Intracellular Calcium Binding
and Release in Frog Heart

SAUL WINEGRAD

From the Department of Physiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19174

ABSTRACT   The capacities and affinities of intracellular calcium-binding sites have been studied in frog ventricles, in which the concentration of Ca^{++} in the sarcoplasm can be controlled as a result of treatment with EDTA. The total calcium content of calcium-depleted and nondepleted muscles at rest and muscles generating considerable tension was 0.8, 1.4, and 5.4 $\mu mol/g$ of muscle, respectively. Net movement of calcium into or out of the cells occurred without change in tension when the sarcoplasmic concentration of Ca^{++} was either of two values, less than 10^{-7} M or approximately 5×10^{-7} M. These data can be explained by the presence of two groups of intracellular calcium sinks which compete with the contractile proteins, one with a capacity of about 0.6 $\mu mol/g$ and an affinity constant greater than 10^{7} M^{-1} and a second with a capacity of 4.0 $\mu mol/g$ and an affinity constant of about 2×10^{6} M^{-1}. The higher affinity calcium is released by anoxia, oligomycin, or abrupt changes in sarcoplasmic Ca^{++}. Muscles soaked in Sr-Ringer's contain electron densities in the sarcoplasmic reticulum and to a lesser extent in the mitochondria.

The treatment of frog ventricular muscle with EDTA has been shown to increase markedly the permeability of the cells to Ca^{++} (Thomas, 1960) permitting direct and rapid control of the Ca^{++} concentration in the sarcoplasm (Winegrad, 1971). A gradual reversal of the high permeability can be initiated by a brief exposure to Ca^{++} at a concentration greater than 10^{-5} M. The relationship between the tension developed and the concentration of Ca^{++} in the sarcoplasm has been established in EDTA-treated hearts, and therefore it is possible to infer the sarcoplasm concentration of Ca^{++} at any instant from the tension (Winegrad, 1971).

The extent of the increase in permeability produced by EDTA can be varied. In the most permeable preparations the entire Ca^{++}-buffering system consisting of Ca-EGTA and Ca^{++} moves across the membrane and the sarcoplasmic pCa (-log (Ca^{++})) rapidly equilibrates with that of the bath in spite of calcium binding by intracellular structures such as the contractile proteins. When the enhancement of permeability is less, the cells are not readily per-

meable to Ca-EGTA, and the only component of the Ca^{++}-buffering system that seems to enter the cell is Ca^{++}. This conclusion has been inferred from the fact that under these conditions the rate of rise of tension but not the final level of tension can be increased by raising the concentration of Ca-EGTA and EGTA without altering the concentration of ionic Ca^{++} in the soak solution (Winegrad, 1971). Sarcoplasmic pCa responds more slowly to an alteration of the pCa of the bath because of the absence of Ca^{++} buffering, and the rate of change of intracellular pCa depends on both the flux and the rate of intracellular binding of Ca^{++}. In the following study this dependence has been used to characterize intracellular Ca^{++}-binding sites.

METHODS

Opened ventricles or strips of ventricle from medium-sized, male frogs (*Rana pipiens*) were suspended in continuously oxygenated Ringer's solution with one end fixed and the other end connected to either an Endevco 8170-02 semiconductor transducer (Endevco Div., Becton, Dickinson & Co., Pasadena, Calif.) or a Statham G10B±0.15 strain gauge transducer (Statham Instruments, Inc., Oxnard, Calif.) for continuous measurement of tension with a Brush Mark 220 or Mark 280 recorder. Before the start of each experimental procedure the muscles were stimulated at a rate of 10/min for about 1 h with changes of solution every 15 min. Throughout the experiment the suspending rods were blotted dry between changes in the bathing medium to prevent the mixing of consecutively used solutions. All experiments were performed at room temperature.

At the end of each experiment the total sodium and calcium content of each solution was measured with a Perkin Elmer 290 atomic absorption spectrometer (Perkin-Elmer Corp., Boller & Chivens Div., South Pasadena, Calif.). In solutions to which neither Na^{+} nor Ca^{++} had been added the values were consistently about 3 μM and 5 μM, respectively. Total calcium content of some ventricles was measured by drying the tissue, ashing at 500°C, dissolving the remains in 0.1 N HCl, and analyzing the final solution with the Perkin Elmer 290. Opened ventricles rather than strips were used in these experiments to increase the total mass of tissue and the ratio of undamaged to damaged tissue.

