were to undergo KCl contractures in salines containing 24 mM of external calcium, the nonradioactive standard salines were replaced by radioactive standard lobster salines and the muscles remained in the latter solutions for 15 min. At the end of this equilibration period in the radioactive standard salines, the muscles were immediately transferred to radioactive stimulation salines which contained 100 mM KCl (the KCl was added hyperosmotically to the radioactive standard saline). The muscles invariably produced contractures in the latter salines, and were kept in these salines for a total of 15 min. In addition to depolarizing the muscle fibers, the hyperosmotic KCl salines caused small transient decreases in the volumes of the muscle fibers. These decreases in volumes reached their peaks (about 7–9% smaller than the control volumes) in 2 min, and the cell volumes returned to their control (isosmotic) values in less than 15 min. An equal number of unstimulated muscles, which served as controls for these experiments, were simultaneously placed in radioactive standard salines for a total of 30 min. In all of these experiments the exposure times of the muscles to the radioactive salines were kept as short as possible, in order to minimize the effects of ^{45}Ca back flux which would reduce the estimate of calcium influx (see Bianchi and Shanes, 1959, for a discussion of this problem). 15 min was chosen as the minimum exposure time of the muscle to a given radioactive saline, since the K^+ contracture of lobster muscle often lasted this long. Furthermore, several calcium influx experiments were done in salines containing less than the standard concentrations of extracellular calcium and it took about 15 min for the extracellular spaces of these muscle bundles to equilibrate with the salines containing less than 24 mM Ca^{++} (i.e. 0.1 mM or 4.0 mM Ca^{++}).

After incubation of the muscles in the radioactive salines for the required times, the muscles were removed from the salines, blotted on Whatman no. 5 filter paper, and then were separated from their remaining exoskeletons. These muscles, which were still attached to a fragment of their central tendons, were then transferred to separate vials each of which contained 5 ml of a nonradioactive saline which was identical in composition to the last radioactive saline that the muscles had been exposed to. The muscles were soaked in this nonradioactive saline for 5 min. After two more identical washes in the nonradioactive salines (this sequence of washes for a total of 15 min removed essentially all of the extracellular ^{45}Ca from the muscle bundle), the muscles were then blotted on filter paper, separated from their tendons, and weighed on a torsion balance. The muscle weights ranged between 15–45 mg. After weighing, the muscles were transferred to individual screwcap tubes, and 0.1 N HCl was added to each tube to a final total volume of 5 ml. The tubes were tightly capped, and their contents were heated to about 90°C for 1 hr, after which the tubes were allowed to cool to room temperature. This treatment was found adequate for complete extraction of the electrolytes from the muscle. After standing for 2 weeks at room temperature, a 1 ml aliquot was transferred from each tube to a scintillation vial (done in replicate). To each vial 10 ml of a ^{45}Ca scintillation medium (Kumar, 1967) was added. The vial was tightly capped and vigorously shaken. The samples were counted in a Tri-Carb Liquid Scintillation Spectrometer (Packard, Model 3375, Packard Instrument Corp., Downers Grove, Ill.) at a standard deviation of less than 1.5%. Aliquots of each of the radioactive salines were prepared similarly and these samples were also counted for their radioactivity. All counts were corrected for quench with the aid of the external standardization mode of the spectrometer. Comparison of the measured specific activity of the saline calcium with the ^{45}Ca uptake found for a known weight of muscle, allowed for the conversion of the ^{45}Ca uptake data to moles of calcium actually transferred across the cell membrane. In order to calculate resting fluxes, the surface areas of the muscle fibers were computed from the weights of the muscles, assuming an extracellular space of 10% (Dunham and Gainer, 1962), an average cell diameter of 150 μ (Gainer and Grundfest, 1968), and a muscle fiber specific gravity of 1.06. The specific gravity of lobster muscle fibers was calculated from the known per cent fiber water (80%) of lobster muscle (Dunham and Gainer, 1962) and the assumption that the residual 20% of dry material had a specific gravity equal to that of myosin, 1.3 (Parrish and Mommaerts, 1954). The influx of calcium for the stimulated preparations was determined from the total influx during the 15 min of stimulation minus the resting influx of the controls over the same period of time.

RESULTS

Properties of Lobster Muscle Contractures

In order to compare the contractile responses of different modes of stimulation, it was first necessary to adopt a procedure which would account for the variations in muscle sizes and recording geometries between the preparations. The procedure which was used to characterize each muscle preparation is depicted in Fig. 1 (line 1). After a routine equilibration of the muscle bundle in stand-

ard lobster saline (*c*), the muscle was exposed to an isosmotic test saline (*t*) containing 50 mM K^+ (in which the product of the external K^+ and Cl^- concentration was kept constant). When the contracture in response to the *t*-saline reached its peak, the *t*-saline was immediately replaced by the *c*-saline thereby causing rapid relaxation to the original resting tension. Each

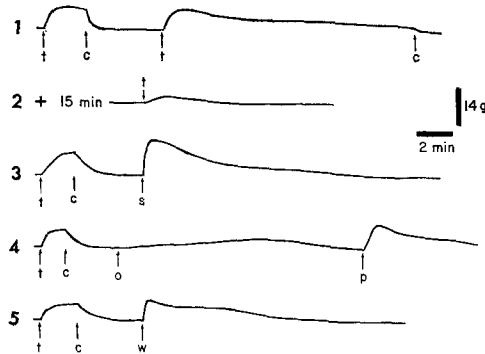


FIGURE 1. KCl and caffeine induced contractures in lobster muscle. In each experiment the muscle was first soaked for 15 min in standard 10 mM K^+ , 24 mM Ca^{++} saline (*c*), and then placed into a 50 mM K^+ (isosmotic, constant product) test saline (*t*). When the test contracture reached its peak, the *t*-saline was replaced by the *c*-saline thereby causing immediate relaxation. All subsequent contractures produced in this muscle were thereafter expressed relative to the test contracture (see Table I A): 1. After the first contracture in the *t*-saline and the return to the standard saline (*c*), the muscle was again placed into the *t*-saline. The second contracture was allowed to relax spontaneously, after which the muscle was returned to the *c*-saline. Note that the peaks of the first and second *t*-contractures were identical, which indicated that the muscle was not refractory after the initial test contracture. 2. The spontaneously relaxed muscle (in 1) was allowed to recover for 15 minutes in *c*-saline and the *t*-saline was then reapplied. The diminished response was due to the relaxation-decoupling process (see text for discussion). 3. On another muscle preparation, after a test contracture (*t*) and relaxation (*c*), the muscle was stimulated to contract by immersion in a 100 mM K^+ (hyperosmotic) saline (*s*). 4. In a fresh muscle preparation after the initial test procedure (*t-c*), the muscle was placed into a chloride-free (10 mM K^+ , isosmotic) saline (*o*). Note the slow chloride withdrawal contracture. When relaxation was complete the muscle was placed into a 100 mM K^+ (zero chloride, hyperosmotic) saline (*p*). 5. In a new muscle preparation, after the initial test procedure (*t-c*), the muscle was stimulated by a standard saline (10 mM K^+ , 24 mM Ca^{++}) containing 5 mM caffeine (*w*). The complete quantitative results of these experiments are presented in Table I.

