Contracture of Slow Striated Muscle during Calcium Deprivation

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ABSTRACT When deprived of calcium the slow striated muscle fibers of the frog develop reversible contractures in either hypertonic or isotonic solutions. While calcium deprivation continues because of a flowing calcium-free solution the muscles relax slowly and completely. Restoration of calcium during contracture relaxes the muscle promptly to initial tension. When relaxed during calcium lack the return of calcium does not change tension and the muscle stays relaxed. When contractures are induced by solutions containing small amounts of calcium relaxation does not occur or requires several hours. The rate of tension development depends upon the rate at which calcium moves outward since the contractures develop slower in low concentrations of calcium and are absent or greatly slowed in a stagnant calcium-free solution. Withdrawal of calcium prevents the contractile responses to ACh, KCl, or electrical stimulation through the nerve. Muscles return to their original excitability after calcium is restored. Origin of the contractures is unrelated to nerve activity since they are maximal during transmission failure from calcium lack, occur in denervated muscles, and are not blocked by high concentrations of *d*-tubocurarine, procaine, or atropine. The experiments also indicate that the contractures do not originate from repetitive activity of muscle membranes. The findings are most simply explained by relating the outward movement of calcium as a link for initiating contraction in slow type striated muscle.

INTRODUCTION

In slow type skeletal muscle fibers of the frog the well maintained contracture accompanying depolarization by acetylcholine or potassium does not develop unless calcium is included in the external fluid (Denton, 1948; Schaechtelin and Lüttgau, 1961; Brecht and Pauschinger, 1962). In a twitch muscle fiber system of this animal potassium also depolarizes but fails to produce contraction in a calcium-free solution (Frank, 1960, 1962). Without calcium the frog heart does not beat and remains in diastole although the electrical response stays essentially unchanged (Ringer, 1883; Locke and Rosenheim, 1907; Mines, 1913). In several invertebrate species heart muscle contracts upon

withdrawal of calcium (Prosser and Brown, 1961). External calcium is essential for contraction in smooth muscle and removal of calcium is followed by a small increase in tension (Hurwitz *et al.*, 1960; Edman and Schild, 1962). The dependence upon calcium for function of intact cells of cardiac, smooth, and skeletal muscle is therefore well established. Much less is known concerning contractile events in muscle which may precede loss of contractility because of the absence of calcium.

The present study describes the occurrence of contractures in slow skeletal muscles when the calcium in the external fluid is removed or reduced. The contractures develop during calcium deprivation and before the muscle loses its ability to contract in response to depolarizing stimuli. A contractile event in this kind of muscle thus precedes the uncoupling of excitation and contraction which occurs in the absence of calcium. Experiments are described which indicate that the contractures are not of neural origin.

METHOD

All experiments were performed from April through December on muscles of *Rana pipiens* obtained from Wisconsin. The frogs were stored at 4°C in the dark for varying periods. The fluid of the storage container was changed frequently and usually contained about 1.0 μg/ml chloramphenicol to prevent red leg. Each experiment was performed at room temperature in a 5 ml bath aerated at 10 to 15 ml/min. with 5 per cent CO_2 and 95 per cent O_2. Control solutions and solutions containing less than the control amount of calcium were flowed through the bath at about 30 ml/min. The direction of the flow was from below upward. The muscle pulled downward on the gauge and the attached nylon thread, opposite to the direction of the fluid flow. A rapid change from no flow to 30 ml/min. did not change the recorded base line tension. Resting muscle tension was adjusted to 1 gm and a four point dose response curve to acetylcholine was obtained at the start of each experiment unless otherwise indicated.

Muscle tensions were obtained using strain gauge outputs amplified by low-drift chopper amplifiers and recorded with pen writers. Periodic trials at similar gains showed no measurable amplifier drift during experimental durations. Each gauge and recording channel was calibrated before each experiment by static loading. Calibration was linear within measurement error.

Two solutions of different ionic content were used as control solutions. The solution most frequently used contained 154.0 mM NaCl, 5.6 mM KCl, 6.0 mM $NaHCO_3$, and 1.9 mM $CaCl_2$ and is called hypertonic control solution in these experiments. This solution had a pH of about 7.4, a tonicity of 336.9 milliosmols, and was 1.3 times the tonicity of solutions usually referred to as "frog Ringer's." The second control solution contained 117.0 mM NaCl, 3.0 mM KCl, 4.0 mM $NaHCO_3$, and 2.7 mM $CaCl_2$ and is called isotonic control solution (256 mOsM). This solution is the same as that used by Swift *et al.* (1960), is similar to solutions usually designated as "frog Ringer's" and had a pH of about 7.2. When calcium was omitted it was replaced by an osmolar equivalent of NaCl. The solutions were made from NIH distilled water in containers

cleaned periodically with acid. Since chelating agents were not used the "calcium-free" solutions may have contained small amounts of calcium. Reference to calcium-free indicates that no calcium was added.

Muscles stimulated through nerves were freed under a dissecting microscope and mounted horizontally in a 20 ml bath. The entire nerve trunks were freed back to the spinal cord and removed with the muscle. The rectus muscle is often supplied by several small nerve branches visible under low power. The two nearest the pelvis were suitable for stimulation and one of these was used in each experiment. Since each of the nerve branches innervates only a part of the muscle the tension developed after nerve stimulation was not from the entire muscle.

