

# Studies of Cardiac Muscle with a High Permeability to Calcium Produced by Treatment with Ethylenediaminetetraacetic Acid

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**ABSTRACT** Thin strips of frog ventricle were isolated and bathed for 15 min in a solution containing 140 mM KCl, 5 mM Na<sub>2</sub>ATP, 3 mM EDTA, and 10 mM Tris buffer at pH 7.0. The muscle was then exposed to contracture solutions containing 140 mM KCl, 5 mM Na<sub>2</sub>ATP, 1 mM MgCl<sub>2</sub>, 10 mM Tris, 3 mM EGTA, and CaCl<sub>2</sub> in amounts to produce concentrations of free calcium from 10<sup>-4.8</sup> M to 10<sup>-9</sup> M. The muscles developed some tension at approximately 10<sup>-8</sup> M, and maximum tension was achieved in 10<sup>-5</sup> M Ca<sup>++</sup>. They relaxed in Ca<sup>++</sup> concentrations less than 10<sup>-8</sup> M. The development of tension by the EDTA-treated muscles was normalized by comparison with twitch tension at a stimulation rate of 9 per min before exposure to EDTA. In 10<sup>-5</sup> M Ca<sup>++</sup> tension was always several times the twitch tension and was greater than the contracture tension of a frog ventricular strip in KCl low Na-Ringer. Tension equal to half-maximum was produced at approximately 10<sup>-6.2</sup> M Ca<sup>++</sup>. Intracellular recording of membrane potential indicated that after EDTA treatment the resting potential of cells in Ringer solution with 10<sup>-5</sup> M Ca or less was between 5 and 20 mv. Contracture solutions did not produce tension without prior treatment with EDTA. The high permeability of the membrane produced by EDTA was reversed and the normal resting and action potentials restored in 1 mM Ca-Ringer. Similar studies of EDTA-treated rabbit right ventricular papillary muscle produced a similar tension vs. Ca<sup>++</sup> concentration relation, and the high permeability state reversed with exposure to normal Krebs solution.

The "skinned" muscle fiber preparation, one in which the surface membrane and some of the more superficial myofibrils have been mechanically removed,

was first developed by Natori (1954) and has been extensively studied by Natori, Podolsky and his coworkers (Hellam and Podolsky, 1969), and Endo et al. (1970). By removing the surface membrane and either suspending the muscle in oil or bathing it with a simulated intracellular medium it has been possible to study the response of the contractile apparatus to controlled changes in the composition of the fluid directly surrounding the components of the cell. These studies have produced considerable information not only about the contractile proteins but also about the sarcoplasmic reticulum.

Although a mechanically skinned cardiac muscle cell preparation would be useful in studying the properties of the heart, the small size of the cells appears to preclude this possibility. Another approach to the development of a cardiac muscle preparation analogous to the skinned skeletal muscle fiber is the use of a chemical procedure for increasing the permeability of the surface membrane in a way that does not impair the function of the intracellular structures. The observation (Thomas, 1960 *a, b*) that EDTA greatly increased the intracellular content of calcium and sulfate in frog ventricles suggests a method for increasing membrane permeability at least to small ions and molecules without obvious impairment of contraction. The experiments to be described were conducted to study the EDTA-treated heart as a possible analogue to the mechanically skinned skeletal muscle cell.

#### METHODS

Thin strips were cut from the ventricles of medium sized, male frogs (*Rana pipiens*) and connected for continuous measurement of tension to either an Endevco 8107-2 semiconductor transducer or a Statham G 10 B  $\pm$  0.15 strain gauge transducer. The muscles were bathed in Ringer solution (117 mM NaCl, 1.0 mM CaCl<sub>2</sub>, 2.4 mM K<sub>2</sub>HPO<sub>4</sub>, 0.6 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.0) and stimulated at 9 per min until a steady contraction tension was achieved. This period of stimulation was never more than a few minutes so the hearts never reached the hypodynamic state (Clark, 1913). At this point the bathing medium was changed, and a solution containing 3 mM EDTA (disruption solution) replaced it. After 15 min the muscle was passed through a series of 2–5 ml aliquots of solutions containing calcium buffered with EGTA (contracture solutions). The muscle remained in each solution until a steady tension was reached. At the end of each experiment the total calcium content of each solution was measured with a Perkin-Elmer 290 atomic absorption spectrometer to eliminate possible errors due to calcium contamination from reagents or chambers.

The transmembrane potentials of fibers in several muscle strips were measured with microelectrodes and recorded on a Tektronix 502 oscilloscope and a Brush 280 recorder.

Several muscles were prepared for electron microscopy by glutaraldehyde fixation followed by osmium fixation, lead staining, and Epon embedding. Gold and silver sections of these muscles were examined with a Hitachi HU11E electron microscope (Perkin-Elmer Corp., Palo Alto, Calif.).

The special solutions used were:

Disruption	Relaxation	Contracture
3 mM EDTA	3 mM EGTA	3 mM EGTA
5 mM Na <sub>2</sub> ATP	5 mM Na <sub>2</sub> ATP	5 mM Na <sub>2</sub> ATP
10 mM Tris buffer	10 mM Tris buffer	10 mM Tris buffer
140 mM KCl	140 mM KCl	140 mM KCl
pH 6.5-7.0	1 mM MgCl <sub>2</sub> pH 6.5-7.0	1 mM MgCl <sub>2</sub> 0-2.85 mM CaCl <sub>2</sub> pH 6.5-7.0

The association constants for CaEGTA are very sensitive to pH, and the values that were used in calculating the concentration of ionic Ca<sup>++</sup> were  $4.9 \times 10^5 \text{ M}^{-1}$  at pH 7.0 and  $4.9 \times 10^5 \text{ M}^{-1}$  at pH 6.5 (Ringbom, 1963).

Some experiments were performed on the right ventricular papillary muscles of rabbits. In these studies the standard predisruption solution was prepared with 2.5 mM Ca<sup>++</sup> at pH 7.4, as described by Krebs and Henseleit (1932). It was bubbled with 95% O<sub>2</sub>-5% CO<sub>2</sub>. The disruption, contracture, and relaxation solutions were the same as those used in the experiments on frog ventricle. ATP was obtained from Mann Research Labs., Inc. (New York) and the EGTA was a gift of the Geigy Chemical Corp. (Ardsey, N.Y.). All reagents were American Chemical Society grade. Solutions were prepared weekly, with the exception of the ATP, which was prepared each day from a frozen, concentrated solution. The pH of all solutions was checked to  $\pm 0.01$  with a Beckman expandomatic meter. All experiments were done at room temperature except for one series in which the influence of temperature was examined.

## RESULTS

### A. Introduction

Since the purpose of this project was to develop a preparation in which the surface membrane was very permeable at least to small molecules and ions, especially calcium, and in which the state of the contractile apparatus was rapidly responsive to changes in the bathing medium, it was necessary to establish some criteria for determining the permeability of the surface membrane. The relation between tension and the concentration of ionic calcium bathing isolated contractile proteins has been well-established (Weber and Herz, 1963) and is considerably different from the relation between extracellular calcium and the tension of intact heart cells. In the former system, maximum tension is developed at a much lower concentration of free calcium. A muscle was not considered to have a high permeability to the calcium buffer system (Ca<sup>++</sup> + EGTA + CaEGTA) unless its tension-calcium concentration relation resembled that of isolated myofibrils and the rate of change of tension occurred with a time course consistent with the absence of major diffusion barriers. The membrane potential was also used as a criterion of permeability.

The presence of a normal resting potential meant restricted diffusion across the cell membrane of ions such as  $\text{Na}^+$  relative to that of  $\text{K}^+$  and  $\text{Cl}^-$  and therefore, a relatively intact membrane.