Strontium localization studies were performed in two ways. In the first, the original Somlyo and Somlyo procedure was employed (1971). Ventricles were soaked first in 140 mM KCl + 10 mM $SrCl_2$ + 10 mM Tris buffer at pH 7.0 for 45 min and then for an additional 15 min in a standard Ringer's solution without added calcium. The tissues were fixed with glutaraldehyde, postfixed with osmium tetroxide, embedded in Epon, and examined with a Hitachi HU-11 electron microscope (Hitachi Ltd., Tokyo, Japan). When it was clear that electron opacities were produced by this procedure, the experiment was performed in a way analogous to the calcium studies. Ventricular strips were exposed to EDTA for 15 min, soaked for 45 min in a solution that was 140 mM KCl, 10 mM Tris buffer at pH 7.0, 5 mM ATP, 1 mM $MgCl_2$, and 10 mM $SrCl_2$ and then exposed for an additional 15 min to the same solution with no Sr. Fixation and embedding were the same as in the first procedure.

The association constant for Ca-EGTA used to calculate ionic Ca^{++} was 4.9 $\times$ 10^5 M^{-1} at pH 6.5 (Ringbom, 1963). The EGTA was a gift of Geigy Chemical Corporation (Ardsley, N. Y.), and the other reagents were American Chemical Society Grade.

The solutions that were used are shown in Table I.

TABLE I*

SOLUTIONS USED IN EXPERIMENTAL PROCEDURES ON FROG VENTRICULAR MUSCLES

	NaCl	KCl	K_2HPO_4	KH_2PO_4	$CaCl_2$	Tris	EDTA	EGTA	$MgCl_2$	ATP	Sucrose	pH
Ringers	117	0	2.4	0.6	1.0	0	0	0	0	0	0	7.0
EDTA	0	140	0	0	0	10	3	0	0	5	0	6.5
Ca-EGTA I	0	140	0	0	0-100	10	0	3	1	5	0	6.5
Ca-EGTA II	0	140	0	0	0-2.85	10	0	3	1	5	150	6.5
Ca-EGTA III	0	290	0	0	0-1.0	10	0	3	1	5	0	6.5

* All concentrations in mM.

RESULTS

Capacity of Calcium-Binding Sites

Frog ventricles were treated with a solution containing EDTA to enhance the membrane permeability to Ca^{++} and to facilitate both the depletion and the addition of Ca^{++} to the cells. 15 muscles with a low permeability to Ca-EGTA, as indicated by the slow rise in tension in a pCa of 6.0, were chosen to avoid the direct effects of EGTA on the intracellular calcium. Once the tissues had been treated with EDTA and relatively depleted of calcium they were divided into three groups. The total calcium content of the first group was measured immediately after the treatment with EDTA (Fig. 1 Point A). The second group was pulsed briefly with high Ca^{++} to replete the binding sites with a high affinity and then returned to a bath with a very low concentration of Ca^{++}. After sufficient time for a return to resting tension, and therefore presumably for equilibration of the extracellular Ca^{++} with the new bath, the total calcium content of the second group was measured (Fig. 1 Point B). Ventricles in the third group were pulsed with high Ca^{++} twice because tension decayed very slowly after the second pulse as a result of the decrease in the permeability of the membrane to calcium, and consequently tissue calcium could be measured when tension was high but extracellular Ca^{++} very low. The amount of calcium in these muscles was measured 5 min after the second pulse, when the sarcoplasmic concentration of Ca^{++} was still very high, but the extracellular concentration was very low (Fig. 1 Point C). The biphasic rise of tension in the second pulse of high Ca^{++} that is apparent in Fig. 1 is considered below.

The calcium content of the first group, 0.8 $\pm$ 0.1 μmol/g of muscle wet
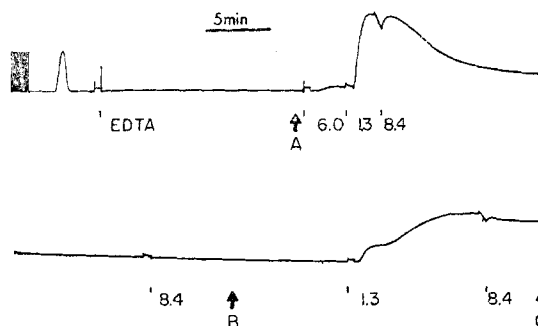
Fig. 1.   Tension record of a frog ventricle exposed to EDTA solution and Ca-EGTA solutions (Ca-EGTA I solution in Table I) with different pCa as indicated by the numbers under the records. A, B, and C indicate three different points at which experiments were terminated in order to measure total calcium of the ventricles. The prolonged artifacts on the tension record mark the changes of solution and were produced by careful blotting of the rod and the clip suspending the muscle. The control twitches at the beginning of the record are used to normalize the tension generated during the contractures as the heterogeneous orientation of the muscle fibers within the strip makes absolute values less useful. Stimulation rate during control period was 9/min. The last control twitch was recorded at 60 × paper speed. The biphasic rise in tension during the second exposure to high Ca^{++} always occurred.