muscle bundle was characterized by this test procedure and all subsequent responses of a particular muscle bundle to various experimental treatments were recorded relative to the tension developed in the *t*-saline for that same muscle bundle. In this way the responses of different muscle preparations to various types of stimulation could be compared. Muscles which underwent this test procedure showed no refractoriness or changes in peak tension re-

sponses to further stimulation. This can be seen in Fig. 1 (line 1) where the muscle, after recovery from the test stimulus, was again placed into *t*-saline. The second contracture was allowed to relax spontaneously, after which the muscle was returned to the *c*-saline. Since the peak tensions of the two *t*-contractures were the same, it appeared safe to conclude that there were no detrimental effects of the test procedure on the muscle's ability to develop tension. However, when the muscle was allowed to relax spontaneously in the *t*-saline (second contracture in Fig. 1, line 1), it did become refractory to further stimulation (line 2, Fig. 1). This diminished response persisted despite a 15 min soak in the recovery saline (*c*). This refractory period of potassium contractures did not occur with the test procedure used in this study, since the muscles were always placed into the *c*-saline as soon as they reached peak

TABLE I
POTASSIUM, CHLORIDE WITHDRAWAL, AND CAFFEINE CONTRACTURES

Conditions	Number of muscles	Relative tension (Mean \pm SEM)	P*
A. Potassium contractures			
100 mM K_o^+ (isosmotic, constant product)	5	1.59 \pm 0.16	—
100 mM K_o^+ (hyperosmotic KCl)	8	1.65 \pm 0.06	0.217
100 mM K_o^+ (hyperosmotic, zero Cl_o^-)	4	1.52 \pm 0.07	0.452
B. Chloride-withdrawal contractures			
10 mM K_o^+ (isosmotic, zero Cl_o^-)	4	0.43 \pm 0.09	0.008
C. Caffeine (5 mM) contractures			
Control (10 mM K_o^+ , 24 mM Ca^{++})	4	1.17 \pm 0.23	—
Relaxation decoupled (10 mM K_o^+ , 24 mM Ca^{++})	4	1.31 \pm 0.17	0.443

* P was determined by the Mann-Whitney U test. The significance level (α) equaled 0.05 (Siegel, 1956).

tension in the *t*-saline, and consequently spontaneous relaxation never took place.

Similar refractory periods following potassium contractures which were dependent upon spontaneous relaxation have been reported for crayfish muscle fibers (Zachar and Zacharova, 1966) and frog muscle fibers (Hodgkin and Horowicz, 1960). This refractoriness was not due to changes in the membrane potential, since the depolarizations during the conditioning and test contractures were essentially the same (Zachar and Zacharova, 1966; and confirmed in the present report on lobster muscle fibers). The refractoriness is also not due to any deficit in the contractile mechanism since the caffeine contractures induced in such relaxation-decoupled preparations did not significantly differ from untreated control preparations (Table I C; Fig. 1, line 5; also Foulks et al., 1965). It appears that this refractoriness is related to the excitation-contraction coupling mechanism. Various hypotheses have

been presented in the literature to explain this decoupling phenomenon (Zachar and Zacharova, 1966; Hodgkin and Horowicz, 1960).

Fig. 2 presents the relation between contracture tension and membrane potential in lobster muscle fibers (filled circles). The results are similar to those found in other muscle systems (Hodgkin and Horowicz, 1960; Orkand, 1962; Hoyle and Smyth, 1963; Edwards et al., 1964; Zachar and Zacharova, 1966), in which a sigmoid relationship between membrane potential and tension

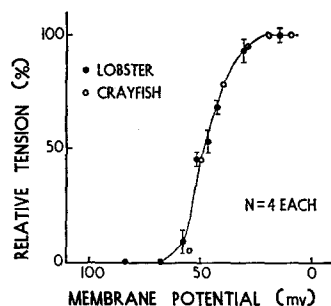


FIGURE 2

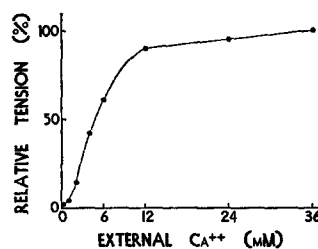


FIGURE 3

FIGURE 2. Correlation of relative (peak) tension with membrane potential. The membrane potentials were instantaneously reduced by placing the muscles in salines containing various increases in K^+ concentration (isosmotic, constant product). For lobster muscle bundles the mean of four experiments (filled circles) \pm SEM (vertical bars) is plotted for each level of membrane potential (mv). The peak contracture in the most depolarized condition (-15 mv) was arbitrarily set at 100%. All the other peak tensions were then calculated relative to the -15 mv value. Results from similar experiments on crayfish single muscle fibers (open circles) as reported by Zachar and Zacharova (1966) are plotted for comparison with the data from lobster muscle bundles.

FIGURE 3. Correlation of the relative (peak) tension with the calcium content of the medium. Each point represents an experiment on a separate muscle bundle from the same lobster. Before producing the 100 mM K^+ (hyperosmotic) contractures at a given external Ca^{++} concentration, the muscles were first soaked in 10 mM K^+ salines for 15 min at the specific levels of external Ca^{++} to be used in the stimulation salines. The peak contracture tension at 36 mM Ca^{++} was arbitrarily set equal to 100%. All the other peak tension values were then calculated relative to the 36 mM Ca^{++} value.

was found, and where no tension was recorded until the membrane potential reached a level of about -60 mv. Crayfish muscle fiber data, which were obtained under comparable conditions, are included in Fig. 2 (open circles) for comparison with the lobster muscle data. The average maximum potassium contracture tension obtained in this study with lobster muscle was equal to 6.2 kg/cm², whereas in frog muscle (Hodgkin and Horowicz, 1960) and crayfish muscle (Zachar and Zacharova, 1966) the maximum tensions obtained were 3.6 kg/cm² and 8.2 kg/cm², respectively. The data from all three species were taken from muscles that had been induced to contract in high

potassium salines in which the product of $[K^+]_o \times [Cl^-]_o$ was maintained constant.