In 2 × 2 crossover experiments two experimental conditions were simultaneously observed in a pair of muscles from one frog. After recovery the experimental conditions were reversed to the opposite muscles and observed again. In 4 × 4 double crossover experiments two pairs of muscles from two frogs were used so that both the single muscles and the pairs could be evaluated in a crossover design. The latter design permitted the evaluation of four experimental conditions at the same time with crossover data gathered from each of four muscles and from each pair of muscles. Muscles used in the crossover experiments remained in calcium-containing solutions for 40 minutes before contractures were induced by calcium-free solutions. Contractures were observed for at least 30 minutes or until maximal contracture. Durations were constant for all muscles in a given experiment.

Muscles were denervated by division of all the nerve trunks on one side through a dorsal opening near the urostyle. The small lateral nerve branches to the rectus abdominis muscle were cut after reflecting the abdominal skin, and the soft tissues adjacent to the sternum were divided. In some frogs the two rectus muscles were separated along the linea alba leaving the blood supply intact; this procedure was later found to be unnecessary for denervation. Muscles were considered to be denervated when (*a*) the sensitivity to ACh was greatly increased and (*b*) electrical stimulation of the peripheral nerve trunk at numerous frequencies failed to elicit movement of the muscle when viewed under the dissecting microscope. Denervation was incomplete for many weeks when the frogs were stored at 4°C but occurred in each frog tested after 14 days at room temperature. Before use the frogs with denervated muscles were stored at 4°C for 1 or 2 days.

RESULTS

Development of Contractures in Hypertonic Calcium-Free Solutions

Contractures developed in slow muscle fibers of the frog when calcium was removed from the external fluid in which the muscles were kept. The top trace in Fig. 1 shows the development of a contracture of a rectus abdominis muscle typical of those seen when the fluid flowing through the bath was changed from a calcium-containing to a calcium-free solution. Contractures of this kind have been observed repeatedly; within 1 to 5 minutes tension begins to develop and reaches a maximum in 10 to 30 minutes after removal of calcium. Maximal tension after calcium withdrawal is less than maximal tension after

acetylcholine (ACh) stimulation. The contractures were completely reversible and could be repeated many times by changing the fluid in the bath alternately from calcium-containing to calcium-free. Contractures did not develop or were markedly reduced when the calcium-free solution stood stagnant in the bath or flowed through at only 1 ml/min. or less. In certain experiments the rate of contracture development was the same for 15 or 30 ml/min. rates

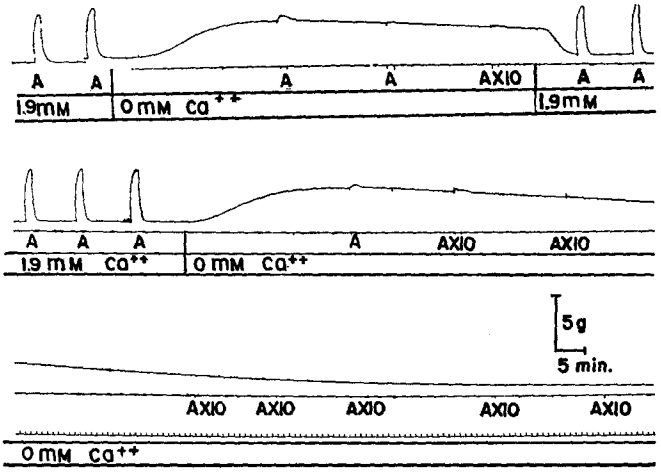


FIGURE 1. Contracture during calcium deprivation in a frog rectus muscle. Fluid flowed over muscle at 30 ml/min. *A*, acetylcholine chloride at 4×10^{-6} gm/ml left in muscle bath for 1 minute. Note that without calcium the response to 4×10^{-5} ACh ($A \times 10$) is prevented during contracture and relaxation. Restoration of calcium relaxes the muscle and returns the ACh response (continuous record).

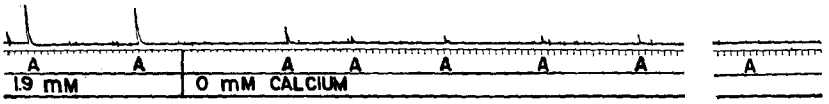


FIGURE 2. Absence of contracture and loss of response to ACh during calcium depletion in a fast fiber muscle (sartorius) of the frog. *A*, 8×10^{-5} gm/ml ACh in the bath for 1 minute. Time in minutes. Tension in response to ACh during 1.9 mM calcium was about 1 gm. Break in record represents 60 minutes

of flow. The 30 ml/min. rate of flow used was therefore probably not rate-limiting for contracture development.

Fig. 2 shows that contractures did not develop in a muscle containing predominantly twitch fibers, the sartorius, when a fluid flowing through the bath was also changed from calcium-containing to calcium-free. Sartorius muscles can be left in a calcium-free medium far longer than is necessary for maximal contracture in the rectus muscle with no more than transient changes in resting tension. There is thus a distinct contrast between the twitch and the slow