weight, was thought to indicate the content of a moderately calcium-depleted muscle. The value in the second group, 1.4 ± 0.1 μmol/g, indicated an amount of calcium that the ventricles could contain without a significant increase in resting tension. The difference between the first and the second values, 0.6 μmol/g, should equal the capacity of cellular calcium-binding sites and any very slowly exchanging connective tissue sites with affinity constants greater than that of contractile proteins, but it will underestimate the true value by an amount dependent on the incompleteness of the removal of calcium during the EDTA soak. A third value, 5.4 ± 0.3 μmol/g, was measured in muscles which were still producing a large amount of tension after 5 min (over five half-times for the washout of the extracellular space) in a solution with Ca^{++} concentration of less than 10^{-8} M. The difference between the second and the third values is a measure of the capacity of calcium-binding sites within the ventricle with affinities roughly similar to that of the contractile proteins. Treatment with the EDTA solution for 15 min after either the first pulse or the second pulse lowered tissue calcium only to 1.0 ± 0.1 μmol/g and not to the original amount observed after the first exposure to EDTA.

An additional group of muscles was exposed twice to high Ca^{++} and then bathed in a pCa greater than 8.0 for 30–60 min. In these experiments the tension and the total calcium content decreased slowly, but the rates of

decline in the several muscles varied considerably. A rough correlation, however, existed between the loss of tension and of calcium.

Energy Requirements for Ca Binding

A group of EDTA-treated ventricular strips was incubated in solution that had no ATP, a pCa of 8 and had been deoxygenated by continuous bubbling of N_2. This procedure did not alter resting tension for at least 30 min even when 0.5 mM iodoacetate had been added to the bathing medium. In, however, strips that had been pulsed once with high Ca^{++} after the EDTA treatment, resting tension rose slowly over the 30 min of anoxia. Normal resting tension could generally be restored by oxygenating the EGTA solution. These data suggested that calcium added during the brief pulse could be released by blocking energy metabolism. Oligomycin had a similar effect on resting tension in well oxygenated muscles. The addition of 2 μg of oligomycin per ml of solution bathing EDTA-treated ventricles produced no change in resting tension unless the ventricles had been previously pulsed with high Ca^{++}. In the latter instances, however, resting tension rose temporarily to an average of 50% of twitch tension and then declined within 30 min (Fig. 2). This rise in tension could be completely inhibited by the addition of 5 mM phosphoenolpyruvate to the bathing solution but not by 5 mM fumarate. Although no statistically significant reduction in calcium content from treatment with oligomycin was observed, losses of up to 0.2 μmol/g of muscle could have occurred without detection because of the variability of the results.
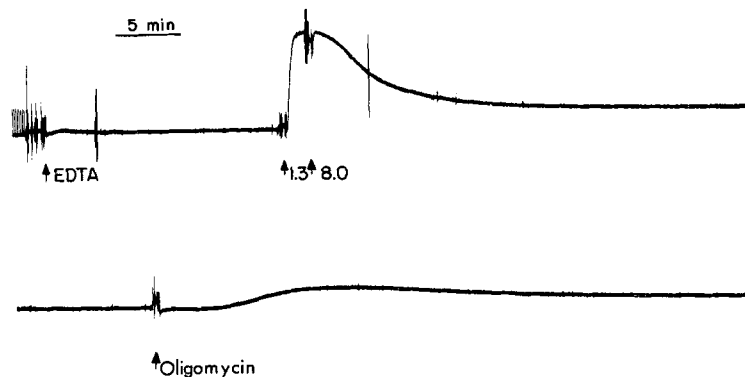

FIGURE 2. Tension of a frog ventricular strip exposed to EDTA solution and then briefly to Ca-EGTA solution containing 50 mM Ca. After a prolonged period in very high pCa, 2 μg/ml oligomycin was added to the bathing solution. Tension rose about 5 min later and after reaching a level equal to about half-twitch tension, it slowly declined (see text). No ATP was present in the oligomycin-containing solution.