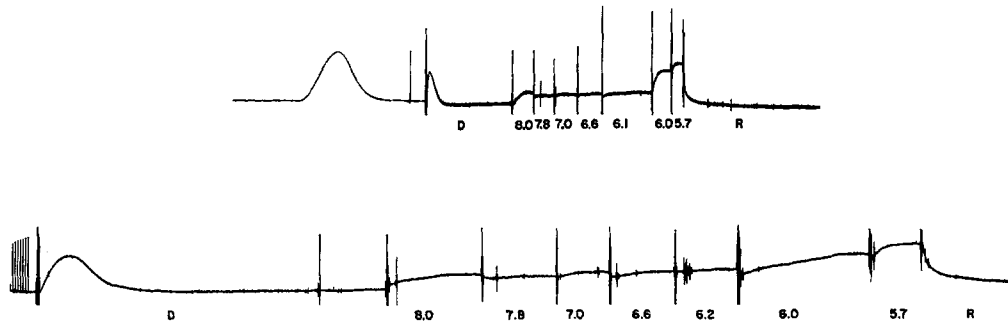


FIGURE 1. Records from two experiments with two frog ventricular strips showing effect of decreasing pCa. In each tracing a control contraction stimulated at 9 per min in Ringer solution (at fast paper speed in upper tracing) is followed by disruption solution (*D*) contraction solutions with the concentration of ionic  $\text{Ca}^{++}$  given as pCa, and finally relaxation solution. The large artifacts on the tracing are due to the change in bathing medium. Time in *D* in each experiment was 15 min and provides time calibration for each tracing (except for control contraction in upper tracing).

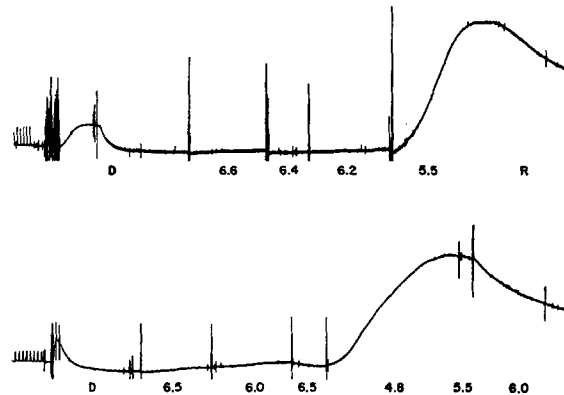


FIGURE 2. Records from experiments with two frog ventricular strips showing effect of decreasing pCa. Time in *D* in each experiment was 15 min and provides time calibration for each tracing. See legend of Fig. 1 for details.

### B. The Effect of EDTA Treatment

Thin strips of frog ventricle were bathed in the membrane disruption solution for periods of 5–30 min. The replacement of normal Ringer solution with disruption solution always produced a contracture that lasted about 1 min. Since omission of calcium from the Ringer solution completely inhibited the

contracture, the increase in tension was probably the result of depolarization of the cell membrane by  $K^+$  before depletion of the extracellular ionic calcium by the EDTA. After the exposure to the disruption solution, the tissue was passed through a series of contracture solutions with different concentrations

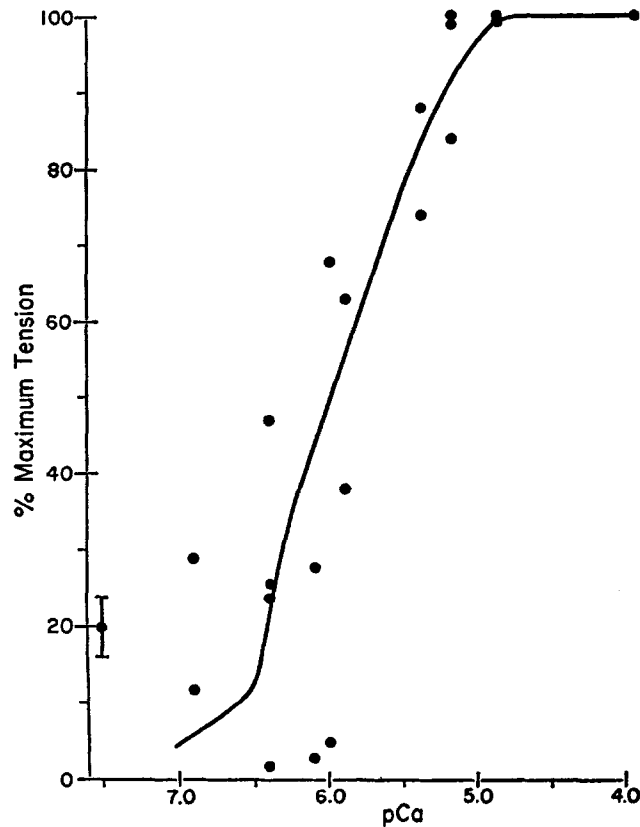


FIGURE 3. Plot of relative tensions produced by frog ventricular strips in contracture solutions containing different pCa. Each point represents a single value derived from a group of seven muscles except for the point with standard error brackets at 20% maximal tension. The latter is the average ratio of contraction tension to maximum tension in seven muscles, and it was used to normalize the values from all the experiments. pH 6.5. The line was drawn by eye through the points.

of ionic calcium produced by different ratios of  $CaCl_2$  to EGTA. The results of two such experiments, in which the muscles were first bathed with disruption solution for 15 min and then in different contracture solutions, are shown in Fig. 1. As the fibers in a frog ventricular strip are not parallel, a meaningful comparison of the performance of different muscles on the basis of absolute tension or tension per unit cross-sectional area was not possible, but normaliza-

tion, by means of contractions that had occurred under identical circumstances before membrane alteration, was feasible. The muscles therefore were stimulated at a rate of 9 per min in Ringer solution before exposure to the chelating agent.

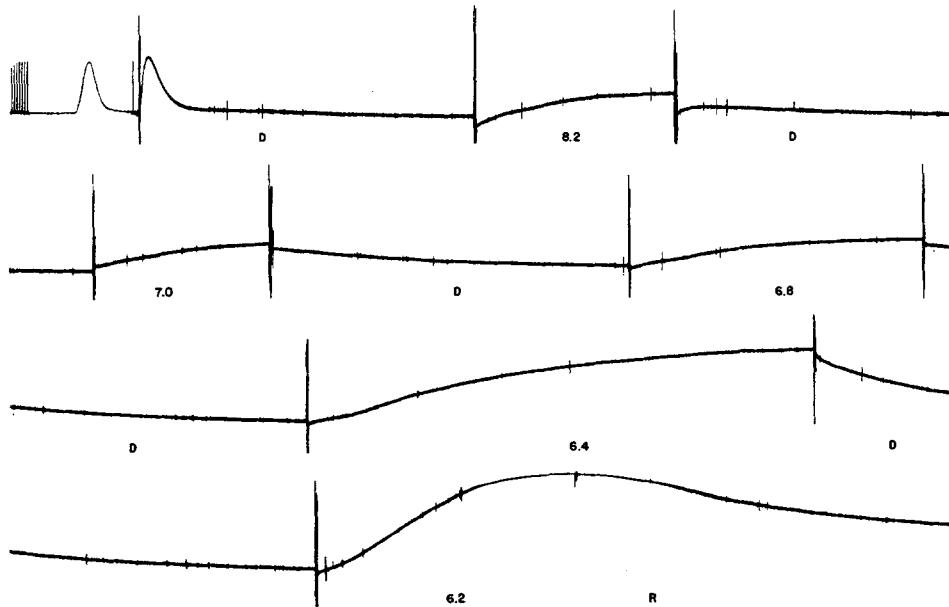


FIGURE 4. Record of a frog ventricular strip bathed alternately in disruption solution and contracture solutions with decreasing pCa. Duration of first exposure to *D* was 15 min and provides time calibration for the entire record except last control contraction. See Fig. 1 for details.