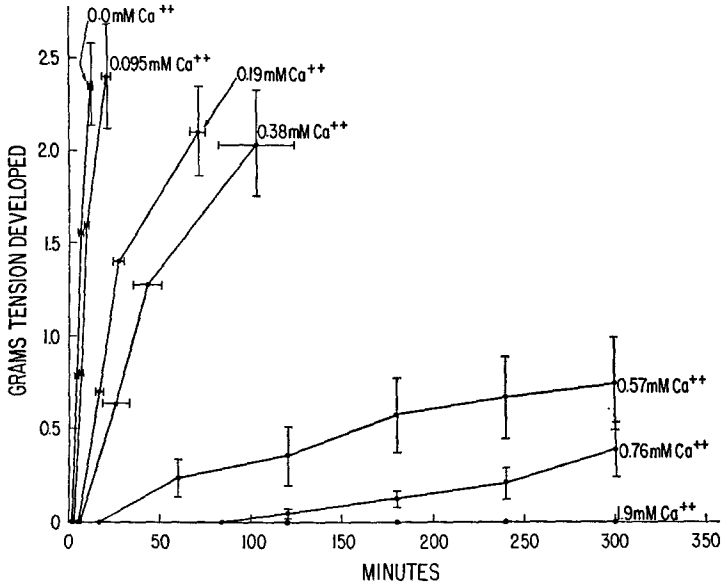


FIGURE 3. The onset and rate of contracture development in frog rectus during calcium lack. The top points in the upper four curves indicate peak tensions. Vertical and horizontal bars indicate standard error of the means. The experiments were performed in the hypertonic control solution flowing at 30 ml/min. Each line represents at least ten muscles.

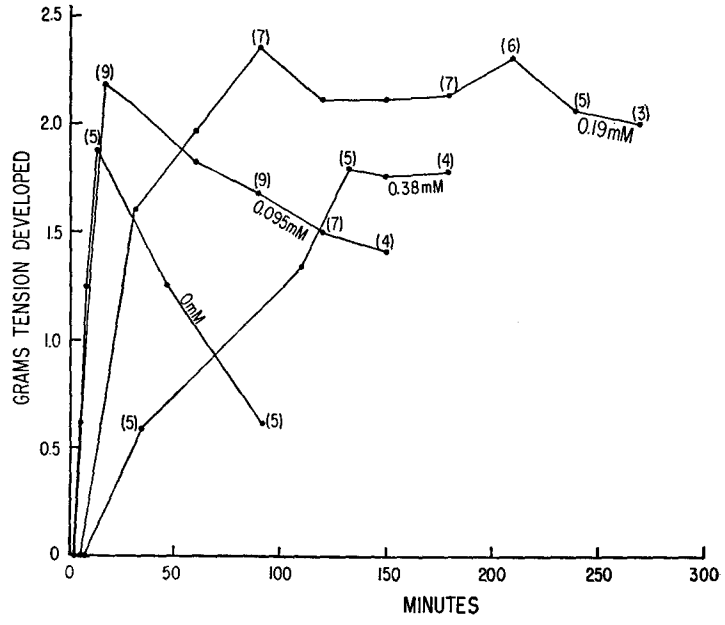
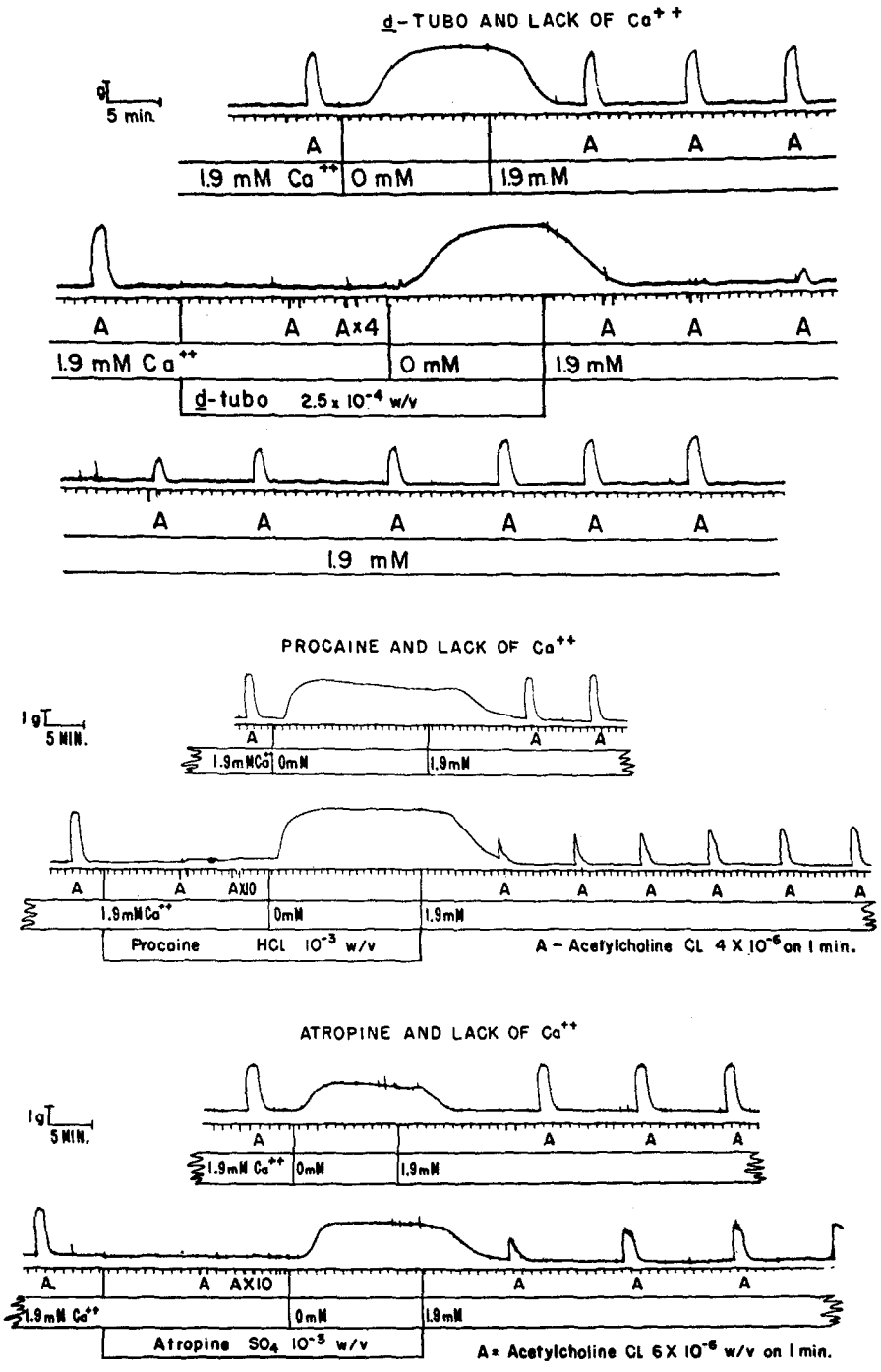