Histochemical Studies

Attempts at direct localization of intracellular calcium sinks by electron microscopical examination of fixed tissues previously loaded with calcium were unsuccessful. Strontium, a more electron-opaque metal, however, is known to activate myofibrillar ATPase (Edwards et al., 1966) and to substitute for Ca^{++} in excitation-contraction coupling of heart muscle (Nayler, 1965). In vascular smooth muscle it is accumulated by the sarcoplasmic reticulum and the mitochondria (Somlyo and Somlyo, 1971).

Three frog ventricular strips were therefore exposed to a strontium-substituted, modified Ringer's solution and examined with the electron microscope to determine in which structures the strontium had been deposited. During the soak in the high K^+-Sr^+ solution tension rose and was maintained at a level approximately equal to twitch tension. In sections of these tissues electron densities were frequently found in the sarcoplasmic reticulum and to a lesser extent in the mitochondria (Fig. 3). Similar localization of electron densities was found when the tissues were exposed to EDTA before the strontium (see Methods). The electron densities in the sarcoplasmic reticulum resembled those observed in stained sections of heart muscle by Sommer and Johnson (1969) and by Page and Niedergerke (1972), but they were never seen in the unstained controls, which had not been exposed to strontium. Studies are currently in progress to try to relate the concentration of free Sr^{++} to the appearance of electron densities in the sarcoplasmic reticulum and the mitochondria.

Calcium Pulsing

The presence of the internal calcium sink inferred from the previous experiments is indicated by another type of experiment. Muscles with limited calcium permeability show two phases of rise and fall in tension when bathed in a medium of the appropriate pCa.

Ventricular strips were exposed to EDTA for only 10 min, a length of time known to have a submaximal effect on the permeability of the membrane, and the degree of permeability was verified by the response of the tissue to changes in the extracellular concentration of Ca and Ca-EGTA. In this state the muscle developed very little tension during brief exposures to contracture solutions with pCa equal to 6.0 and 5.4.

The muscle was exposed very briefly to a very high concentration of calcium, 100 mM, and then placed in a Ca^{++} concentration of less than 10^{-3} M.