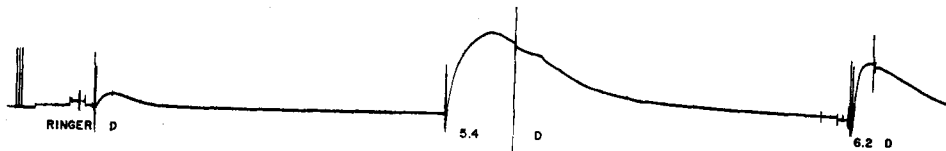


FIGURE 5. Record of a frog ventricular strip bathed alternately in disruption solution and contracture solutions with increasing pCa. Duration of first exposure to *D* is 15 min and provides time calibration for the entire record. See Fig. 1 for details.

These experiments show that some tension developed at a concentration of ionic calcium as low as  $10^{-8}M$  ( $pCa = 8.0$ ) and a large amount of tension, at about  $10^{-6}M$   $Ca^{++}$  ( $pCa = 6.0$ ). In the two experiments shown in Fig. 1, tension equivalent to the control contractions was produced, after the EDTA treatment, in solutions with pCa between 6.0 and 5.7. The contractures were reversed by lowering the  $Ca^{++}$  concentration to less than  $10^{-8}M$ .

The effect of the EDTA treatment depended on the duration of exposure to the disruption solution. Exposure times of 12–20 min were followed by the greatest relative tension at a given pCa and therefore considered to be optimal. EDTA was more effective in increasing membrane permeability than EGTA,

TABLE I  
HEALING OF DISRUPTED CELLS

Time <i>min</i>	Solution	R. P.			A. P.		Twitch
		<i>mv</i>	<i>mv</i>	<i>msec</i>	<i>mv</i>	<i>msec</i>	
0	1 mM Ca Ringer	60–65	115	400			Strong twitch
1	0 mM Ca Ringer						
3		60			None		None
11	Disruption solution						
26	KCl Ringer $10^{-6}$ M Ca	10			None		None
46	NaCl Ringer $10^{-6}$ M Ca	20			None		None
72		30			None		
76		40			None		
86		50			None		
88		50	50	60			None
106	1 mM Ca Ringer	50	50	60			None
107		60	60	80			Very weak
114		60	60	150			Very weak
128		60	110	250			Weak
135		60	125	375			Weak

R.P. and A.P. stand for resting potential action potential, respectively.

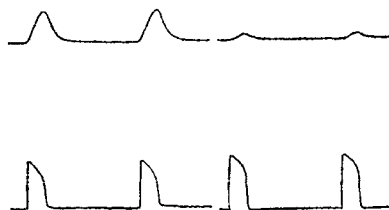


FIGURE 6. Contraction tension (upper trace) and transmembrane potential (lower trace) before disruption (left) and during healing from disruption (right, at time 135 min in Table I).

and its effect was enhanced when  $K^+$  was the predominant cation of the bathing medium. This judgment was based on both the rate of rise of tension and the final tension achieved at given concentrations of ionic Ca. When EGTA was the chelator used in the disruption solution or when EDTA was used in the absence of high  $[K^+]$ , the rate of rise of tension in contracture solutions was no greater than one-fourth to one-fifth and the final tension was never more than one-half of that reached by a muscle after treatment with EDTA plus high  $[K^+]$ . Changes in pH between 6.5 and 7.0 and in temperature be-

tween 25 and 30°C did not alter the response to EDTA. Substitution of a phosphate buffer for the Tris buffer in the disruption solution completely inhibited the production of the high Ca permeability although it had no special effect in the contracture solutions. Muscles never produced tension in the contracture solutions unless they had been previously exposed to the disruption solution. Muscles not previously treated with disruption solution required more than  $10^{-5}$  M  $\text{Ca}^{++}$  to produce tension in a high  $\text{K}^+$  low  $\text{Na}^+$  solution.



FIGURE 7. Frog ventricular strip exposed to contracture solutions with same pCa but different  $\text{Ca}^{++}$  buffer concentrations (see text). Half-times for rise of tension in 0.001 M EGTA and 0.01 M EGTA were 50 and 15 sec, respectively.

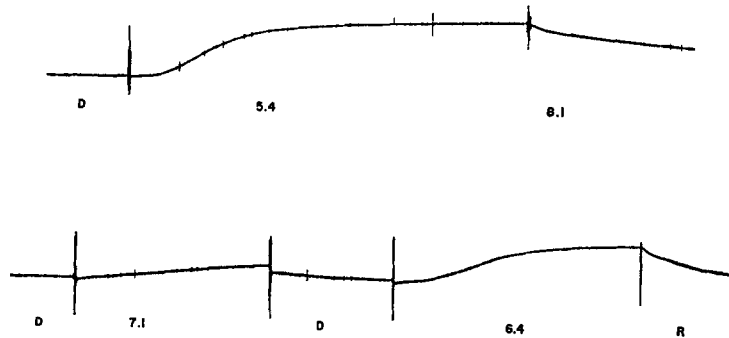


FIGURE 8. Two frog ventricular strips exposed to disruption solution and contracture solutions with stated pCa. Note delay in rapid rise of tension in upper trace and in pCa of 6.4 in lower trace. Duration of exposures to solutions with pCa of 5.4, 7.1, and 6.4 were 35, 17, and 21 min, respectively.

### C. *The Relation between Tension and pCa*

The tension developed by the ventricular strips over the range of pCa from 8.0 to 5.0 is shown in four different preparations in Figs. 1 and 2. Maximum tension developed at pCa equal to 5.0-5.5 and was always significantly more than the tension of the predisruption contractions, often by as much as four- or fivefold. The rate of relaxation from a contracture at a pCa of 5 frequently was slower than from weaker contractures at high pCa's (compare Figs. 1 and 2).

The relation between pCa and developed tension derived from studies of several muscles is given in Fig. 3. The sigmoidal shape is similar to that which has been described for other muscle model systems (Weber and Herz, 1963;



Hellam and Podolsky, 1969), and the midpoint is approximately the same as it is for skinned skeletal fibers (in which Hellam and Podolsky used the same dissociation constant for CaEGTA). The relation was independent of pH between 6.5 and 7.0.

A second procedure for testing the tension-pCa relation involved alternate periods in disruption solution and contracture solutions of different pCa instead of a single exposure to disruption solution (Fig. 4). The tension developed at a given pCa was the same after single and multiple exposures to EDTA and was independent of the order in which contracture solutions had been applied (Fig. 5).

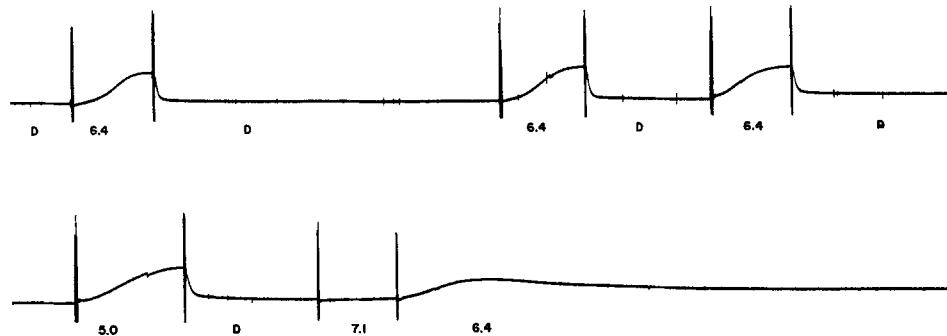


FIGURE 9. The effect of exposure of a frog ventricular strip to a solution with a low pCa on the mechanical response to a solution with higher pCa. Note the reproducibility of the first three mechanical responses in pCa of 6.4 and the different response after an exposure to pCa of 5.0. Duration of exposure to pCa of 6.4 each of first three times was 7 min.

#### D. *The Effect of EDTA Treatment on the Membrane Potential*

In order to determine the effect of the disruption solution on the membrane potential, the simulated intracellular solutions containing 140 mM KCl were replaced with a solution containing the normal extracellular concentrations of Na, K<sup>+</sup>, and Cl<sup>-</sup>. In a solution with only 5 mM K<sup>+</sup> the resting membrane potentials measured with an exploring microelectrode were between +10 and +15 mv, and no action potential could be produced by electrical stimulation. The resting potential remained very small for at least 10–15 min when the bathing solution contained 10<sup>-6</sup> M Ca<sup>++</sup> or less. In 10<sup>-5</sup> M Ca-Ringer, the resting potential gradually increased and eventually a poor action potential could be elicited, while in 10<sup>-3</sup> M Ca-Ringer the normal resting and action potentials were restored.