FIGURE 4. Relaxation after contracture in low concentrations of calcium. Parentheses indicate number of observations. Conditions as in Fig. 3.


d-TUBO AND LACK OF Ca++
5 min.
A
A
A
A
1.9 mM Ca++
0 mM
1.9 mM
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A Ax4
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A
A
1.9 mM Ca++
0 mM
1.9 mM
d-tubo 2.5 x 10-4 w/v
A
A
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A
1.9 mM
PROCAINE AND LACK OF Ca++
1g
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A
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1.9mM Ca++
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A
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AX10
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A
1.9mM Ca++
0mM
1.9mM
Procaine HCl 10-3 w/v
A - Acetylcholine CL 4 X 10-6 on 1 min.
ATROPINE AND LACK OF Ca++
1g
5 MIN.
A
A
A
A
1.9mM Ca++
0mM
1.9mM
A
A AX10
A
A
A
1.9mM Ca++
0mM
1.9mM
Atropine SO4 10-3 w/v
A= Acetylcholine CL 6 X 10-6 w/v on 1 min.

FIG. 5

type muscle fibers in response to a calcium-free solution; the slow fibers develop contractures, the twitch fibers do not.

When fluids with less than the control amounts of calcium flowed through the bath instead of calcium-free solutions the onset of contracture was delayed, the time necessary to reach maximal contracture was lengthened, and the tension of the maximal contracture diminished (Fig. 3). Contractures did not occur when enough calcium was included in the fluid flowing through the bath. A contracture developed in each muscle kept in a flowing bath of 0.38 mM calcium or less. In the presence of more calcium 0.57 or 0.76 mM, some muscles failed to develop tension. These zero values are included in the averaged data of Fig. 3 but preclude a statistical evaluation of time at these two calcium concentrations. Tensions were recorded for 5 hours in these latter two series and none of the muscles had reached a maximum tension. In addition to the muscles observed during this study, hundreds of muscles used for bioassay in our laboratory remained relaxed for as long as 16 hours when kept in the same hypertonic solution (1.9 mM Ca^{++}) mentioned in Methods. Solutions which contain 1 mM calcium are in prevalent use and frog muscles apparently do not contract at this or higher concentrations of calcium.

Relaxation after Contracture

The relaxations at various low calcium concentrations are shown in Fig. 4. When rectus muscles were left in the calcium-free solution the maximal contracture was not maintained and they completely relaxed. The original resting tension reestablished itself within about 2 to 3 hours. At low concentrations of calcium relaxation became less rapid. Only slight relaxation occurred at 0.19 mM and no relaxation was observed at 0.38 mM. Thus at an optimum concentration of calcium relaxation did not occur, or was greatly delayed, and the muscles maintained tension for long periods. Muscles in contracture because of a reduced amount of calcium relaxed promptly upon addition of calcium. In muscles which had previously contracted and relaxed in a calcium-free solution restoration of calcium did not produce a contracture (Fig. 1). Relaxation in the absence of calcium was slower in the isotonic control solution than in the hypertonic.

Lack of Calcium and Loss of Contraction to ACh Stimulation

The rectus and the sartorius muscles are similar in loss of ability to contract to ACh stimulation when calcium is absent (Figs. 1 and 2) in the bathing fluid.

FIGURE 5. Frog rectus contractures during calcium deprivation with and without *d*-tubocurarine Cl, procaine HCl, or atropine SO_4. *A*, acetylcholine chloride challenges (1 min.) at final bath concentration as indicated or at (10^{-6} w/v). Each of the drugs blocked the muscle to several times the concentrations of ACh necessary for a nearly maximal contracture before the bath fluid (30 ml/min.) was made calcium-free. Tension traces are essentially continuous for each drug. Only one drug was used for each muscle.

As the rectus muscle contracts after calcium withdrawal the response to ACh gradually diminishes and disappears. This occurs even though the muscle is not maximally contracted and under control conditions is capable of greater contraction. Concentrations of ACh much higher than needed for maximal stimulation also fail to produce contractures unless calcium is present (Fig. 1). The restoration of calcium, regardless of whether a muscle is relaxed or contracted in the absence of calcium, is followed by a prompt return of the ability of the muscle to respond to ACh stimulation. The sartorius muscle also contracts in response to ACh after readmission of calcium. The return of contractility with the restoration of calcium is more rapid than either the development of contracture or loss of ACh response following a change into calcium-free fluid.

