Studies on the in Vitro Interaction of Electrical Stimulation and Ca^{++} Movement in Sarcoplasmic Reticulum

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ABSTRACT Sarcoplasmic reticulum fragments (S.R.F.) were isolated from skeletal and heart muscles. These fragments were found to take up Ca^{++} very actively from media. When monophasic square waves were passed through the S.R.F. suspension, the Ca^{++} uptake by S.R.F. was decreased. When the suspension was stimulated electrically after the Ca^{++} was taken up by S.R.F., the initiation and the cessation of the stimulation were followed by the release and re-uptake of Ca^{++} by S.R.F., respectively. The degree of inhibition of the Ca^{++} uptake as well as of the Ca^{++} release by electrical stimulation was dependent on the voltage and the frequency of stimulation. The presence of inorganic phosphate or oxalate modified the influence of electrical stimulation on the release and the uptake of Ca^{++} by S.R.F. Attempts were made to observe the release of Ca^{++} by electrical stimulation from unfractionated sarcoplasmic reticulum remaining in myofibers, and the interaction of the released Ca^{++} with myofibrils in vitro. For this purpose, the glycerol-extracted fiber was selected as a muscle model, since it contains both sarcoplasmic reticulum and myofibrils. It was found that electrical stimulation of skeletal and heart glycerol-extracted fibers resulted in the contraction of fibers. It appeared that the contraction of glycerol fibers by electrical stimulation was caused by the Ca^{++} release from sarcoplasmic reticulum by stimulation.

The role of calcium as a mediator of the so-called excitation-contraction coupling in muscle and the identification of the "calcium pump" mechanism of the sarcoplasmic reticular system as the cause of relaxation appear to have been well documented (Ebashi, 1961 *b*; Ebashi and Lipmann, 1962; Fanburg et al., 1964; Hasselbach and Makinose, 1963; Martonosi and Feretos, 1964; Muscatello et al., 1962; Revel, 1964; Shanes, 1958; Weber et al., 1964). According to the current concept of excitation and contraction coupling, elec-

trical excitation of muscle membrane releases the Ca^{++} from the sarcoplasmic reticulum which initiates muscular contraction. After the contraction, the sarcoplasmic reticulum (or relaxing factor) again takes up Ca^{++} from the cytoplasm causing the relaxation of myofibrils. The above concept has wide support from the following findings; first, the very close anatomical relationship of sarcoplasmic reticulum and myofibrils as shown by electron microscopic studies (Edwards et al., 1956; Fawcett and Revel, 1961; Franzini-Armstrong and Porter, 1964; Palade, 1956; Porter, 1956 and 1961; Porter and Armstrong, 1965; Porter and Palade, 1957; Revel, 1964); second, the essential requirement of Ca^{++} for contraction of myofibrils (Ebashi, 1961*a* and 1961*b*; Podolsky and Constantin, 1964; Seidel and Gergely, 1963; Weber and Winicur, 1961; Weber and Herz, 1963; Weber et al., 1963); third, and the most important, the very active uptake of Ca^{++} by isolated fragments derived from sarcoplasmic reticulum and a good correlation of this Ca^{++} uptake mechanism and its relaxing activity (Ebashi, 1961*a*; Fanburg et al., 1964; Hasselbach and Makinose, 1961, Weber et al., 1963 and 1964). However, at the present time, no experimental evidence is available which directly supports the concept that there is a release of Ca^{++} from the sarcoplasmic reticulum following membrane excitation and a re-uptake of Ca^{++} following the cessation of the excitation. In the present investigation, attempts have been made to demonstrate in vitro that the electrical stimulation of isolated sarcoplasmic reticulum fragments (S.R.F.) releases Ca^{++} from S.R.F. and cessation of the stimulation is followed by the re-uptake of Ca^{++} by the S.R.F. For this purpose, S.R.F. from skeletal and heart muscle were isolated and the effect of electrical stimulation on the Ca^{++} uptake of the isolated S.R.F. was studied. In addition, the effect of electrical stimulation on glycerol-extracted fibers was studied to supplement the findings obtained with isolated sarcoplasmic reticulum.