The detailed results of one experiment in which the resting potential completely recovered are given in Table I. When the muscle was transferred from normal Ringer solution to 0 Ca-Ringer the resting potential remained unchanged but the action potential increased considerably in duration as has

been described by Garnier et al. (1969). After 15 min in disruption solution and another 20 min in  $10^{-5}$  M Ca-KCl solution, the membrane potential was very low, but in  $10^{-5}$  M Ca-Ringer a slow recovery of the resting potential occurred. Complete restoration of the normal resting and action potentials and of the mechanical response to stimulation did not occur until the muscle

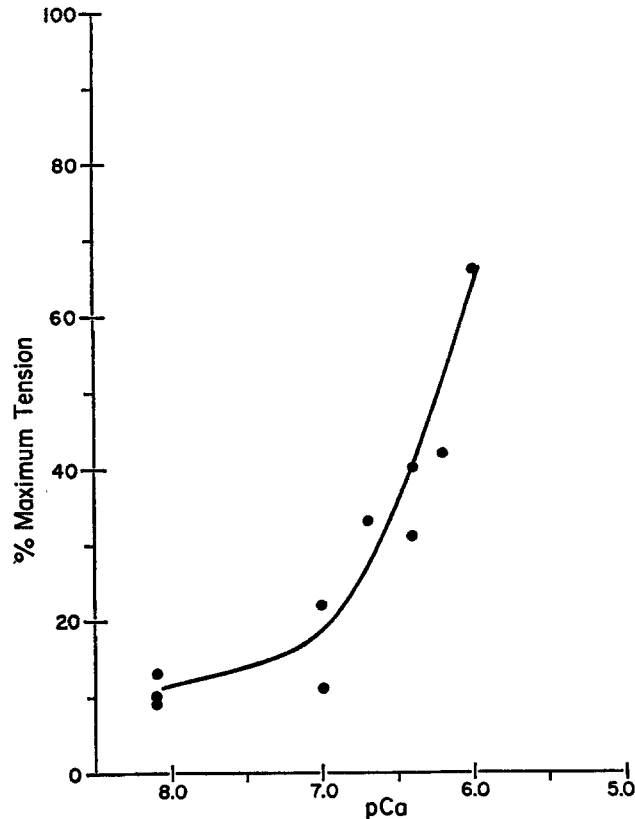


FIGURE 10. Plot of relative tensions produced by frog ventricular strips in contracture solutions containing different pCa at pH 7.0. Each point represents one measurement from one of four muscles.

had been exposed to 1 mM Ca-Ringer for over 30 min (an example of complete recovery of the twitch after disruption solution is shown in Fig. 11 C). Mechanical and electrical records of both the control twitch and the twitch at 135 min after the beginning of the experiment are given in Fig. 6. The action potential was actually somewhat higher at 135 min although the threshold for its production was still abnormally high.

It was not possible to say whether the weak contractile tension, in spite of normal action potentials, was due to a recovery of the membranes of only some of the cells or a generally reduced contractility. Attempts were made with

the microelectrode to explore the state of cells throughout the ventricular strip but most of the measurements were limited to the more superficial fibers. Within these limitations of sampling, however, the resting and action potentials did not vary over a wide range. This point is of interest in determining whether there was a stage in recovery when a normal action potential was associated with a weak contraction.

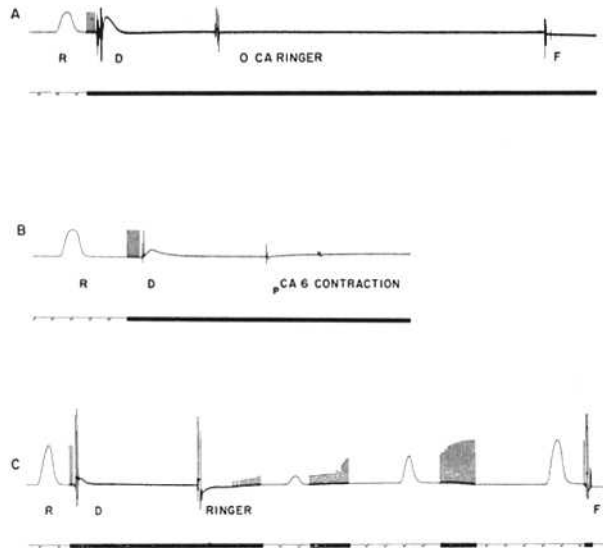
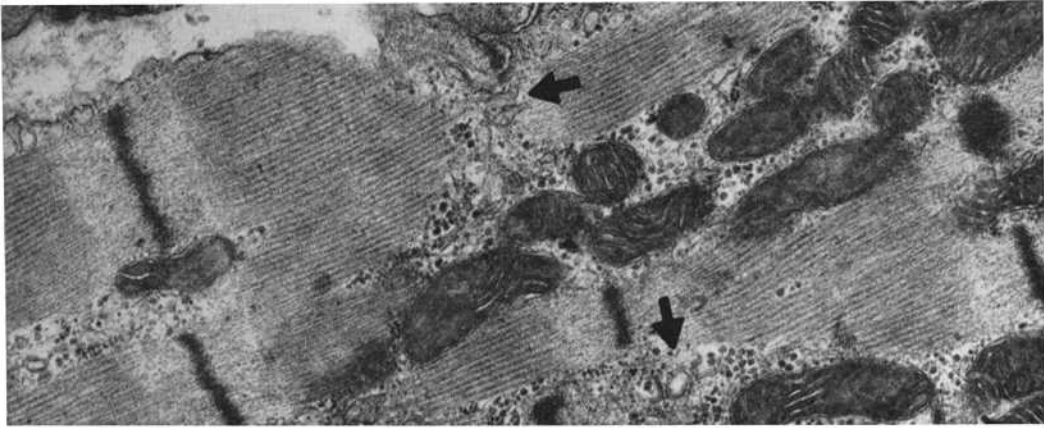


FIGURE 11. Records from three frog ventricular strips exposed to disruption solution for 15 min in each case and ultimately fixed in glutaraldehyde. *R* is normal Ringer solution and *F* is fixation solution.

#### E. *The Rate of Development of Tension and the Rate of Relaxation*

Although the half-times for the development of tension were often as short as 20 sec, a value consistent with relatively unrestricted diffusion, in some preparations half-times as long as 5 min were observed. In order to determine the cause of the slow rise in tension, a muscle that exhibited the slow rise was exposed to two contraction solutions with the same pCa but different concentrations of the  $\text{Ca}^{++}$  buffer system (Fig. 7). If diffusion of the buffer system was limiting the rate of equilibration of sarcoplasmic  $\text{Ca}^{++}$  with  $\text{Ca}^{++}$  in the bath, a higher concentration of the  $\text{Ca}^{++}$  buffer should cause a more rapid rise of tension to the same final level. The data in Fig. 7 support this notion. The striking difference in the rates of relaxation following these two contractions may have resulted from the use of a  $\text{Mg}^{++}$ -free disruption solution for relaxation. The dilute calcium buffer system probably permitted a faster decrease in the concentration of  $\text{Ca}^{++}$  than in the concentration of  $\text{Mg}^{++}$ . The opposite was probably true when the more concentrated calcium buffer



system was used. The depletion of  $Mg^{++}$  before the washout of  $Ca^{++}$  in the latter solution would have interfered with the relaxation of the calcium-activated contraction (Weber et al., 1969).