TABLE I

CONTRACTURES IN CALCIUM-FREE SOLUTIONS WITH AND WITHOUT *d*-TUBOCURARINE Cl, PROCAINE HCl, OR ATROPINE SO_4

No. of muscles	No. of observations	Onset	Maximal contracture		Comment
		min.	*min.*	*gm.*	
14	14	1.47	12.6	2.35	Without *d*-tubocurarine 1st responses only
4*	7	1.6	12.9	2.66	With *d*-tubocurarine 5×10^{-4} w/v
	7	1.8	12.4	2.47	Without *d*-tubocurarine
6*	6	2.1	10.1	2.30	With procaine 10^{-3} w/v
	5	3.0	10.5	1.80	Without procaine
4*	4	1.6	8.3	1.83	With atropine 10^{-3} w/v
	3	2.2	10.0	1.63	Without atropine

* 2×2 crossover experiments.

Contractures in the Presence of d-Tubocurarine

The contractures developing during deprivation are unaffected by high concentrations of *d*-tubocurarine. Fig. 5 shows an experiment in which *d*-tubocurarine (2.5×10^{-4} gm/ml) prevented a muscle response to four times as much ACh as produced almost maximal tension but did not prevent contracture during calcium withdrawal. Challenges of the muscle with ACh after replacement of the calcium demonstrated that the muscle was blocked to ACh stimulation throughout the experiment.

Experiments such as those shown in Fig. 1 using a pair of rectus muscles from the same frog and a 2×2 crossover design gave the data in Table I and emphasize the lack of effect of *d*-tubocurarine on contracture development. Since the development of contractures has been observed in some muscle prepara-

tions during rapid stimulation of the nerve in the presence of *d*-tubocurarine we determined whether this occurred with frog rectus muscle as used in this study. Fig. 6 shows a nerve-muscle preparation experiment demonstrating that *d*-tubocurarine blocks the nerve activity of the rectus muscle at a variety of stimulation frequencies. In one part of the experiment the nerve was stimulated in the presence of *d*-tubocurarine for 9 minutes at 60 per second. No contraction or contracture developed. In this experiment 5×10^{-4} gm/ml *d*-tubocurarine was used to block the nerve activity. This high concentration of *d*-tubocurarine blocked these preparations for long periods to any further stimulation through the nerve. The block was maintained throughout the experiment since periodic stimulation at 1, 10, and 100/sec. did not produce any tension increase. Removal of calcium from the external medium produced a contracture the same as in an unblocked muscle. Stimulation of the muscle

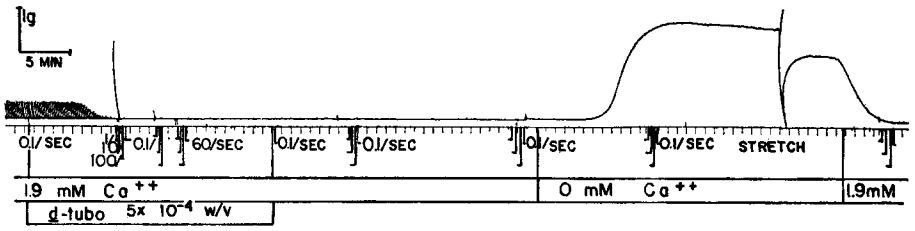

FIGURE 6. A rectus abdominis nerve-muscle preparation showing absence of contracture development during prolonged and rapid stimulation (60/sec. for 9 min.) in presence of *d*-tubocurarine and calcium and subsequent contracture development in response to calcium deprivation. The step marks downward indicate stimulation frequency at 0.1, 1.0, 10, and 100/sec. Failure of nerve stimulation to produce tension after calcium restoration to the bath indicates a transmission block by *d*-tubocurarine throughout the experiment.

by another series of stimuli after the calcium was replaced demonstrated that the muscle was completely blocked to nerve activity during the development of the contracture. The experiments thus determined that the slow muscle fiber system does not develop contracture when stimulated at rapid rates in the presence of *d*-tubocurarine and that repetitive activity of nerve is completely blocked by *d*-tubocurarine.

Contractures in the Presence of Procaine or Atropine

Fig. 5 shows an experiment in which 10^{-3} gm/ml of procaine in the control solution failed to prevent the contracture during calcium deprivation although the ACh responses were completely abolished. Data from crossover experiments showing the lack of procaine effect are given in Table I.

An experiment in which calcium was omitted during slow stimulation of the nerve is shown in Fig. 7. The top trace of the figure shows that as calcium is

withdrawn a contracture develops during the failure of transmission between nerve and muscle. Contracture is maximal while the stimulation of nerve is completely ineffective in producing muscle tension. Upon restoration of calcium, transmission is reestablished and the muscle relaxes. When 10^{-3} gm/ml procaine was flowed through the bath transmission also failed and contracture developed when the procaine solution was made calcium-free. After readmission of calcium to the procaine solution of the bath the muscle did not respond to direct electrical stimulation or ACh.