METHODS

Preparation of Isolated Sarcoplasmic Reticulum Fragments

Skeletal and cardiac sarcoplasmic reticulum fragments were prepared from rabbit skeletal and dog heart muscle, respectively, using the same procedure as described previously (Lee et al., 1965*c*) with following minor modifications. All extraction media contained α-tocopherol, 0.2 mM, since it was found that the presence of this agent in the extraction medium preserved the stability of Ca^{++} uptake function in the S.R. from bovine heart (Inesi et al., 1964) and the last precipitate (S.R.F.) was resuspended in 0.02 M Tris-maleate buffer pH 6.5 instead of histidine-KCl solution used previously. Electron microscopic examination (kindly done by Dr. L. Hermann, Department of Pathology, State University of New York, Downstate Medical Center) showed that both cardiac S.R.F. and skeletal S.R.F. preparations consisted almost

exclusively of isolated sarcoplasmic reticulum fragments similar to those shown previously by Ebashi and Lipmann (1962).

Assay of Uptake and Release of Ca^{++} by S.R.F.

S.R.F. was suspended in the medium containing Ca^{45} and other components as indicated in the text and figures. In some experiments, mostly with long incubation periods, the suspension medium was incubated in a test tube and

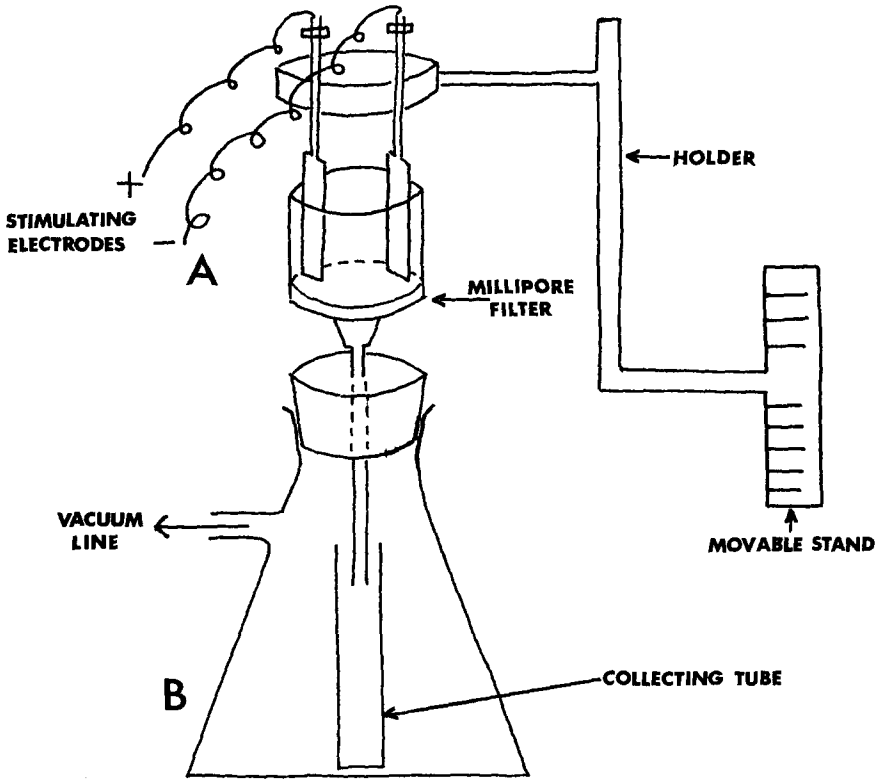

FIGURE 1. Apparatus for electrical stimulation and filtration of sarcoplasmic reticulum.

samples taken from the suspension were placed in the syringe space (*A* in Fig. 1). The sample was filtered through a Millipore filter (Millipore Filter Corporation, Bedford, Mass.) by gently applying a negative pressure in the bottom flask (*B* in Fig. 1), and the filtrate collected in a small vial inside the bottom flask. In other experiments, the suspension was directly placed in the syringe tube space (*A*), incubated for the desired period, and then filtered through the Millipore filter. Millipore filters used were 0.45 μ and 0.35 μ pore size depending on the amount of S.R.F. used. The radioactivity of the filtrate was determined and the calculation was made from these values for the

amount of Ca^{++} taken up by or released from S.R.F. All reactions were performed at 25°C and started by adding S.R.F. last unless specified otherwise.

Electrical Stimulation of S.R.F.

For stimulation of the suspension medium containing S.R.F., a pair of platinum electrodes (4 mm wide, 7 mm long, 3 mm apart) was inserted in the syringe tube space as shown in Fig. 1. When monophasic electrical stimulation was applied, square wave impulses of 10 msec duration with varying voltages and frequencies were passed through the suspension. The voltage, frequency, and the duration of stimulation recorded in tables and figures are those indicated on the Model S4 Grass stimulator. In order to electrically stimulate larger quantities of S.R.F. suspensions, larger platinum electrodes (4 mm wide, 10 mm long, and 10 mm between the two electrodes) were used in 30 ml beakers. In some experiments, a pair of silver-silver chloride electrodes (1 mm diameter, 7 mm long, 3 mm apart) was used in the syringe tube space shown in Fig. 1.