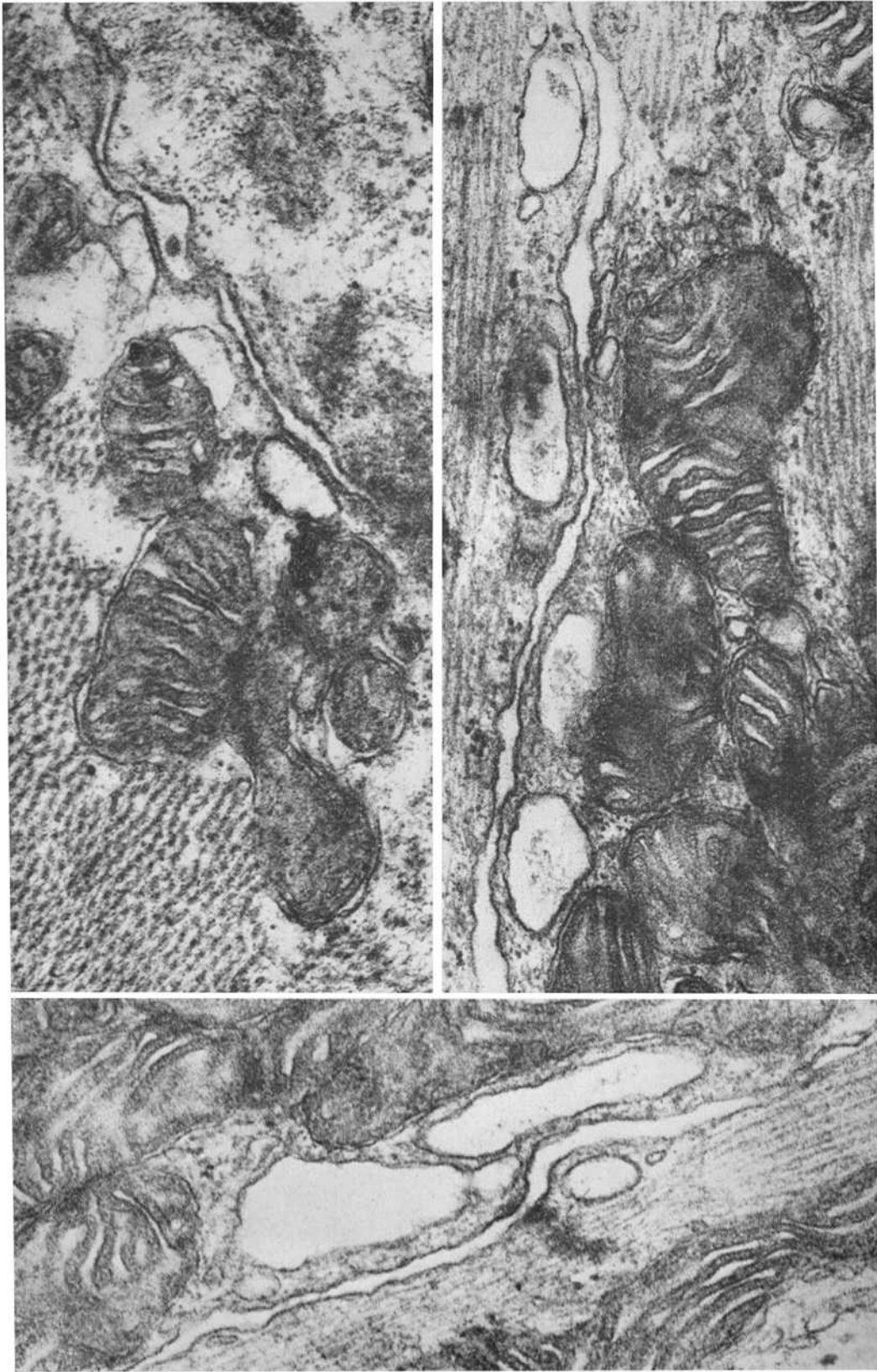
In several experiments there was a break in the rising portion of the tension curve when the muscle was exposed to intermediate or higher concentrations of  $Ca^{++}$ . Two examples of this are shown in Fig. 8. In the upper tracing, the initial increase in tension in pCa of 5.4 was slow, but after about 90 sec the rate of rise increased markedly. In the lower trace the onset of tension in pCa of 7.1 and 6.4 was very similar but after about 90 sec the rate of rise of tension in pCa of 6.4 increased substantially.

The rate of relaxation varied as did the rate of development of tension from speeds fast enough to be accountable for by simple diffusion of the  $Ca^{++}$  buffer system out of the cells (Fig. 1) to very much slower rates (Fig. 4). The slower rates of relaxation were much less common from weak or intermediate contractures in solutions of pH 6.5 than from the stronger contractures at pH 7.0. Measurements of resting potentials had indicated that a slow decrease in the permeability of the membrane occurred in  $10^{-5}$  M  $Ca^{++}$ -Ringer (Table I), and the slow relaxation seen in muscles contracted in pCa of 5 or less might have been due, at least in part, to the slow washout of the calcium buffer from cells in which the leakiness of the membrane was being reversed. The experiment shown in Fig. 9 supports this interpretation. A muscle was alternately exposed three times to contracture solution with pCa of 6.4 and disruption solution to demonstrate the reproducibility of the contracture. In a pCa of 5.0, however, although the developed tension was greater, the rates of rise and fall of tension were somewhat slower in contrast to the greater speeds that would have been expected if the concentration gradient of the buffer system had been the sole rate-limiting factor. A subsequent exposure to a pCa of 6.4 produced a much weaker contracture in which the tension rose slowly and was not sustained. The tension dropped without the rapid phase seen in the previous contractures. The inability of the ventricular strip to sustain tension was not due to depletion of the energy supply for the contractile system, as equal or greater tension had been maintained under other circumstances for longer periods of time (Fig. 8).

These observations are consistent with the initiation of a significant decline in calcium permeability of the membrane in pCa of 5.0 and a continuation of the decline in pCa of 6.4. The effect of pH on this process was pronounced. In contracture solutions at pH 7.0, this healing process appeared to occur at pCa as high as 6.0, and consequently the tension-pCa curve at pH 7.0 gave

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FIGURE 12. Electron micrographs of normal frog ventricle. Note the presence of sarcoplasmic reticulum in both upper and lower (arrows). In upper there are two subsarcolemmal cisternae. Upper,  $\times 29,000$ ; lower,  $\times 23,000$ .



lower values of tension for pCa less than 6.0. The curve was the same for pCa greater than 6.0 (Fig. 10).