The contractile response of lobster muscle to the removal of chloride from the external saline appears to be similar to the chloride-withdrawal contractures reported for frog toe muscles (Foulks et al., 1965). This contracture (Fig. 1, line 4) which was generated by the Cl^- free saline (*o*-saline) was out of phase with the rapid membrane potential changes which took place in this muscle. The membrane potential rapidly decreased to about -60 mv and then repolarized to the original resting potential in less than 5 min (Gainer and Grundfest, 1968). The chloride-withdrawal contracture, on the other hand, reaches peak tension about 8 min after stimulation in the Cl^- free saline (Fig. 1, line 4). Thus, the peak tension occurs approximately 8 min after the peak of depolarization, which is very different from the time course of tension responses observed in the muscles depolarized instantaneously by high potassium salines (Fig. 1). Similar results have been found for frog toe muscles (Foulks et al., 1965), where 48% of the maximum potassium contracture tension could be obtained by chloride-withdrawal contractures. In lobster muscle the average response to chloride-withdrawal was about 28% of the maximum potassium contracture tension (Table I B).

The results of the experiments presented in Table I A indicate that the magnitudes of chloride fluxes do not directly control the sizes of potassium contractures in lobster muscle. The muscles were stimulated to contracture by three different types of saline containing 100 mM K^+ . The first high K^+ saline was isosmotic and the product of $[K^+]_o \times [Cl^-]_o$ was kept constant with reference to the saline bathing the resting cells, so that only a small KCl flux would occur during the rapid depolarization under these conditions. The second high K^+ saline contained hyperosmotic KCl , so that the KCl influx during depolarization would be maximal. The third high K^+ saline, which was chloride-free contained hyperosmotic potassium propionate, and was applied to preparations that had been equilibrated in chloride-free standard lobster salines, so that the absolute chloride fluxes during depolarization equalled zero. Each of the salines produced the identical equilibrium membrane potential (i.e. about -28 mv). As can be seen from the data in Table I A, there were no statistically significant differences between the relative tensions developed by these independent modes of potassium contractures. Similar findings have been reported for crayfish muscle fibers at physiological (13.5 mM) calcium levels (Zachar and Zacharova, 1966). At calcium levels less than 13.5 mM, Reuben et al. (1967) were able to demonstrate a significant effect of external chloride on contraction.

Effects of External Calcium Levels on Potassium Contractures

Fig. 3 shows the relationship between evoked tension of lobster muscle and the calcium content of the medium. The tension developed in response to 100

mm K^+ (hyperosmotic) salines increased with increasing external calcium ion levels. "Physiological" lobster saline contains 24 mm Ca^{++} , and hence the lobster muscle would under normal conditions be contracting close to maximum.

The effects of low calcium treatment on lobster muscle contractures were further examined by the various types of experiments described in Fig. 4.

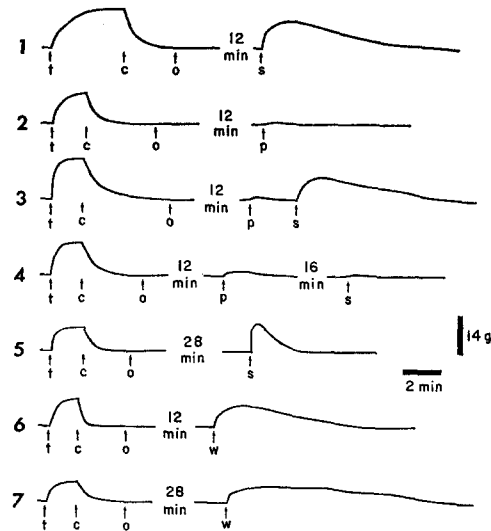


FIGURE 4. The effects of low calcium on lobster muscle contractures. Each experiment was done on a separate muscle bundle which was first characterized by the test contracture procedure (*t-c*). After complete recovery from the test contractures, the muscles were placed in a low calcium (10 mm K^+ , 0.1 mm Ca^{++}) saline (*o*) for either 12 or 28 min. Following this treatment the muscles were stimulated by various combinations of salines: 1. 100 mm K^+ , 24 mm Ca^{++} (hyperosmotic) saline (*s*). 2. 100 mm K^+ , 0.1 mm Ca^{++} (hyperosmotic) saline (*p*). 3. *p*-saline for 2 min, followed by *s*-saline. 4. *p*-saline for 16 min, followed by *s*-saline. 5. *s*-saline (after 28 min in *o*-saline). 6. 10 mm K^+ , 24 mm Ca^{++} saline containing 5 mm caffeine (*w*). 7. *w*-saline (after 28 min in *o*-saline). See Figs. 5 and 6 and text for further description and discussion of these and other experiments in low calcium.

In these experiments, the muscle preparations were first characterized by the usual test procedure, and then were placed into a standard 10 mm K^+ saline which contained a low (0.1 mm) concentration of Ca^{++} . The fibers were bathed in this saline for either 12 or 28 min, and then were subjected to various combinations of specific stimulation salines. The various experimental procedures are shown in Fig. 4, and the results of these and other similar experiments are presented in Figs. 5–8. It can be seen in Fig. 4 (lines 1 and 2) that, after pretreatment of the muscle fibers with low Ca^{++} saline, there was a large reduction in the evoked potassium contracture. The evoked contracture was

considerably larger if the stimulation saline contained a high Ca^{++} level (line 1, Fig. 4), but even in this case there was substantial depression of the tension developed. Reduction of the potassium contractures in the lobster muscles was not due to depolarizing effects of the low Ca^{++} salines on the resting membrane potentials of the muscle cells as has been found for other muscle systems (Luttgau, 1963). The data in Table II show that 2 hr of soaking the lobster muscles in salines which were severely altered in pH (Table II A) or which contained zero calcium (Table II B) did not change the membrane potentials of these cells from control preparations. Even the addition of EDTA (2 mM) to the zero calcium salines did not cause depolarization after 2 hr of incubation (Table II B). Similar findings have been reported for crayfish muscle fibers (Zacharova and Zachar, 1967).

TABLE II
EFFECTS OF pH AND CALCIUM ION ON THE RESTING POTENTIAL
OF LOBSTER MUSCLE

Condition*	Number of cells	Resting potential	Potential
		(Mean \pm SEM)	difference of means from paired control
		mv	mv
A. pH changes			
pH = 8.0	10	81 \pm 0.94	0
pH = 10.7	10	81 \pm 1.27	0
pH = 3.7	10	81 \pm 1.77	0
B. Low calcium			
Calcium free	20	82 \pm 1.09	0
Calcium free plus 2 mM EDTA	20	80 \pm 0.78	0

* The muscle fibers were soaked for 2 hr in each condition and were then compared to an equal number of control muscle fibers which were soaked for 2 hr in standard lobster saline (24 mM Ca^{++} , pH = 7.4).