FIGURE 3.   Two electron micrographs from frog ventricular strips fixed after 45 min in a solution containing 10 mM Sr^{++} and 140 mM K^+. Note densities in the sarcoplasmic reticulum and the mitochondria.
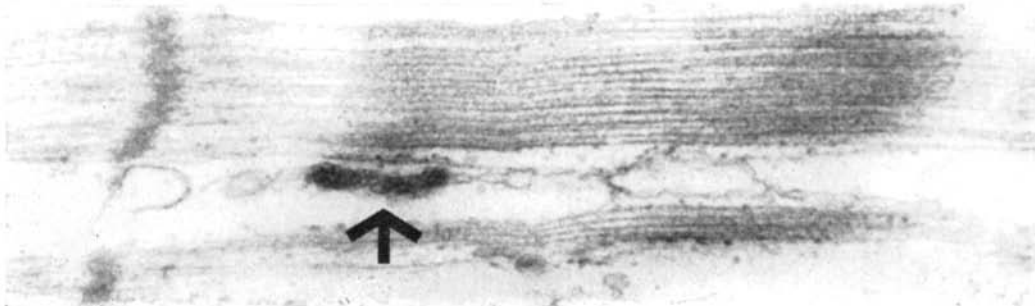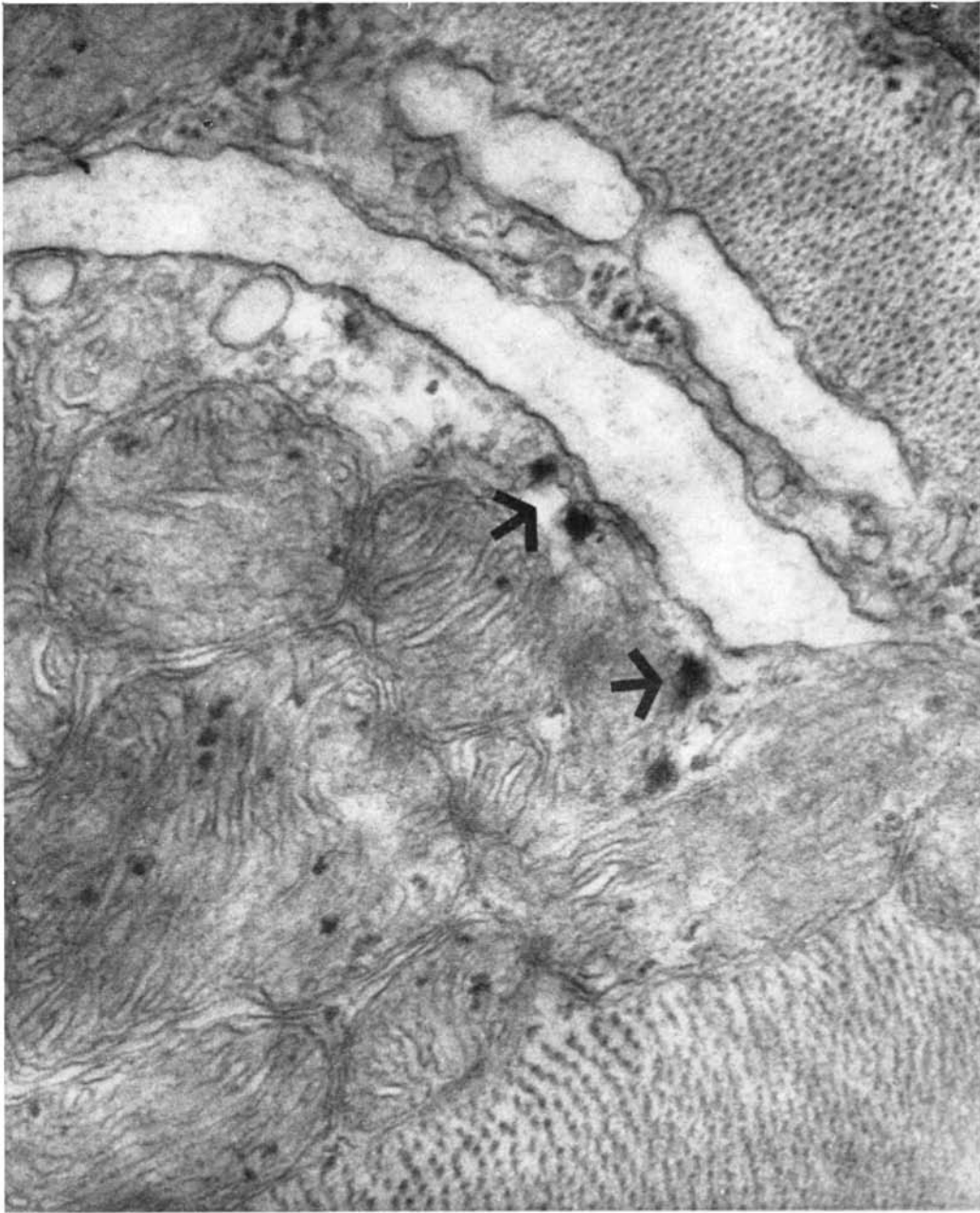
The tension rose very quickly in the high calcium and levelled off at a value equal to about four times twitch tension. The tension fell abruptly when the pCa was lowered to 8.1, but when the tension had dropped to about 160% of twitch tension the rate of decline of tension slowed markedly for about 1 min and then at about 125% of twitch tension the rate increased considerably. Tension ultimately fell to the resting level, and at this point a solution with a pCa of 5.4 produced more tension at a faster rate than it had before the brief exposure to high Ca^{++}. The muscle was again exposed to high Ca^{++} briefly, and tension rose sharply although not quite as rapidly as during the first exposure. There was a shoulder on the rising phase at about the same level of tension as on the falling phase of the previous contracture. The shoulder was not always as flat as seen in the experiment in Fig. 4 but it never had a negative slope, i.e., it never included a dip in tension. The reason for the shoulder on the rising phase of the second and not the first contracture was probably the decline in the influx of Ca^{++} caused by the first exposure to high Ca^{++}. At the peak of tension the solution was again changed to one with a pCa of 8.1 and tension initially fell rapidly. When the rate of decline of tension began to slow, as it had done after the first exposure to high Ca^{++}, a solution with a pCa of 5.4 replaced the one with pCa of 8.1. This time the tension rose more rapidly than on either of the two previous occasions in pCa of 5.4 and reached a level equal to about 300% of twitch tension. Lowering the Ca^{++} quickly lowered the tension. Any shoulder on the falling phase of the second contrac-
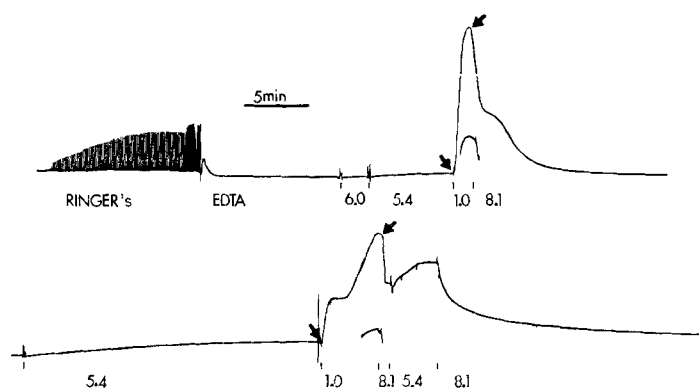