#### F. *Electron Microscopy of Muscles Treated with EDTA Solution*

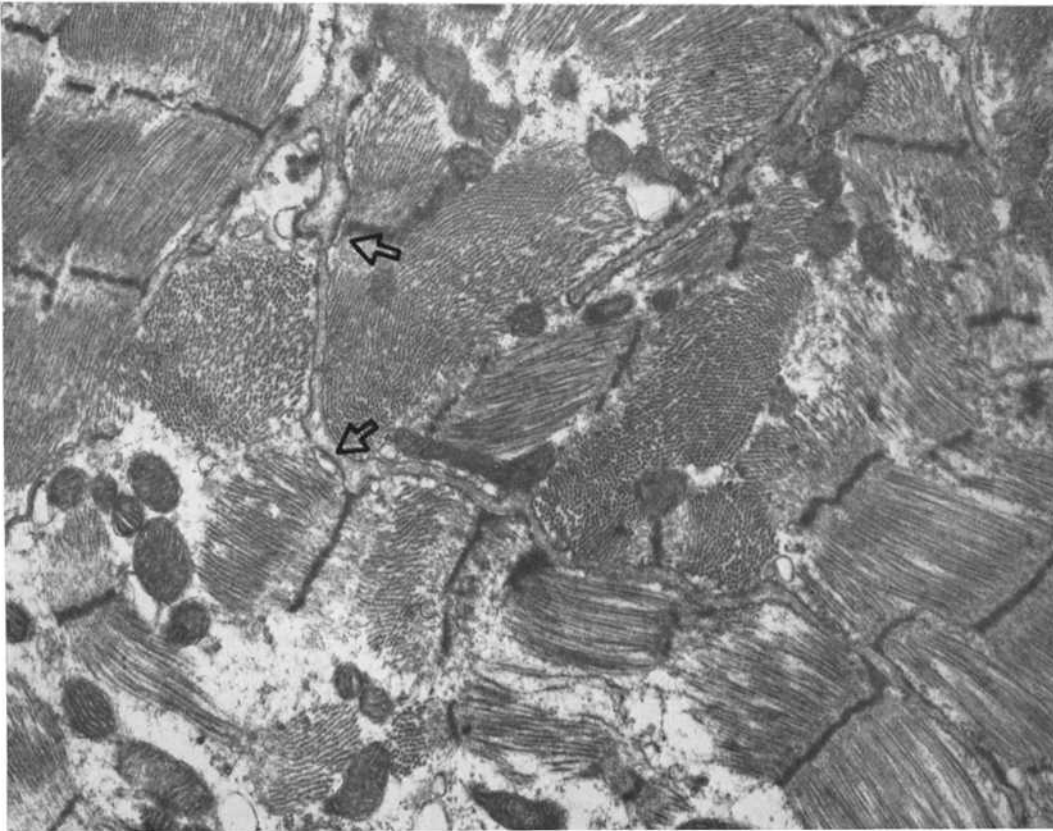
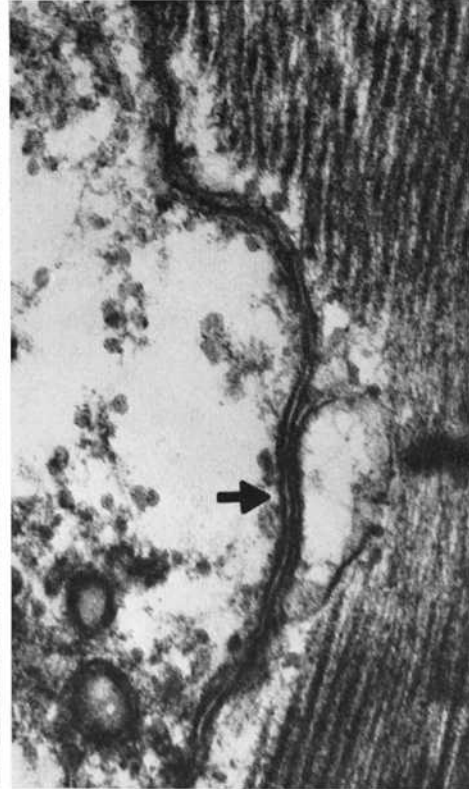
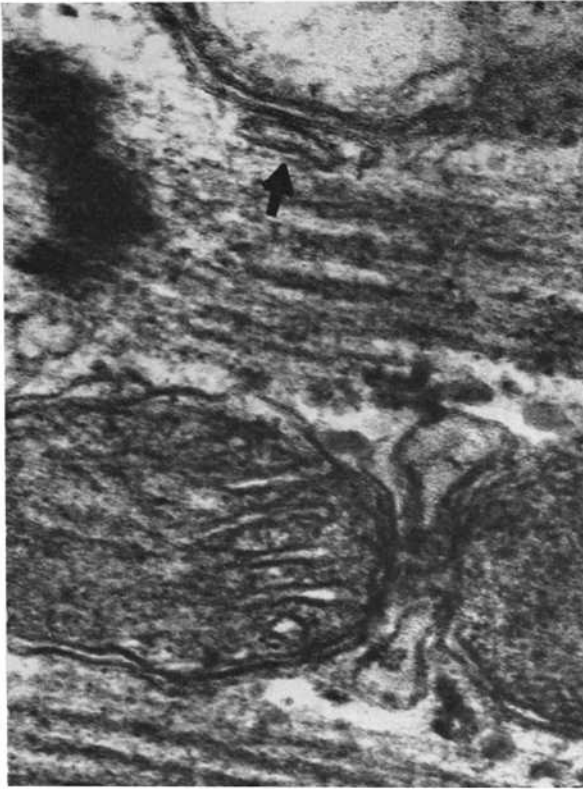
The morphology of the cells in four heart strips treated with disruption solution was examined under the three different conditions shown in Fig. 11. Two muscles were exposed to disruption solution for 15 min and then washed out in Ringer solution with no added calcium ( $\text{Ca}^{++}$  less than  $10^{-7}$  M) for 30 min before fixation in glutaraldehyde and osmium. It was not possible to fix a muscle directly after exposure to disruption or relaxation solution because the embedding media did not polymerize properly at very low  $\text{Ca}^{++}$  concentrations unless the chelating agent had been washed out of the tissue before fixation. A third muscle was exposed to disruption solution for 15 min and then fixed after several minutes in a contraction solution with a pCa of 6.0. The fourth muscle was exposed to disruption solution for 15 min and fixed after the mechanical response to electrical stimulation in normal Ringer solution had returned to control levels.

Electron micrographs of control frog ventricular strips fixed in normal Ringer without any prior exposure to disruption solution are shown in Fig. 12. A small amount of sarcoplasmic reticulum was present, and the flattened reticular tubules (subsarcolemmal cisternae) just beneath the sarcolemma that have been described in frog and avian hearts (Sommer and Johnson, 1969) and mammalian hearts (Fawcett and McNutt, 1969) were visible. The dark spots in the I band near the Z line described by Staley and Benson (1968) were not seen. The muscles that had been disrupted are shown in Fig. 13. In these cells, the sarcolemma, mitochondria, and contractile filaments had the same appearance as that of the control muscles, but the sarcoplasmic reticulum was substantially different. Most of the reticulum in the treated cells appeared to be swollen. Subsarcolemmal cisternae were less frequent and they were swollen, but to a smaller extent than the rest of the reticulum. The reticulum in the muscles fixed in Ringer with less than  $10^{-7}$  M  $\text{Ca}^{++}$  appeared to be swollen to a greater degree and to have fewer subsarcolemmal cisternae than the muscle fixed in contraction solution with a pCa of 6.0, but more work is necessary to confirm this impression.

The appearance of cells which had been disrupted and then completely repaired as judged by their contractility was much closer to that of control cells (Fig. 14). The extreme degree of swelling of the sarcoplasmic reticulum seen in the muscles fixed in pCa greater than 7.0 was not present, and although

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FIGURE 13. Electron micrographs of frog ventricular strips treated with disruption solution and bathed in 0 Ca-Ringer before fixation (see text). Note swollen sarcoplasmic reticulum and swollen subsarcolemmal cisternae.  $\times 59,000$ .





some subsarcolemmal cisternae were swollen, the frequency of their occurrence was about the same as in control muscles.

#### G. EDTA Treatment of Rabbit Right Ventricular Papillary Muscle

The effectiveness of the disruption solution in increasing the permeability of mammalian heart muscle to calcium was tested on rabbit right ventricular papillary muscle. The change in tension that occurred in the disruption solution was more complex than that seen with frog ventricle (Figs. 15 and 16). As with the frog, there was an initial rise in tension that was eliminated by a relatively short presoak in  $\text{Ca}^{++}$ -free Krebs-Henseleit solution, but in addition a slower second rise and an even slower decline occurred. In some experiments

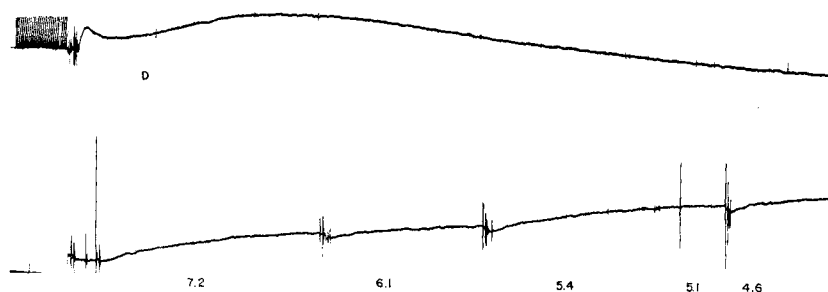


FIGURE 15. Rabbit right ventricular papillary muscle exposed to disruption solution and then contracture solutions with stated pCa. Prior to disruption, muscle was stimulated at 20 per min in Krebs solution with 2.5 mM  $\text{Ca}^{++}$ . Duration of exposures to D, 7.2, 6.1, 5.4, 5.1, and 4.6 was 75, 20, 14, 18, 4, and 10 min, respectively.

it took over an hour for the tension to stabilize in the disruption solution, and consequently the duration of exposure to EDTA was greater than occurred with frog ventricle. Relaxation from the second rise in tension was accelerated by transferring the muscle from disruption solution to a contracture solution with a pCa of 8.0 (Fig. 16). The second rise of tension could be eliminated by either a moderately long soak in Ca-free solution before disruption or by vigorous oxygenation of the contracture solutions.