Fig. 5 A presents the results of experiments in which muscles were soaked for varying lengths of time in low (0.1 mM) calcium salines and were then exposed to 100 mM K^+ salines which contained either 24 mM (filled circles) or 0.1 mM (open circles) of calcium. The resultant evoked tensions were plotted in Fig. 5 A against the soaking times in the low calcium salines. Similar experimental procedures were followed for Fig. 5 B except that the stimulation salines contained 5 mM caffeine instead of the 100 mM K^+ . The four different experiments described in Fig. 5 all showed decreased tension with increased times of soaking in low Ca^{++} salines. However, they differed considerably in their kinetics. The potassium contractures declined much more rapidly than the caffeine contractures. It is of interest to note that the potassium contractures induced in the presence of 24 mM Ca^{++} (filled circles) were diminished at a lower rate than the potassium contractures produced in the low calcium (open

circles) salines. The rates of decline of the caffeine contractures showed no dependency on the Ca^{++} levels of the stimulation salines, although the initial caffeine contractures in low Ca^{++} were about 25% less than the caffeine contractures evoked in standard lobster saline (24 mM Ca^{++}). Frank (1960) found in frog muscle that caffeine can produce undiminished contractures after degrees of calcium depletion which substantially depress potassium contractures. Frank's interpretation of these data was that caffeine contractures involve the mobilization of calcium from sites which are deep in the fiber and

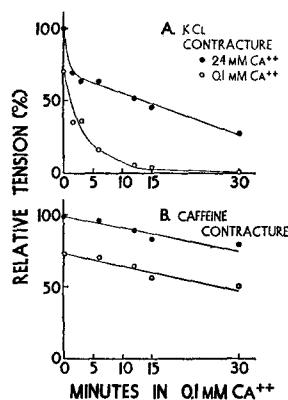


FIGURE 5

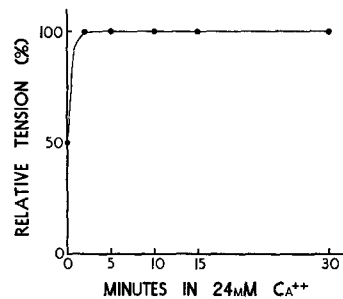


FIGURE 6

FIGURE 5. Effects of soaking the muscles in low (0.1 mM) calcium on KCl and caffeine contractures. *A.* KCl contractures. Muscles were first soaked in standard lobster salines (10 mM K^+ , 24 mM Ca^{++}) for 15 min, and were then placed into low calcium saline (10 mM K^+ , 0.1 mM Ca^{++}) for various lengths of time, after which they were immersed into stimulation salines containing either 100 mM K^+ , 24 mM Ca^{++} (filled circles) or 100 mM K^+ , 0.1 mM Ca^{++} (open circles). The values for the peak tensions of the KCl contractures were expressed as relative to the tension produced in the muscle by the 100 mM K^+ , 24 mM Ca^{++} saline, in which the muscle had not been soaked in the low calcium saline (at 0 min in the figure, filled circle). *B.* Caffeine contractures. The experimental procedure was identical to that described in *A* except that the stimulation salines used were either 5 mM caffeine in low calcium (10 mM K^+ , 0.1 mM Ca^{++}) saline (open circles) or 5 mM caffeine in standard (10 mM K^+ , 24 mM Ca^{++}) lobster saline (filled circles). The values for all the peak tensions of the caffeine contractures were expressed as relative to the tension produced in the muscle by the 5 mM caffeine (10 mM K^+ , 24 mM Ca^{++}) saline, in which the muscle had not been soaked in the low calcium saline (filled circle at 0 min). See text for discussion.

FIGURE 6. Recovery from low calcium salines. Each point represents an experiment on an individual muscle preparation from the same lobster. Each muscle had been soaked for 15 min in low calcium saline (10 mM K^+ , 0.1 mM Ca^{++}). After this 15 min treatment, the muscles were placed in standard lobster salines (10 mM K^+ , 24 mM Ca^{++}) for varying lengths of time, and then were immersed into 100 mM K^+ , 24 mM Ca^{++} (hyperosmotic) salines. All the tension values were plotted relative to the 30 min value (100%). Note the rapid recovery of tension in this experiment, in contrast to the slow decay of tension in the reverse experiment shown in Fig. 5 *A* (filled circles).

therefore would be depleted more slowly than the potassium contracture sites which are superficial. This two-site model also seems applicable to the lobster muscle fiber data presented in Fig. 5. After 15 min of soaking in 0.1 mM Ca^{++} , the contractile response to the 100 mM K^+ , 0.1 mM Ca^{++} saline was almost completely abolished (Fig. 5 A, open circles), while the response to the 5 mM caffeine, 0.1 mM Ca^{++} saline was still about 55% of the value found before calcium depletion (Fig. 5 B, open circles). Since under this condition the deep (caffeine) sites which were still well loaded with Ca^{++} could not be mobilized by high potassium saline, it would appear that the superficial sites only were mobilized by the potassium contractures. Furthermore, stimulation under the same conditions with the 100 mM K^+ , 24 mM Ca^{++} saline produced almost 50% of the tension measured before calcium depletion (Fig. 5 A, filled circles). This rapid partial recovery of the potassium contracture suggests that the depleted superficial sites were able to reabsorb the Ca^{++} rapidly from the external medium into a readily mobilizable form. The increase in tension in 24 mM Ca^{++} in Fig. 5 B (filled circles) indicates that about 25% of the caffeine contracture tension can also be attributed to superficial sites. The continuous and rapid decline with time of the potassium contractures in 24 mM Ca^{++} (Fig. 5 A, filled circles) when compared to the trivial reductions in tension of the comparable caffeine contractures (Fig. 5 B, filled circles); indicates that with prolonged soaking in the low calcium salines the superficial sites become more refractory to recovery.

Evidence for the rapid recovery of calcium depleted fibers is shown in Fig. 6. Each muscle preparation was soaked for 15 min in low (0.1 mM) Ca^{++} saline. After this treatment, the muscles were placed in standard lobster salines (24 mM Ca^{++}) for various lengths of time, and then were immersed into 100 mM K^+ , 24 mM Ca^{++} salines. The muscle which was stimulated to produce a potassium contracture without any time for recovery in the 24 mM Ca^{++} saline (at 0 min), responded with a tension that was 50% maximal. 2 min of soaking in normal Ca^{++} saline was sufficient to return the contracture response to 100% maximal. Hence the recovery process was much faster than the depletion process examined in Fig. 5 A. Since the total potassium contracture is often 15 min long, it was of interest to consider why the potassium contracture in this experiment at zero time did not reach 100% tension. In this case too, the high potassium saline contained 24 mM Ca^{++} which should have had sufficient time (i.e. 2 min) to completely occupy the superficial sites and therefore to induce the maximal contracture. Obviously the simultaneous addition of the high K^+ and the Ca^{++} inhibited the "loading" of the Ca^{++} on the superficial sites and therefore reduced the contractile response. This possibility that the high K^+ in the saline was antagonistic to the Ca^{++} loading of the superficial sites was investigated and the results are presented in Fig. 7.