FIGURE 4.   Tension record of a frog ventricular strip stimulated in Ringer's solution and then, after 10 min in EDTA solution, exposed to a series of Ca-EGTA solutions (Ca-EGTA solution I in Table I) with the pCa value indicated on the record. Note the different tension developed in pCa of 5.4 on each occasion. A portion of the tracing of tension in pCa of 1.0 has been drawn from a record made simultaneously at a lower gain on the second channel on the recorder. The original records at low gain for the portion of the tracing that has been drawn have been superimposed. The arrow indicates the point of change in solution. Control stimulation rate successively 9, 30, and 60/min. (See text for further details.)

ture has been obscured by the slower decline of tension due to lower Ca^{++} permeability of the membrane after a second pulse of high Ca^{++}.

The simplest interpretation of these experiments is that during the plateau in tension, pCa remains constant in spite of a continuing influx of calcium. It cannot be due to a cessation of Ca influx because tension continues to rise again later. It seems that at a certain rate of Ca inflow a steady state can be reached at some intracellular pCa where intracellular pCa remains constant. The average level of tension at which this steady state occurred in five experiments was 119% of twitch tension. A reasonable explanation for this steady state is based on the assumption that at the pCa an intracellular sink other than the myofibrils removes Ca from the cytoplasm as rapidly as it enters the cytoplasm from the outside. Shoulders observed during the washout may be explained by a constant cytoplasmic pCa while the internal sink empties into the cytoplasm.

The results of this type of experiment were essentially the same when variations in ionic strength and osmolarity were eliminated by the appropriate additions of KCl and sucrose to the solutions used (Table I). A similar effect could be produced without using the very high concentration of calcium by pulsing the ventricular strip several times with only a moderate concentration of Ca^{++} such as 0.1 mM (Fig. 5) instead of a single pulse with a very high concentration. After four pulses in 0.1 mM Ca^{++} and an exposure to EDTA to reverse any decline in the permeability of the membrane to Ca^{++} produced by the 0.1 mM Ca^{++}, the response to pCa of 6 was much larger than it had been before the pulses of 0.1 mM Ca^{++}. After the fifth exposure to 0.1 mM Ca there was a shoulder in the relaxation curve at about 120% of twitch tension that was similar to but less pronounced than that seen in Fig. 5. The same results occurred when the second exposure to EDTA was eliminated.
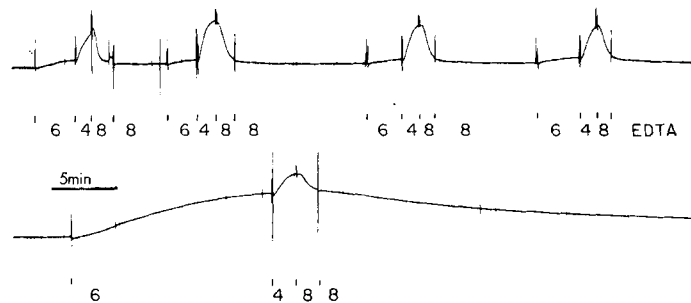

FIGURE 5. Tension record of a frog ventricular strip exposed to EDTA solution and Ca-EGTA solutions (Ca-EGTA solution I in Table I) with the pCa values indicated. Notice the higher tension developed in pCa of 6.0 during the fifth exposure in comparison with that during the first exposure. Artifacts on record indicate time of change of solutions. (See text for further details.)