The rate of rise of tension was always slow but the tension-pCa relation at pH 6.5 or 7.0 was not significantly different from that seen in the frog ventricle at pH 6.5 (Fig. 17). In the papillary muscle all fibers are parallel and it is possible to estimate the tension developed per unit cross-sectional area. The

FIGURE 14. Electron micrographs of frog ventricles treated with disruption solution and then exposed to normal Ringers' until the contraction returned to normal. Note much less swelling of sarcoplasmic reticulum than in Fig. 13 and the presence of both normal (arrow, *upper left*) and swollen (arrow, *upper right*) subsarcolemmal cisternae. Upper left and upper right,  $\times 73,000$ ; lower,  $\times 12,500$ .

maximum tensions observed were in the range of 1.0-1.5 kg/cm<sup>2</sup>. In contrast to observations of frog heart, the higher pH did not favor healing, and there was no change in the rate of rise of tension with a 10-fold increase in buffer concentration (Fig. 18). In the latter experiments, however, the tension was not completely stable before each testing with a pCa of 5.2. Nevertheless, it was clear that the slow rise of tension was not due simply to an insufficient concentration gradient of calcium buffer between the bath and the cell. Vigorous bubbling of O<sub>2</sub> greatly increased the otherwise slow rate of relaxa-

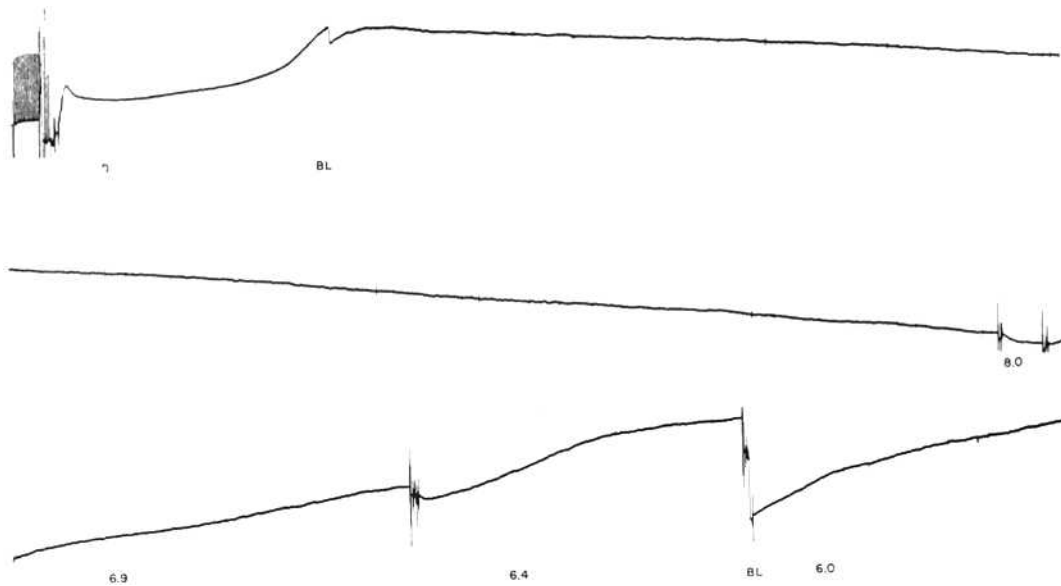


FIGURE 16. Rabbit right ventricular papillary muscle exposed to disruption solution and contracture solutions of stated pCa. *BL* indicates a shift of base line. Duration of exposures to *D*, 8.0, 6.9, 6.4, and 6.0 was 158, 4, 36, 29, and 28 min, respectively.

tion from the tension developed in the contracture solutions. The mechanical response to electrical stimulation returned to control values after a 30-60 min exposure to Krebs-Henseleit solutions with 2.5 mM Ca<sup>++</sup>.

The studies on rabbit heart, though relatively preliminary, are included to indicate the applicability of the technique to the mammalian heart.

#### DISCUSSION

The contractile system of frog ventricular strips that had been treated with a solution containing EDTA and a high concentration of KCl behaved as if the Ca<sup>++</sup> in the sarcoplasm equilibrated rapidly with Ca<sup>++</sup> in the bathing media. The relation between the Ca<sup>++</sup> concentration in the bathing solution and the tension developed by the tissue was similar to that observed in isolated myo-

fibrils (Weber and Herz, 1963; Katz, 1969) and in mechanically skinned skeletal muscle (Hellam and Podolsky, 1969). The speed of the response to a new concentration of  $\text{Ca}^{++}$  was relatively high if the concentration of the  $\text{Ca}^{++}$  buffer system in the bath was adequate and when the rate of rise of tension was relatively slow it could be substantially accelerated by an increase

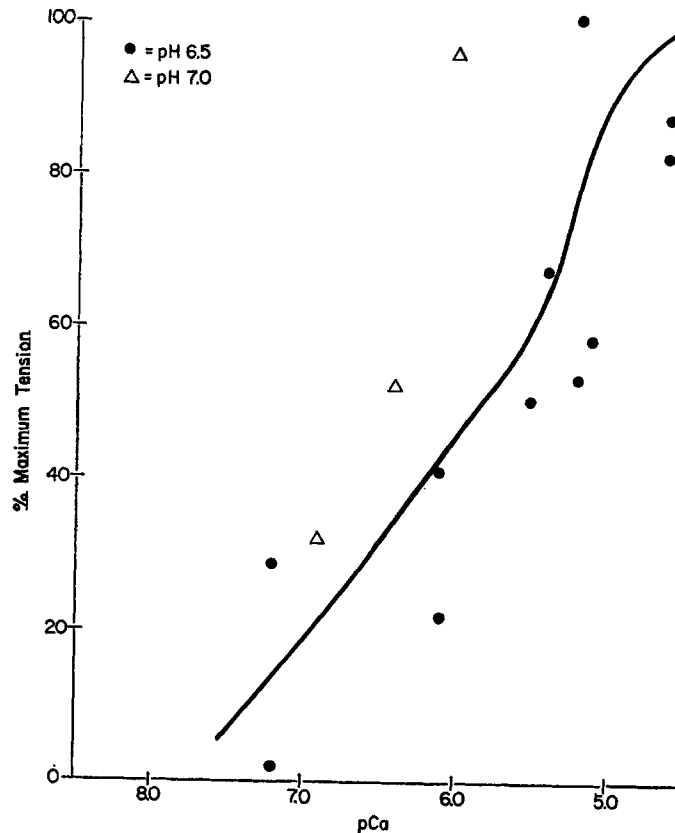


FIGURE 17. Plot of relative tension produced by rabbit right ventricular papillary muscles in contracture solutions containing different pCa. Each point represents one measurement from one of five muscles. pH of contracture solutions either 6.5 or 7.0 as indicated.