In the experiments depicted in Fig. 7, the muscles were first soaked in low

calcium salines (10 mM K^+ , 0.1 mM Ca^{++}) for 15 min, and then were placed into 100 mM K^+ , 0.1 mM Ca^{++} (prestimulation) salines. The mean contractile response of the muscles due to this first stimulation is shown in the figure (filled square) at 0 min. After various times of soaking in the 100 mM K^+ , 0.1 mM

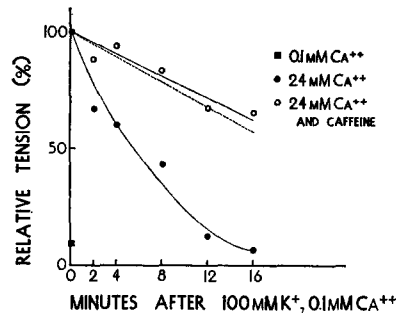


FIGURE 7

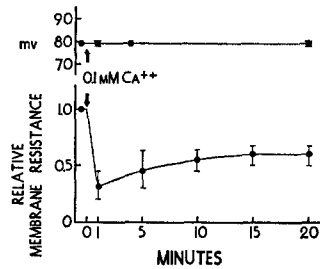


FIGURE 8

FIGURE 7. Contracture tensions produced by the addition of Ca^{++} (24 mM) or of Ca^{++} (24 mM) and caffeine to solutions containing 100 mM K^+ immediately after exposure of the muscles to solutions containing 100 mM K^+ and 0.1 mM Ca^{++} for various lengths of time. In these experiments the muscles were first soaked in low calcium saline (10 mM K^+ , 0.1 mM Ca^{++}) for 15 min, and were then placed into 100 mM K^+ , 0.1 mM Ca^{++} salines. The mean contractile response of the muscles due to the first stimulation is shown in the figure (filled square) at 0 min. After various times of soaking in the 100 mM K^+ , 0.1 mM Ca^{++} saline, the muscles were immersed into either 100 mM K^+ , 24 mM Ca^{++} salines (filled circles), or salines containing 5 mM caffeine, 100 mM K^+ , and 24 mM Ca^{++} . All the peak tensions were expressed relative to the tension produced by the muscle preparation that was stimulated by the 100 mM K^+ , 24 mM Ca^{++} saline, and which (after 15 min in 10 mM K^+ , 0.1 mM Ca^{++} saline) had undergone no prestimulation in the low calcium saline (filled circle at 0 min). Note the difference in the kinetics of the relative tension reduction with time between the KCl and caffeine contractures. The broken line represents the expected drop in tension for the KCl contracture due to prolonged soaking in 0.1 mM Ca^{++} alone (without the prestimulation). The broken line was calculated from the 15–30 min data in Fig. 5 A (filled circles). The difference in kinetics between the broken line and the solid line (filled circles) indicates that the prestimulation in low Ca^{++} causes the muscle fibers to become refractory to further stimulation by 100 mM K^+ , 24 mM Ca^{++} salines. See text for discussion.

FIGURE 8. The effects of low calcium on membrane potentials and resistances of lobster muscle fibers. Individual isolated muscle fibers were soaked for 15 min in standard lobster saline (10 mM K^+ , 24 mM Ca^{++}), and their resting membrane potentials and effective membrane resistances were measured. The standard salines were then replaced by 10 mM K^+ , 0.1 mM Ca^{++} salines and the electrophysiological measurements were continued for 20 min under these conditions. The results are plotted as means of five individual fibers (filled circles) and the vertical bars represent the ranges of variation. Where the vertical bar is absent (upper graph), it was because all five cells had the identical resting membrane potential. The effective membrane resistances were plotted relative to the initial values in standard lobster saline (10 mM K^+ , 24 mM Ca^{++}) which were set equal to 1.0. Note that the membrane potential remained unchanged while the membrane resistance underwent large transient and steady-state reductions.

Ca^{++} salines the muscles were immersed into either 100 mM K^+ , 24 mM Ca^{++} salines (filled circles), or salines containing 5 mM caffeine, 100 mM K^+ , and 24 mM Ca^{++} (open circles). All the peak tensions were expressed relative to the tension produced by the muscle preparations that was stimulated by the 100 mM K^+ , 24 mM Ca^{++} saline, and which (after 15 min in 10 mM K^+ , 0.1 mM Ca^{++} saline) had undergone no prestimulation in the low calcium saline (filled circle at 0 min). There was a marked difference in the kinetics of the relative tension reduction with time between the KCl and caffeine contractions. The broken line represents the expected drop in tension for the KCl contraction due to prolonged soaking in 0.1 mM Ca^{++} alone (without the prestimulation). The broken line was calculated from the 15–30 min data in Fig. 5 A (filled circles). The difference in kinetics between the broken line and the solid line (filled circles) demonstrated that the prestimulation in low Ca^{++} causes the muscle fibers to become refractory to further stimulation by 100 mM K^+ , 24 mM Ca^{++} salines. It was not possible from these data to determine the mechanism of the refractoriness produced by the high K^+ . One possible explanation is that the depolarization of the fibers by the increased external K^+ triggered a slow inactivation of the Ca^{++} release mechanism. Another explanation might be that there was a competition between the K^+ and Ca^{++} for the superficial sites and that it required a long time (greater than 16 min) for the K^+ to completely occupy these sites. Regardless of the specific mechanism underlying the potassium induced refractoriness, the caffeine experiments demonstrated that the decrease in the evoked tension was not due to a depletion of internal Ca^{++} from the fibers; but rather from a decreased efficiency of superficial sites which were involved in the mobilization of extracellular calcium.

The effects of the low calcium salines on membrane potentials and resistances of lobster muscle fibers are shown in Fig. 8. As was noted earlier (Table II B), there were no effects on the membrane potentials in response to placing the fibers into low (0.1 mM) Ca^{++} salines. However, there was an immediate and large decrease in effective membrane resistance which slowly (over 15 min) rose to a level about one-half the initial value in the standard lobster saline. While the decrease in effective membrane resistance was typical for muscle fibers of other species when placed in low calcium salines, the slow transient rise in resistance was intriguing. It is of interest to note that the time course of the transient resistance increase in Fig. 8 was similar to the time course of Ca^{++} depletion of the superficial sites shown in Fig. 5 A (open circles). From these data one might speculate that the slow loss of Ca^{++} from the superficial sites was forcing a membrane reorganization which was reflected in the effective membrane resistance. Since both the plasma membrane and the membranes of the transverse tubular system are known to contribute to the effective membrane resistance of muscle fibers (Falk and

Fatt, 1964), it is then feasible that the superficial sites are located in one or both of these membrane systems.