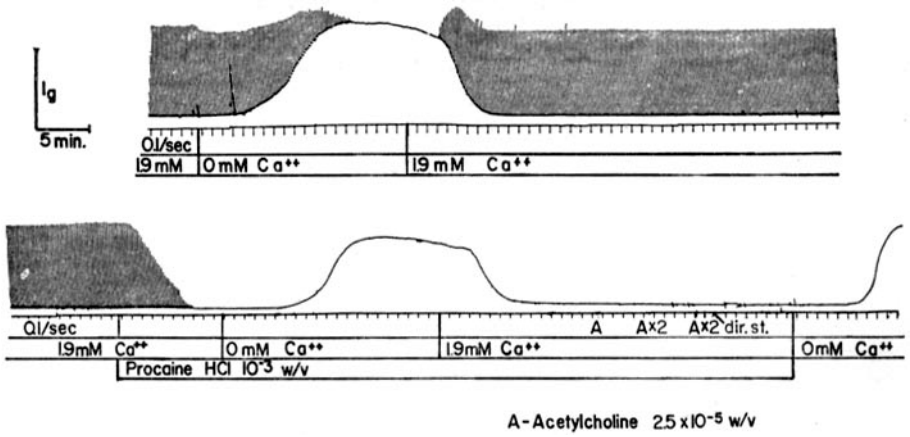

FIGURE 7. Transmission failure in the iliofibularis muscle during contracture development in response to calcium deprivation. Continuous stimulation of the nerve at 0.1/sec. throughout the experiment except at "dir. st." where an attempt was made to stimulate the muscle directly at different frequencies (0.1 to 100/sec.) for a few seconds at about 50 ma. Bottom trace shows contracture development in response to calcium deprivation during transmission failure from procaine.

The contractures in response to calcium depletion also occur when muscles are blocked to ACh stimulation by atropine SO_4 (10^{-3} gm/ml) (Fig. 5). Data from crossover experiments using atropine are shown in Table I.

Calcium Deprivation and Contracture Development in Denervated Muscles

Fig. 8 shows an example of contracture development during calcium deprivation in a denervated muscle. Electrical stimulation of the degenerated peripheral nerve trunks in these preparations did not produce muscle movement. The use of *d*-tubocurarine (5×10^{-4} gm/ml) or procaine (10^{-3}) with the denervated muscles eliminated contractile responses to ACh but did not prevent contractures induced by either isotonic or hypertonic calcium-free solutions. In some denervated muscles small random fluctuations of tension indicative of membrane instability were superimposed on the steadily developing contracture. These were more prevalent in muscles that developed less than the

average amount of tension. The small tension fluctuations disappeared with repeated use of calcium-free solutions and were prevented by procaine. The abolition of these small tensions by procaine or repeated use of the muscle did not appreciably affect the contractures occurring during calcium deprivation.

Contractures in Various Ionic Solutions

Except for some observations with denervated muscles the experiments described until now were performed using the hypertonic control solution mentioned in Methods. The contractures which occurred in calcium-free hypertonic solution always developed steadily and without fluctuations in the tension trace or visible indication of random activity of muscle fibers. In the

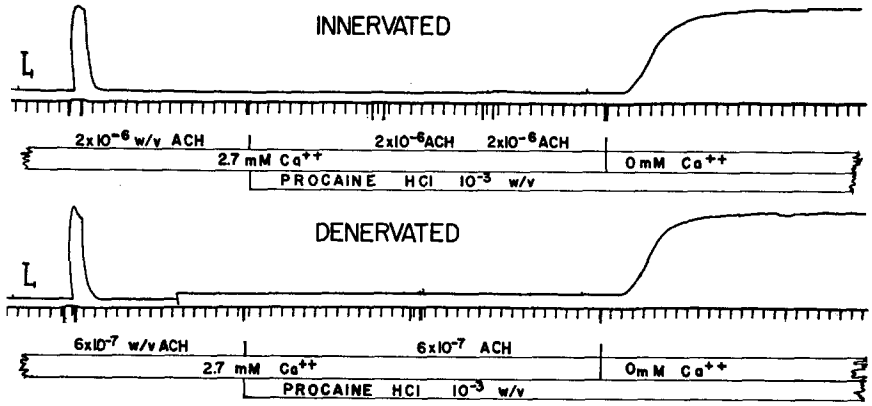

FIGURE 8. Contracture development in a denervated rectus muscle during calcium deprivation in the presence of procaine. The nerves to one muscle of the pair were cut 14 days before the experiment. Electrical stimulation of the degenerated nerve trunk failed to produce muscle movement. Calibration mark indicates minutes and grams. The experiment was performed in isotonic Ringer's flowing at 30 ml/min.

control isotonic solution without calcium some muscles showed small rapid contractions and relaxations superimposed at random upon the prolonged contracture. Other muscles in calcium-free isotonic solution developed tension as steadily as in the hypertonic solution. Both *d*-tubocurarine and procaine blocked the randomly occurring small tension changes observed in isotonic calcium-free solution without affecting the sustained contracture.

The contractures developed more slowly in the calcium-free isotonic than in the hypertonic solutions. In addition to other differences the isotonic solution contained more calcium than the hypertonic one. Table II shows data from crossover experiments performed to determine what accounted for the slower development of the contractures. The experiments indicate that the difference in the amount of calcium in the hypertonic compared to the isotonic solution failed to influence appreciably either the amount or rate of de-

velopment of tension. Replacement of KCl with NaCl in the hypertonic solution did not change the onset or development of the contractures. A 4 × 4 crossover experiment indicated that tension developed more slowly in isotonic solutions; the amount of tension was essentially unchanged. The slower rate in the modified hypertonic control solution with the tonicity reduced to 256 milliosmolar was nearly the same as the rate in the isotonic control solution. This indicates that the slower contractures were due solely to a change in