in the concentration of the  $\text{Ca}^{++}$  buffer. Even when the tension rose more slowly, its final relative value at a given pCa was approximately the same as the tension developed more rapidly at the same pCa in another preparation. The slower development of tension was probably due to a lower permeability of the membrane to CaEGTA and the competition of both the sarcoplasmic reticulum and the mitochondria with the contractile proteins for  $\text{Ca}^{++}$  diffusing into the sarcoplasm (Brierley et al., 1964; Chance, 1965). The delayed

rise in the rate of development of tension in some preparations could have been due to a relative saturation of the  $\text{Ca}^{++}$  binding sites competing with the contractile proteins during the early portion of the exposure to the contracture solution. These conclusions are supported by tracer studies of frog ventricles treated with Ringer solution containing added EDTA (Thomas, 1960 *a, b*). The cells of the EDTA-treated rabbit ventricle, however, did not appear to be

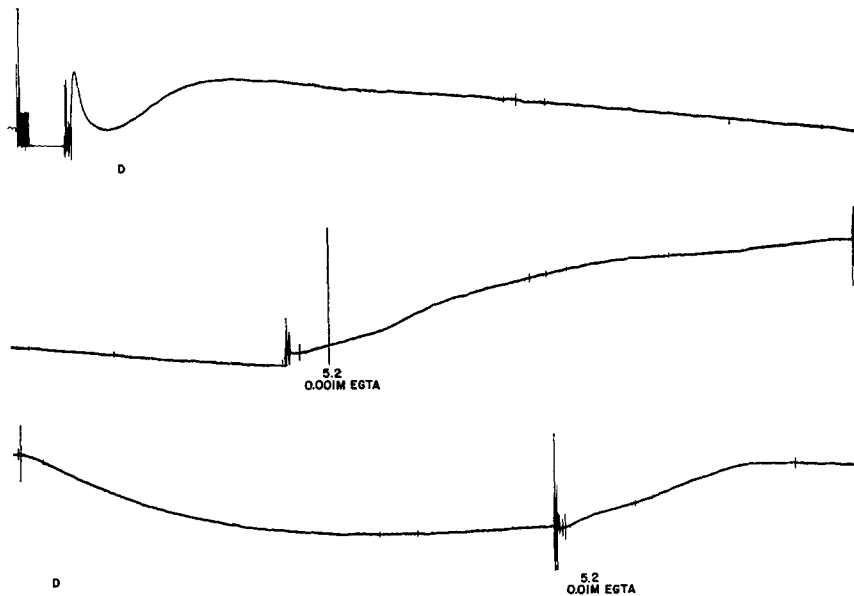


FIGURE 18. The effect of Ca buffer concentration on the mechanical response of rabbit right ventricular papillary muscle. Note the absence of any increase in the rate of rise of tension with an increase in the concentration of buffer (compare with Fig. 7). Duration of exposures to D, 0.001 M EGTA and 0.01 M EGTA was 92, 98, and 28 min, respectively.

permeable to CaEGTA as the rate of rise of tension was always slow, and it was not accelerated by a 10-fold increase in the concentration of the calcium buffer system in the bathing medium. These results suggest that a relatively high permeability to ionic calcium was a fairly constant property of the preparation but the permeability to another component of the  $\text{Ca}^{++}$  buffer system, CaEGTA, varied from a relatively low to a relatively high value. The steady-state tension at a given pCa depended only on permeability to  $\text{Ca}^{++}$  but the rate of change of tension depended on the permeability to the larger molecules in the  $\text{Ca}^{++}$  buffer system.

The membrane permeability to other ions was also markedly enhanced by the disruption solution. The almost complete disappearance of a resting potential demonstrates that the selective barrier to the flow of small univalent

ions had disappeared, and the impairment of relaxation in certain instances by Mg-free solutions indicates that the sarcoplasmic  $Mg^{++}$  had approached equilibration with bath  $Mg^{++}$  (Weber, 1969). Although tracer studies will be necessary to determine whether ATP crossed the membrane, it is clear that at least part of the rabbit ventricle was not adequately supplied by ATP from the bathing solution because of a diffusion barrier and/or hydrolysis in the superficial region of the tissue. The abrupt increase in the rate of relaxation of contracted rabbit heart produced by oxygenation suggested that the tissue was ATP deficient and that the mitochondria were functionally intact with adequate endogenous substrate for ATP synthesis. ATP is a larger molecule than CaEGTA and from simple considerations of both size and charge, one would expect ATP to be less permeant than CaEGTA.

There appear to be at least two functionally different types of interactions of calcium with the membrane, a cooperative one that is important in the general permeability of the membrane and involves calcium bound in the range of  $10^{-5}$  M or less, and a more specific one concerned with the conductance changes of the action potential (Frankenhaeuser and Hodgkin, 1957). The reason for describing the first as cooperative comes from the experiment shown in Fig. 8. Although no restoration of the low permeability of the membrane to  $Ca^{++}$  normally occurred at pCa of 6.4, if the recovery process had been first initiated at a pCa of 5.0 it continued at a pCa of 6.4. Apparently, after some of the calcium had been restored to the membrane, further accumulation did not require as high a concentration of  $Ca^{++}$ . Such a mechanism would enhance membrane stability, as the initial removal of calcium would require a very low  $Ca^{++}$  concentration.

The ratio of tension in electrically stimulated contractions to the maximum tension generated by direct  $Ca^{++}$  activation of the EDTA-treated heart was considerably less than one in both frog and rabbit. In the isolated heart, therefore, and possibly in the intact heart, the degree of activation by calcium during a contraction is generally much less than maximal. In the rabbit papillary muscle, in which the cell geometry permits a meaningful estimate of the density of myofibrils parallel to the axis along which tension is measured, the maximum tension developed was significantly less than one would have expected from maximal activation of the entire tissue. It is likely that all the cells in the treated rabbit heart did not have high permeabilities to  $Ca^{++}$ .

During the EDTA treatment of rabbit ventricle in the absence of oxygenation  $Ca^{++}$  was probably released from the sarcoplasmic reticulum because the sarcoplasmic [ATP] was too low to sustain the calcium pump. A large amount of tension was developed and sustained, however, when the concentration of  $Ca^{++}$  in the bath was raised. It appears as if the activated contractile proteins have a greater affinity for ATP than the calcium pump in the sarcoplasmic reticulum. This inference is the opposite of the data derived from studies of

isolated subcellular systems but consistent with studies of ATP levels of skeletal muscles in rigor (Kushmerick and Davies, 1968).

The reversibility of the increase in membrane permeability offers the attractive possibility of introducing normally impermeant molecules into the intracellular space and healing the membrane to trap the material inside the cell. In this way the intracellular effects of small, normally impermeant molecules might be studied. The permeability of the EDTA-treated frog membrane to CaEGTA, which has a molecular weight of about 400, gives some indication of a minimum limiting size of molecules that might pass through the disrupted membrane.

Although the ultrastructural studies of the cells treated with disruption solution are not yet sufficiently extensive to support detailed conclusions, they do seem to permit certain inferences. In frog ventricles treated with disruption solution subsarcolemmal cisternae were less frequent and the sarcoplasmic reticulum was swollen. The swelling was not reversed by 1 mM MgCl<sub>2</sub>. The frequency of normal subsarcolemmal cisternae increased and the amount of swelling of the reticulum markedly decreased when the cell membranes were bathed in 1 mM Ca-Ringer which restored normal mechanical and electrical activity. The reason for the swelling of the reticulum is not clear but two possibilities are: (a) An impairment of a transport system involved in the extrusion of osmotically active molecules or ions; and (b) an increase in the fixed charge within the reticulum by the removal of bound Ca<sup>++</sup> and/or Mg<sup>++</sup>. Normally the reticulum is bathed by a concentration of Ca<sup>++</sup> below 10<sup>-6</sup> M while the sarcolemma is bathed by 10<sup>-3</sup> M Ca<sup>++</sup>. The swelling of the reticulum in very low Ca<sup>++</sup> is better explained by some primary change in the sarcolemma and secondarily in the reticulum, possibly via a functional junction between the subsarcolemmal cisternae and the sarcolemma, than by a direct effect on most of the surface of the reticulum.

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