Effects of Various Divalent Cation Substitutes for Calcium

It has been demonstrated for frog toe muscle that divalent cations as diverse as Cd, Co, Be, Mg, Sr, Ni, Mn, or Zn, when they were added to calcium free salines, were all able to support potassium contractures with varying degrees of effectiveness (Frank, 1962; and Lorkovic, 1967). This suggested that the requirement for calcium at the superficial sites was relatively nonspecific in

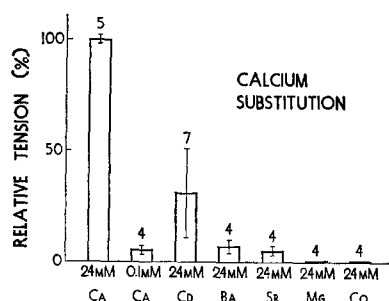


FIGURE 9. The effects of various calcium substitutes on KCl contractures in lobster muscle. The muscle preparations were first soaked for 15 min in 10 mM K^+ salines containing a given concentration of the specific divalent cations. The muscles were then placed into 100 mM K^+ (hyperosmotic) salines which contained the same concentration of divalent cation as the conditioning solution. The heights of the vertical columns represent the mean values of the resultant contractures, each value being expressed relative to the peak tension under standard conditions (24 mM Ca^{++}) which was arbitrarily set at 100%. The number of muscles studied for each condition is shown above the appropriate vertical column, with the S.E.m. of the values represented by the vertical bar immediately beneath the number.

frog muscle, despite the significant quantitative differences found between the different divalent ions (Lorkovic, 1967).

The effects of substituting various selected foreign divalent cations for calcium on potassium contractures in lobster muscle is presented in Fig. 9. In contrast to frog muscle, the excitation-contraction coupling process in lobster muscle appeared to be highly selective for calcium. Potassium contractures in calcium-free salines containing Ba^{++} or Sr^{++} showed no significant increase in tension over low (0.1 mM) Ca^{++} salines, whereas the salines containing Mg^{++} and the Co^{++} as substitutes for calcium produced no tension at all. The only foreign cation which showed any degree of effectiveness as a Ca^{++} substitute was Cd^{++} . However, there was large variation in the data obtained with Cd^{++} (Fig. 9), and the evoked tension ranged between 0–80% of the tension obtained with 24 mM Ca^{++} . The reason for the unique position of Cd^{++} is not clear at present, but in this context it is interesting that Frank

(1962) also found that Cd^{++} was the most effective substitute cation in frog muscle.

Fig. 10 presents the effects of the various divalent cations on the kinetics of depolarization of lobster muscles in response to the addition of hyperosmotic 100 mM K^+ salines. The various cation substitutes (including the low Ca^{++}

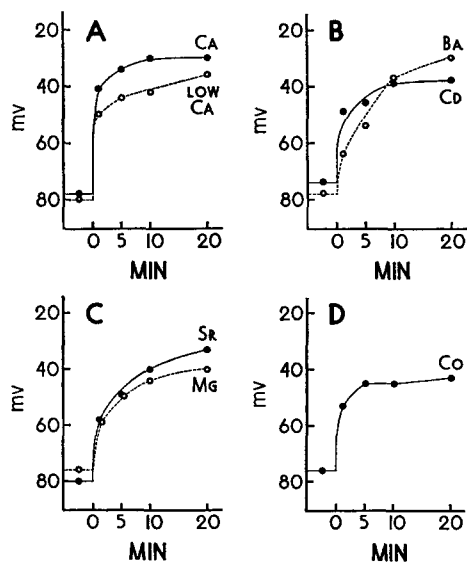


FIGURE 10. The effects of various calcium substitutes on the kinetics of depolarization of lobster muscle fibers by the addition of hyperosmotic KCl . In each experiment the muscle preparation was first soaked for 15 min in a 10 mM K^+ saline containing a given concentration of the specific divalent cation. The membrane potentials of five fibers were measured and (at 0 min) the muscle was placed into a 100 mM K^+ (hyperosmotic) saline which contained the same concentration of divalent cation as the conditioning saline. Membrane potentials of the muscle fibers were measured in this new saline for 20 min. Each point represents the mean value of five fibers. The S.E.m. of all the measurements ranged from 0.58–1.89 mv. *A*. Experiments in standard 24 mM Ca^{++} saline (solid line, filled circles) and low (0.1 mM) Ca^{++} saline (broken line, open circles). *B*. Experiments in 24 mM Ba^{++} saline (broken line, open circles) and 24 mM Cd^{++} saline (solid line, filled circles). *C*. Experiment in 24 mM Sr^{++} saline (solid line, filled circles) and 24 mM Mg^{++} saline (broken line, open circles). *D*. Experiment in 24 mM Co^{++} saline (solid line, filled circles).

salines) tended to decrease the rates of depolarization and in some cases the total level of depolarization (Cd^{++} , Co^{++} , and Mg^{++}). However, these effects alone could not have produced the low tension responses obtained in Fig. 9 (see Fig. 2 for expected tensions at these depolarization levels). Therefore, it was the coupling mechanism which was deficient in the absence of calcium, and not the excitation process. The decreased rate of depolarization in low Ca^{++} salines (Fig. 10 *A*) was also observed by Zacharova and Zachar (1967)

in crayfish muscle fibers. These workers proposed that depolarization of the transverse tubular membranes caused an increased membrane permeability to Ca^{++} , and the resultant influx of Ca^{++} contributed to the depolarization of the muscle fiber and thereby increased the depolarization rate. Therefore, lowering the external Ca^{++} concentration of lobster saline from 24 mM to 0.1 mM would decrease the equilibrium potential of Ca^{++} across the tubular membranes by about 60 mv, and which therefore would decrease the rate of depolarization due to the decreased Ca^{++} current. If this interpretation is correct then it would seem that the activation of contraction in lobster muscle fibers is produced by a massive influx of extracellular calcium. That potassium contractures in lobster muscle are exclusively dependent on superficial sites which are in rapid equilibrium with extracellular calcium was demonstrated by the experiments described in Figs. 5 and 6. The muscle fibers of frog heart and rectus and barnacle muscle also appear to be dependent upon a substantial calcium influx in their excitation-contraction coupling mechanisms (Edwards and Lorkovic, 1967; Ashley, 1967).

Influx of Calcium During Potassium Contractures

The calcium influx in the muscle fibers of the lobster was determined from the rate of entry of ^{45}Ca . In these experiments the exposure times of the muscles to the radioactive salines were kept as short as possible in order to minimize errors due to ^{45}Ca back flux, and the ^{45}Ca content of the control and experimental muscles were measured after a 15 min washout period which insured that the only radioactive Ca^{++} measured was intracellular (for a discussion of this approach see the methods section of this paper; Bianchi and Shanes, 1959; Edwards and Lorkovic, 1967). No corrections were made in this study for the loss of intracellular ^{45}Ca during the 15 min washout period, so the data to be presented probably represent underestimates of the absolute ^{45}Ca influx.