Triggered Release of Stored Calcium

When high calcium pulsing was done with muscles in which EDTA had increased membrane permeability to a smaller extent or in tissues in which the high permeability had been partially reversed by a previous high calcium pulse (Fig. 1), tension changed more slowly, and the shoulders on the rising and falling phases of the contracture were more prominent as a result of the smaller net calcium flux. In these tissues tension and hence sarcoplasmic Ca^{++} were elevated for a longer time by a pulse than in the more permeable muscles. More striking, however, was the gradual appearance of a second and much more prolonged phase of contraction after the removal of the high Ca^{++} (Fig. 6). This phase increased in amplitude with successive calcium pulses, and eventually it even included a rising portion as well as a plateau. Exposure to EDTA did not reverse the effects of previous calcium pulsing on the second phase of contraction. With successive pulses the delay between the withdrawal of high calcium and the onset of relaxation increased, and in some experiments a pronounced delay in the onset of tension in a low pCa appeared after several pulses (Fig. 6). Tissue calcium, which was measured in parallel
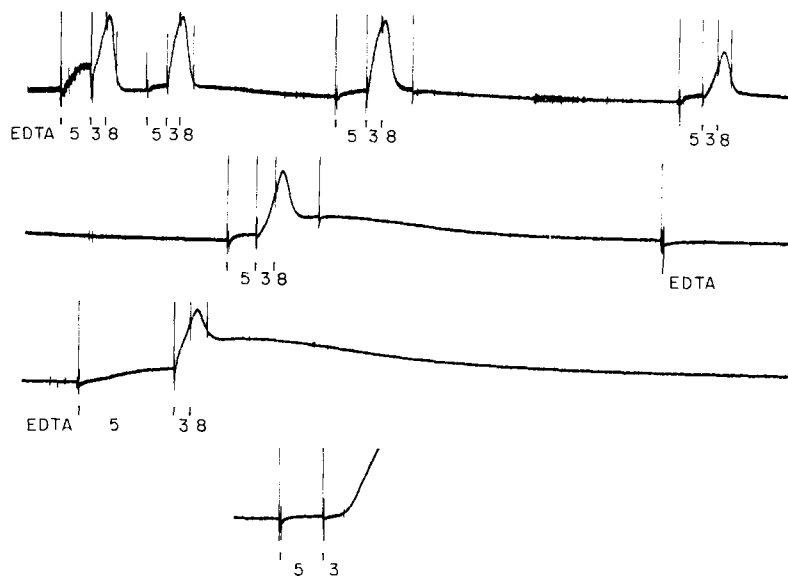

FIGURE 6.   Tension of a frog ventricular strip exposed first to EDTA solution and then to Ca-EGTA solutions I with the pCa indicated underneath the records. Changes in solution are indicated by the artifacts. Note both the gradual appearance of a biphasic contracture with the second phase occurring after the pCa has been raised and the increasing lag in the onset of relaxation after the change to a higher pCa. Exposure to EDTA fails to inhibit the second phase of contracture.
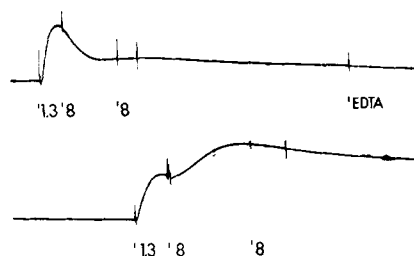
FIGURE 7.   Tension of a frog ventricular strip exposed first to EDTA solution and then to Ca-EGTA solutions I with the pCa indicated. Note that the second phase of the second contracture is larger than the first phase of either contracture.

experiments during the resting phase after each contracture in elevated calcium, was increased with each pulse.

The rate at which the second phase of tension appeared was increased by raising the concentration of Ca^{++} in the pulsing solution and accelerating the accumulation of calcium (Fig. 7). Four or five pulses with 1 mM Ca^{++} but only two pulses with 50 mM were required to produce a second phase. In some experiments the second phase of tension was even larger than the first phase (Fig. 7) and occasionally exhibited damped oscillations.

DISCUSSION

Calcium Binding

In frog ventricles calcium was taken up by two groups of sites other than the contractile proteins even when the extracellular concentration of Ca^{++} was less than 10^{-8} M. One group of sites had a capacity of at least 0.6 μmol of calcium per g of muscle and retained calcium at a sarcoplasmic concentration of Ca^{++} which was too low to support tension, that is less than 10^{-7} M. These sites had therefore a greater affinity for calcium than the contractile proteins and they would compete with troponin for free calcium in the sarcoplasm. A brief exposure to an adequate concentration of Ca^{++} saturated the sites, diminished their competition with the contractile proteins, and enhanced the development of tension in muscles exposed to pCa within the range for activation of contraction. As anoxia or oligomycin caused a reversible rise in resting tension only in tissue containing this "high affinity calcium," its sequestration probably required metabolic energy in the form of ATP. The total amount of calcium actively taken up was not more than 0.2 μmol/g, but it could have exceeded the 0.05 μmol/g that is necessary for activation of all the contractile protein. The minor fraction of the high affinity sites which retained their calcium after exposure to EDTA were presumably separated from most of the high affinity sites by a diffusion barrier.