TABLE II

CONTRACTURES IN DIFFERENT CALCIUM-FREE IONIC SOLUTIONS

Ionic media before Ca^{++}-free contracture				Muscles	Observations	Contracture			Comment
Milliosmol	$CaCl_2$	$NaHCO_3$	KCl	No.	No.	Onset	Maximal		
	mM	*mM*	*mM*			*min.*	*min.*	*gm*	
337	1.9	6.0	5.6	21	21	1.7	12.6	2.2	1st response only
337	1.9	6.0	5.6	2	5	1.5	17.8	2.8	2 × 2 crossover
337	2.7	6.0	5.6		6	1.7	19.4	2.8	
256	2.7	4.0	3.0	2	6	3.0	24.4	4.3	2 × 2 crossover
256	1.9	4.0	3.0		5	3.2	24.8	4.3	
337	1.9	6.0	5.6	4	5	2.1	21.6	3.0	No Ach
256	2.7	4.0	3.0		7	2.2	32.8	3.6	2 × 2 crossover
337	1.9	6.0	5.6	6	6	2.3	24.8	3.0	ACh*
256	2.7	4.0	3.0		6	3.4	57.1	3.1	2 × 2 crossover
337	1.9	6.0	5.6	4	6	2.2	16.3	1.8	No ACh
256	1.9	6.0	5.6		7	2.9	24.2	2.0	4 × 4 crossover
256	2.7	4.0	3.0		6	3.1	26.2	2.2	
337	2.7	4.0	3.0		7	3.5	19.5	2.0	

* ACh challenges given between calcium-free contractures.

tonicity rather than to a difference between the two control solutions in calcium, bicarbonate, or KCl.

The challenging of muscle with ACh between repeated withdrawals of calcium slows the development of the contractures (Table II).

DISCUSSION

Knowledge of the contractures of slow skeletal muscle fibers during calcium depletion may further understanding of electromechanical coupling of muscle, providing the contractures do not originate from spontaneous repetitive activity of nerves or muscle membranes. The function of calcium in the excita-

tion and stability of nerve and muscle has been investigated extensively since the work of Ringer (1883) and excellent reviews are available (Brink, 1954; Shanes, 1958; Hajdu and Leonard, 1960; Shanes, 1961; Rodahl and Horvath, 1962; Leonard and Hajdu, 1962). Nerves become spontaneously active when the calcium concentration is low (Lehmann, 1937; Brink and Bronk, 1937; Brink *et al.*, 1946). Early in this study we questioned whether spontaneous activity of the nerves was the origin of the contractures occurring during calcium deprivation since such activity would perhaps explain the occurrence of the contractures. A careful consideration, however, leads to questioning of this explanation for a number of reasons. Frankenhaeuser (1957) has shown that single fibers of frog nerve fail to conduct impulses within seconds when the fluid bathing a node of Ranvier is made calcium-free. Spontaneous activity of the frog sciatic nerve is greatly reduced in 2 per cent CO_2 (Lorente de Nó, 1947). The present experiments were performed with 5 per cent CO_2. The earliest investigations of excitation of muscle by stimulation through nerve revealed that removal of calcium from the fluid bathing a nerve-muscle preparation leads to a block of transjunctional excitation (Locke, 1894). The end-plate potential decreases as the concentration of calcium is lowered (del Castillo and Stark, 1952) and at less than about one-fourth normal, excitation between nerve and muscle is blocked (Kuffler, 1943; Hunt and Kuffler, 1950; Fatt and Katz, 1952 *a*, 1952 *b*). This failure of frog nerve to elicit contraction in the slow type muscle in the absence of calcium was shown in our experiments by stimulating the nerve and recording muscle tension as a calcium-free solution flowed through the bath. While the contractures developed the nerve-muscle preparation became inexcitable to stimulation through the nerve. At the point where supermaximal stimulation was totally ineffective in eliciting tension the contractures were maximal. That spontaneous firing of neurons would occur while the nerve is inexcitable to electrical stimulation seems unlikely. The temporal relationships between the failure of muscle contraction after nerve stimulation and contracture development are therefore directly opposite to prediction, if the origin of the contractures relates to nerve activity.

Further data supporting the interpretation that nerve activity is not involved came from experiments using procaine. Concentrations of procaine which blocked nerve or ACh stimulation failed to prevent the contractures. Although atropine prevented ACh stimulation of the rectus muscle, it also failed to influence the contractures. In addition, contractures developed in denervated muscles kept in flowing calcium-free solutions, either with or without *d*-tubocurarine or procaine. Because of these findings it appears evident that the origin of the contractures developing during calcium deprivation in slow type skeletal muscles is unrelated to nerve activity.

Whether muscle membranes become spontaneously active without calcium is open to question. For fast type fibers, but not for slow, Kuffler (1943) re-

ported that membranes were spontaneously active in calcium-free solutions. This belief was based on the failure to record back-firing in anterior roots. It is now known that nerve endings release ACh asynchronously, which could activate single muscle fibers without antidromic excitation of an entire neuron. The statements of Kuffler (1943) and Brink (1954) concerning spontaneous activity of muscle fibers in calcium-free solutions in the presence of curare can be traced to the work of Adrian and Gelfan (1933). The spontaneous activity they observed was not blocked by curare because it arose from injured fibers and thus furnishes no pertinent information. Occurrence of spontaneous activity of muscle fibers in calcium-free solutions in the presence of blocking concentrations of *d*-tubocurarine is thus in doubt for fast fibers and has not been demonstrated for the slow type.