The results of various calcium influx experiments on resting and stimulated lobster muscle fibers are summarized in Table III. The resting calcium influx of lobster muscle in physiological (24 mM Ca^{++}) saline (Table III A) was about 100 times greater than that found for frog muscle. Bianchi and Shanes (1959) found that the resting calcium influx in frog muscle was equal to 0.05×10^{-12} mole/cm²-sec. Even in salines with external calcium concentrations (0.1 mM Ca^{++}) one-twentieth that of frog Ringer's, the lobster muscle's resting calcium influx was some 3-4 times greater than for frog muscle. In addition to this large resting influx of calcium in lobster muscle, there was a massive influx of calcium found associated with the potassium contractures (Table III B). In the frog (Bianchi and Shanes, 1959), the influx of calcium per potassium contracture equalled 13 $\mu\text{M}/\text{kg}$ of muscle. The calcium influx in lobster muscle fibers stimulated to contract in low (0.1 mM) Ca^{++} salines (which produced about 3% of maximum contracture tension) equalled 8.8 $\mu\text{M}/\text{kg}$ of

muscle. However, under conditions that produced larger tensions, the calcium influx in lobster muscle equaled 698 $\mu\text{M}/\text{kg}$ (in 4 mM Ca^{++} salines) and 3590 $\mu\text{M}/\text{kg}$ (in 24 mM Ca^{++} salines). The relative tensions developed in 100 mM K^+ , 4 mM Ca^{++} salines and in 100 mM K^+ , 24 mM Ca^{++} salines were about 40

TABLE III
CALCIUM INFLUX IN LOBSTER MUSCLE

Experi- ment number	Conditions*	Number of muscles	Influx†	
			Mean	(Range)
A. Resting influx			10^{-12} mole/cm ² -sec	
1	0.1 mM Ca^{++} saline	5	0.184	(0.129-0.236)
2	4.0 mM Ca^{++} saline	5	1.74	(1.29-2.32)
3	24.0 mM Ca^{++} saline	5	8.55	(5.90-10.85)
B. Stimulated influx (100 mM K^+ contracture—15 min)			10^{-6} mole/kg—contracture	
4	0.1 mM Ca^{++} saline	5	8.80	(7.7-19.6)
5	4.0 mM Ca^{++} saline	5	698	(343-1285)
6	24.0 mM Ca^{++} saline	5	3590	(2060-5500)
7	24.0 mM Ca^{++} saline (after 15 min in 10 mM K^+ , 0.1 mM Ca^{++})	5	980	(598-1062)
8	24.0 mM Ca^{++} saline§ (after 2 min in 100 mM K^+ , 0.1 mM Ca^{++})	5	945	(739-1196)
9	24.0 mM Ca^{++} saline§ (after 15 min in 100 mM K^+ , 0.1 mM Ca^{++})	5	522	(265-945)

* For the resting influx, measurements were made on muscle bundles that were soaked for 30 min in the appropriate ⁴⁵Ca-labeled salines. In the stimulated influx experiments numbered 4-6, the muscles were first soaked for 15 min in a ⁴⁵Ca saline (10 mM K^+) which contained the same Ca^{++} concentration and specific activity as the stimulating solution (100 mM K^+). Experiments numbered 7-9 were first soaked in ⁴⁵Ca-labeled 10 mM K^+ , 0.1 mM Ca^{++} salines for 15 min before stimulation in salines of equal specific activity.

† The influxes of calcium for the stimulated preparations were determined from the total influxes during the 15 min of stimulation minus the resting influxes expected for 15 min determined from experiments numbered 1-3. The surface areas of the muscle fibers were calculated from the weights of the muscle bundles, assuming an extracellular space of 10% (Dunham and Gainer, 1962), an average cell diameter of 150 μ (Gainer and Grundfest, 1968), and a muscle fiber specific gravity of 1.06. The stimulated influxes of calcium are expressed in 10^{-6} mole/kg muscle per 15 min contracture period.

§ In experiment 8 the muscle bundles were presoaked in 10 mM K^+ , 0.1 mM Ca^{++} saline labeled with ⁴⁵Ca, for 15 min and then placed in 100 mM K^+ , 0.1 mM Ca^{++} for 2 min followed by 100 mM K^+ , 24 mM Ca^{++} for 13 min. All the salines contained ⁴⁵Ca at the same specific activity. In experiment 9 the muscle bundles were presoaked for 15 min in 10 mM K^+ , 0.1 mM Ca^{++} , then soaked 15 min in 100 mM K^+ , 0.1 mM Ca^{++} , and finally 15 min in 100 mM K^+ , 24 mM Ca^{++} saline. Each saline contained an equal specific activity of ⁴⁵Ca.

and 92% of the maximum potassium contracture tension for this muscle. The results of these influx studies appear to be consistent with the hypothesis that the potassium contractures in lobster muscles are directly coupled to the influx of extracellular Ca^{++} which increases the internal Ca^{++} concentration and thereby serves to directly activate the contractile protein. Edwards and

Lorkovic (1967) presented ^{45}Ca influx data for another crustacean (barnacle) muscle which suggested that activation in this muscle was achieved, at least in part, by entry of extracellular Ca^{++} . They found that after one minute of potassium contracture, the increment in the ^{45}Ca content of barnacle muscle was about $90 \mu\text{M}/\text{kg}$. The potassium contracture time used for the lobster muscle data in Table III was 15 min, and in the physiological saline (i.e. in $24 \text{ mM } \text{Ca}^{++}$) the increment for 1 min of stimulation over this period averaged about $240 \mu\text{M}/\text{kg}$. This is most likely an underestimate for the first minute, since the first few minutes of contracture would undoubtedly produce a greater influx than the later relaxation phase of the contracture. From experiments in which Ca^{++} was directly injected into crab muscle fibers, Portzehl et al. (1964) found that an increase in internal Ca^{++} concentration equal to $0.3\text{--}1.5 \mu\text{M}$ was sufficient to reach threshold for contraction. Hasselbach (1964) calculated that a $150 \mu\text{M } \text{Ca}^{++}$ concentration inside the muscle fiber should produce maximum activation. Since the stimulated influx values in Table III *B* are well above $150 \mu\text{M}$, these data are then compatible with the view of muscle activation by the entry of extracellular calcium.

Experiments numbered 7–9 in Table III *B* were done in order to test whether the calcium influx would decrease under specific conditions in which reduced tensions were developed. Since the tensions developed in experiments 7, 8, and 9 were about 46, 40, and 5%, respectively, of that developed in experiment 6; it can be seen that the calcium influx rates shown in Table III *B* correlated well with the tension data. Furthermore, since in experiments 6–9 the final stimulation salines were identical, the increases in influx of calcium during stimulation cannot simply be attributed to increased calcium exchange diffusion rates in the high potassium salines. With respect to the possibility of an increase in exchange diffusion in high potassium salines, experiment 9 (in which only a small amount of tension was developed) could be viewed as a control. The increment in Ca^{++} influx in experiment 9 over the resting fiber (in low potassium saline) was $522 \text{ M}/\text{kg}$. This value is about one-seventh the increment in calcium influx found when the muscles produced 92% of the maximum contracture tension (experiment 6). It was not possible to quantitatively analyze these data any further, since there was no independent information available on the total relationship between the absolute internal calcium concentration and developed tension. From the data in Table III *B* one could speculate that a sigmoid relationship might exist, as in the case between membrane potential and tension (Fig. 2).