The second group of sites had a lower affinity and a much larger capacity for calcium. They could have been in direct contact with the sarcoplasm or could have represented a second compartment with access to the sarcoplasm through low affinity binding sites. These sites are probably not important in normal contractions, or even strong ones where cytoplasmic Ca^{++} concentration rises substantially, as contractions requiring sarcoplasmic concentrations of calcium high enough to saturate these sites occur in hearts without enough calcium to satisfy even a small fraction of the total capacity of these binding sites (Sands, 1968). The additional cytoplasmic calcium made available during a contraction therefore must be bound by troponin more rapidly than this lower affinity sink.

The capacities and affinities of the high and moderate affinity sites suggest that the former include the sarcoplasmic reticulum and the latter, the mitochondria. It is possible that also included with the more tightly bound calcium fraction is the small number of high affinity sites present on mitochondria. Histochemical localization of strontium in the sarcoplasmic reticulum and to a lesser extent in the mitochondria indicates that these organelles accumulate divalent cations in the intact heart and lends further support to the suggested localization of calcium-binding sites. The rise in tension in the oligomycin-treated tissues that was caused presumably by the release of calcium from the sarcoplasmic reticulum was not prevented by the mitochondria, indicating the inability of the latter to compete with troponin for calcium during weak contractions. The response of the high affinity sites to metabolic inhibition is similar to that observed in intact skeletal muscle treated with fluorodinitrobenzene (Nauss and Davies, 1966) in which a decrease in ATP produces a release of calcium and a rise in tension. The calcium release is likely to be from the sarcoplasmic reticulum and not from the mitochondria since substrate-supported calcium uptake by the mitochondria can still occur in oxygenated tissues treated with oligomycin. Calcium release from the sarcoplasmic reticulum in cardiac and skeletal muscle is surprising in view of the high affinity of isolated reticulum for ATP, and must be attributed to a difference between the performance of the isolated and intact sarcoplasmic reticulum.

Calcium Release from Cellular Sites

The rising and falling phases of many contractures included abrupt changes in the derivative of tension even when the bathing medium remained the same or was changed to one which would not have been expected to alter tension in the observed direction. These "shoulders" on the tension records could be explained by changes in the rate of delivery or removal of calcium from the contractile proteins due either to simple binding and release dependent only on the binding constants of the ligands or by the more compli-

cated mechanism of active uptake and triggered release. The simple binding and release of calcium could alter the rate of development of tension, but active uptake or triggered release would have been necessary to account for a change in the direction of tension. An alternative hypothesis to explain these shoulders which has the primary effect of calcium in the bath on membrane permeability rather than on the driving force for diffusion is unlikely. The major evidence against it is the progressive increase in amplitude of the second phase of contraction with repeated pulses of moderate or high calcium, which would have been causing a continual decline in membrane permeability.

The simpler mechanism is adequate and seems more appropriate in the muscles where the responses to changes in pCa were immediate and abrupt due to the high permeability of the membrane and the rapid movements of calcium. The levels of tension at which the shoulders occurred were very similar on both the rising and falling phases of the contractures in keeping with simple binding and release. From the level of tension at which these shoulders occur and the tension-pCa relation, the apparent affinity constant of this pool for Ca^{++} is estimated to be $2 \times 10^6 M^{-1}$.

In the muscles with a lower membrane permeability, where unexpected changes in the direction of the development of tension occurred, an uptake with a triggered release of calcium would have been required in addition to simple binding. The exact nature of the trigger is not clear from these experiments, but the release seems to require considerable calcium loading of the high affinity sites of the tissue and possibly a rapid change in the sarcoplasmic concentration of Ca^{++}. In these respects it resembles the triggered release of calcium from the sarcoplasmic reticulum that has been shown in "skinned" skeletal muscle fibers (Ford and Podolsky, 1972 *a* and *b*; Endo et al., 1970).

In one model which can explain the triggered release of calcium in isolated ventricles the inside of the sarcoplasmic reticulum is at a higher electrical potential than the sarcoplasm in accordance with data already in the literature (Costantin and Podolsky, 1966; Winegrad, 1972). Accumulation of calcium by the reticulum increases the calcium equilibrium potential and reduces the potential difference. An abrupt decrease in the concentration of Ca^{++} in the sarcoplasm or an increase inside the reticulum could depolarize the membrane sufficiently to cause some permeability change and an outflux of calcium from the reticulum. It is not clear, however, whether this sort of mechanism would operate in the normal intact frog ventricle as the observed calcium releases required a level of calcium loading of the tissue that was significantly greater than normal.

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