The slow fibers, in which the contractures occur, have markedly different innervation (Tasaki and Mizutani, 1944) and electrical properties from fibers of the fast twitch type (Kuffler and Gerard, 1947; Kuffler *et al.* 1947; Kuffler and Vaughan Williams, 1953 *a*, 1953 *b*; Peachey, 1961). Slow fibers maintain contracture in a graded fashion to depolarizing concentrations of ACh or potassium but the fast ones do not. ACh depolarizes all areas of a slow fiber equally well in contrast to a localized depolarization at the end-plate region in a fast fiber (Burke and Ginsborg, 1956 *a*, 1956 *b*). Whether end-plates are so closely adjacent that their depolarizing areas overlap or whether the entire muscle membrane is equally sensitive to ACh is apparently unknown. Propagated action potentials do not occur in the slow type fibers even when completely depolarized (Burke and Ginsborg, 1956 *a*). Therefore, the slow fibers, in effect, do not have areas in the membranes less responsive to ACh than the end-plates whereas in twitch fibers the muscle membranes away from the end-plates are much less sensitive. It appears that *d*-tubocurarine would stabilize the entire membrane of the slow type fiber since it completely blocks the activity of ACh. However, *d*-tubocurarine does not stabilize all of the membrane to electrical stimulation because both types of muscles contract fully when kept in high concentrations of *d*-tubocurarine and stimulated directly (Kuffler and Vaughan Williams, 1953 *b*; Irwin and Wells, 1959). Although *d*-tubocurarine blocks the ACh response, some areas of the slow fiber membranes could possibly become spontaneously active when calcium is removed. If the contractures arose from firing of the membrane areas not sensitive to *d*-tubocurarine, then a difference in contracture development between experiments with *d*-tubocurarine and those with procaine should have been seen because the stabilizing effects of procaine on muscle membranes are not restricted to ACh-sensitive areas (Shanes, 1958). No such differences were found. The contractures developed equally well in either *d*-tubocurarine or procaine solutions and were not prevented by concentrations of procaine which blocked both direct electrical stimulation and ACh-elicited contractures. It seems un-

likely that muscle membranes stabilized to this extent by procaine would be spontaneously active and produce large contractures.

We have found no published reference to the question of whether slow skeletal muscle fibers depolarize in the presence of procaine when calcium is withdrawn. Small depolarizations occur in twitch muscle fibers during calcium deprivation (Bülbring *et al.*, 1956; Ishiko and Sato, 1957; Koketsu *et al.*, 1962). The amount of depolarization reported seems too small to account for the tension developed in our experiments.

When a slow muscle reaches complete relaxation in a calcium-free solution the return of calcium does not reestablish contracture although the response to ACh or KCl is rapidly restored. A concept may thus be proposed for the slow striated type of muscle relating the outward movement of calcium to contraction. This concept leads to serious questioning of the belief that contraction is in general initiated by the influx of calcium (Sandow, 1952; Frank, 1958; Winegrad, 1961; Shanes, 1961; Bianchi, 1961). In fast type fibers contraction does not occur in response to a rate of calcium deprivation produced by changing the calcium concentration to zero in the external fluid. The rate at which calcium leaves the slow muscle determines the rate of contracture development because (*a*) solutions with low calcium concentrations give a slower rate than calcium-free solutions and (*b*) muscles kept in a stagnant calcium-free fluid fail to develop tension. Thus in a fast muscle, which does not maintain tension during persistent depolarization, the exit of calcium by changing the external fluid may be too slow to initiate contraction. Perhaps a sudden change in membrane polarization would move the calcium outward fast enough. The fact that calcium deprivation does not induce contraction in fast fibers does not necessarily invalidate the concept that the movement of calcium outward initiates contraction. Both calcium efflux and influx increase during muscle contraction (Woodward, 1949; Shanes, 1958, 1961; Bianchi and Shanes, 1959; Shanes and Bianchi, 1960). The experiments measuring calcium influx have led to correlations between contraction and inward movement of calcium. The influx experiments by necessity occurred under conditions which depolarized the muscle membranes. Calcium movement, both inward and outward, as measured by flux experiments may reflect nothing other than increased mobility of ions in both directions across a more porous membrane. This would explain an increased flux measurement in both directions during contraction and why stimulated muscle neither loses nor gains calcium (Fenn *et al.*, 1938; Liu, 1962). An action potential may move calcium between compartments within the muscle fiber; possibly between sarcoplasmic tubules and the myofibrillar contractile proteins. If so, then the measurement of a calcium flux would correlate more with depolarization than with contraction. This concept also avoids the need to explain how less than one molecule of calcium moving into the muscle per molecule of contractile protein

could fully contract a muscle (Winegrad, 1961); also why high concentrations of external calcium decrease contractures produced by depolarization in slow muscle of the frog (Shanes, 1961).

The experiments of Heilbrunn and Wiercinski (1947), Niedergerke (1955), and Podolsky (1962) emphasize the importance of calcium in initiating contraction, but their experimental conditions allowed a saturation of the calcium sites without reference to the direction of calcium movement.

Proposals based on the interaction of calcium with relaxing factor, ATPase, and the contractile proteins may be formulated agreeing with the concept that contracture relates to the movement of calcium outward. Until more information is available on the concentrations and movements of calcium within the muscle fiber compartments, any such proposal would be speculative. Although the experiments reported here suggest that the contracture of calcium deprivation is not related to slow depolarization of the muscle membrane more direct evidence is needed.

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