DISCUSSION

The results of the present study on lobster muscle fibers showed a typical sigmoid relation between absolute membrane potential and tension with an apparent “threshold” for developing tension at about -60 mv (Fig. 2).

Reuben et al. (1967) have pointed out that these data are not sufficient evidence to demonstrate a "critical" membrane potential threshold for tension. By using a variety of experimental conditions, they have demonstrated that the amplitude of tension is a continuous function of membrane depolarization and that under some conditions even hyperpolarization may produce tension. According to their channeled-current model, the transmembrane current and charge displacement, which are continuous functions of the membrane potential, are the relevant parameters in excitation-contraction coupling. Hence, in crayfish muscle fibers depolarization of the plasma membrane of muscle is believed to be accompanied by an influx of chloride across the transverse tubular membrane at the diadic-sarcoplasmic reticulum junction. It is proposed that this outflow of current across the tubules might lead to a local accumulation of cations (presumably Ca^{++}) at strategic intracellular sites for triggering contraction. Several experiments must be accounted for before accepting this model for potassium contractures. For example, no significant differences in evoked tensions were found when crayfish (Zachar and Zacharova, 1966) and lobster (Table I A) muscles were induced to produce potassium contractures in various salines (containing physiological concentration of Ca^{++}) which produced chloride fluxes ranging from zero to maximum values. In contrast to these data are the observations that in low calcium salines the thresholds for contraction produced in crayfish muscle fibers by intracellular currents can be markedly lowered by the presence of external chloride (Reuben et al., 1967, cf. Figs. 17 and 18). The latter data do not necessarily support the channeled-current model which specifically calls for the influx of Cl^- ions. Since potassium contractures in salines with absolutely no chloride can produce maximum tensions (Table I A), then perhaps the role of chloride is to influence the muscle binding sites for Ca^{++} rather than to act as a co-ion during the influx of Ca^{++} . A recent report on the influence of inorganic anions on the Ca^{++} binding by muscle microsomes (Carvalho, 1967) appears to support this mechanism of chloride action. The selective binding of Ca^{++} by microsomes in the presence of ATP and Mg was depressed 40–50% if KCNS or KI replaced KCl in the medium.

It is difficult at this time to come to any definite conclusions as to whether the development of tension is specifically coupled to membrane potential or to transmembrane current. Obviously this problem is confounded by the probable existence of more than one mechanism of inducing tension in muscle fibers, and ample evidence exists in the literature for multiple mechanisms. For example: (a) it is possible to selectively inhibit KCl contractures in a frog muscle and still maintain an intact twitch (Fujino and Fujino, 1964); (b) electrical and mechanical events in muscle may be dissociated experimentally (Axelson and Thesleff, 1958; Kiku-Iri, 1964); (c) caffeine and chloride-withdrawal contractures are more resistant to calcium depletion of the cell than are

KCl contractures (Foulks et al., 1965; Frank, 1960; and text of this paper with reference to Fig. 5 and Table I *B* and *C*); and (*d*) hypertonic solutions inhibit KCl contractures but not caffeine contractures (Caputo, 1966). In view of these diverse modes of stimulation that are able to cause tension in muscle, it is unlikely that any single model of excitation-contraction coupling will be able to explain all of them.

It is clear from Fig. 3 that the potassium contractures of lobster muscle are dependent upon the presence of calcium ions in the external media. This requirement for Ca^{++} is typical for arthropod muscle (Aidley, 1965; Zacharova and Zachar, 1967; Reuben et al., 1967; Edwards and Lorkovic, 1967); and the rapid recovery of maximum contractures of calcium depleted cells by the addition of calcium to the external medium indicates that a superficial site (readily available to the extracellular Ca^{++}) is involved in the coupling mechanism of lobster muscle fibers. The data in Figs. 5 *A* and 6 show that while the depletion process in lobster muscle took more than 15 min to reach completion, complete recovery from Ca^{++} depletion can occur in less than 2 min. Further evidence that a superficial site is involved in the Ca^{++} depletion experiments is demonstrated in Fig. 5 *B*. Caffeine contractures induced in Ca^{++} -depleted cells, which were unable to produce potassium contractures, were only slightly reduced. Hence it appeared that the deep (Ca^{++}) sites of Ca^{++} -depleted fibers were still loaded with calcium, but did not participate in the coupling mechanisms involved in the potassium contractures. It is interesting, however, that about 25% of the tension evoked by caffeine was also dependent upon the extracellular Ca^{++} . The suggestion by Jenden and Reger (1963) that the effect on frog sartorius muscles of low calcium salines was in part due to a fall in resting potential, was not found applicable to lobster muscle fibers (Table II *B*, and Fig. 8).

The Ca^{++} sites in lobster muscle involved in excitation-contraction coupling appear to be highly selective for calcium ions (Fig. 9), and even strontium ions which so closely approximate the roles of calcium in other systems were not effective in restoring contractility to the calcium depleted fibers. Similar findings have been reported by Van der Kloot (1965) for lobster muscle fibers. Part of the release of the calcium ions in potassium contractures may be due to a competition between the increased potassium and the calcium in the media for the site. The experiments in Fig. 7 indicated that raising the external K^+ level inhibited the mobilization (and possibly the binding) of Ca^{++} by the sites and therefore inhibited its role in excitation-contraction coupling. Competition between Ca^{++} and K^+ for binding sites has been demonstrated in fragmented sarcoplasmic reticulum isolated from rabbit skeletal muscle (Carvalho and Leo, 1967).

The implication of superficial sites for Ca^{++} release in potassium contractures raises the possibility that the depolarization produced an increased

influx of extracellular Ca^{++} which itself accounted for the activation of the lobster muscle. In experiments in which the calcium influx was determined from the rate of entry of ^{45}Ca (Table III), it was found that potassium contractures produced a massive influx of extracellular calcium. Potassium contractures that were evoked under conditions that produced 40% (in 4 mM Ca^{++} salines) and 92% (in 24 mM Ca^{++} salines) of the maximal contracture tensions for this muscle, were accompanied by Ca^{++} influxes per contracture (above the resting influx) of 698 and 3590 $\mu\text{M}/\text{kg}$, respectively. Since the estimates of the increase in intracellular calcium necessary to produce maximum activation range between 10 μM (Sandow, 1965) and 150 μM (Hasselbach, 1964), it would appear that the results of these influx studies are consistent with the hypothesis that potassium contractures in lobster muscles are produced by the influx of extracellular Ca^{++} which can directly activate the contractile mechanism. The deep and wide clefts found in crustacean muscle fibers (Peachey, 1967) would facilitate the entering extracellular calcium to reach even the deepest myofibrils in time to produce synchronous activation.

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