Preparation and Tension Recording of Glycerol-Extracted Fibers

Glycerol-extracted skeletal and heart muscle fibers were prepared from rabbit psoas muscle and trabecular muscle from dog ventricle, respectively, according to the method described previously (Lee, 1961). However, in the case of skeletal muscle, fibers were separated manually instead of using blades. The method of recording the tension development of fibers was also the same as described previously (Lee, 1961).

Electrical Stimulation of Fibers

A pair of platinum-stimulating electrodes (4 mm wide, 3 mm long, and separated by 5 mm) were placed in a muscle chamber one on each side of a muscle fiber as shown in Fig. 2 and electrical impulses were passed through the medium across a muscle fiber. A Grass stimulator Model S4 was used and voltages indicated on the stimulator are recorded in the tables and figures.

RESULTS

Experiments Using the Isolated Sarcoplasmic Reticulum

SKELETAL MUSCLE SARCOPLASMIC RETICULUM FRAGMENTS (S.S.R.F.)

S.S.R.F. were found to take up Ca^{++} from the medium very actively in the presence of 3 mM adenosine triphosphate (ATP), 1 mM creatine phosphate (CP), creatinephosphokinase (CP kinase), and other components as shown in Fig. 3. Over 70% of the Ca^{++} was taken up by S.S.R.F. from a medium containing 30 μM $CaCl_2$ in 3 min when no electrical current was passed through the medium. However, when the monophasic square wave of 10

msec duration was passed through the medium at a frequency of 60 per min at varying voltages, the per cent of Ca^{++} taken up by S.S.R.F. during the incubation period fell markedly. The degree of inhibition of Ca^{++} uptake by the electrical stimulation was dependent on the stimulation voltage. Thus, 0.5 v had some effect, but the maximum inhibitory effect of stimulation was obtained at 2 v and a further increase to 4 v did not increase and often even decreased the degree of inhibition. Thus, monophasic square waves of 2 v, 10 msec duration, and 60 per min, inhibited Ca^{++} uptake of S.S.R.F. to about half of that observed without stimulation. It was found that biphasic

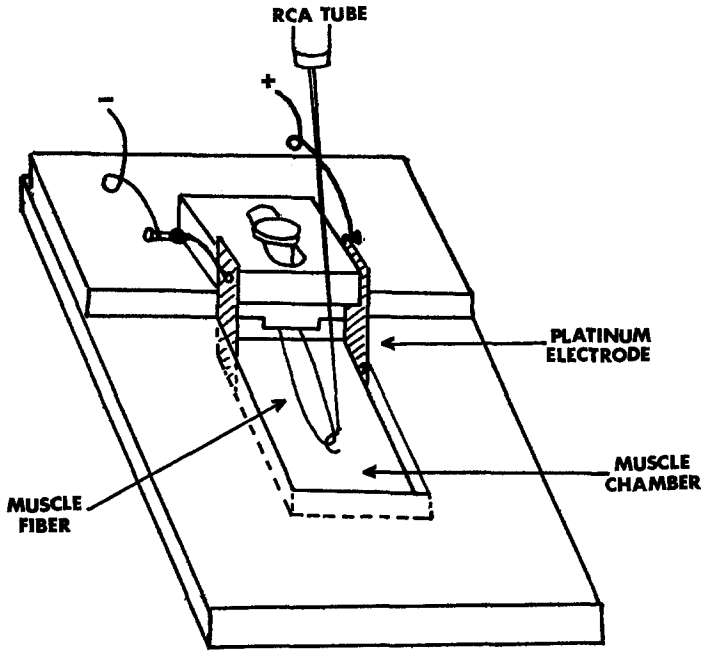

FIGURE 2. Apparatus for recording of tension and electrical stimulation of glycerol-extracted fibers.

electrical stimulation did not show a significant inhibition of Ca^{++} uptake by S.S.R.F. even at 6 v. It should be mentioned that in preparations which showed only a weak capacity of Ca^{++} uptake, it was difficult to demonstrate the inhibition of Ca^{++} uptake by electrical stimulation, although the tendency could be noted. Thus, fresh and potent preparations showed the best results with regard to electrical stimulation.

The effect of electrical stimulation was also frequency-dependent at constant voltage. Thus, the inhibitory effect of stimulation with square waves of 2 v and 10 msec duration with a frequency of 20 per min was less than half of that with a frequency of 60 per min (see Fig. 4). This effect of frequency of

stimulation will be shown more clearly with cardiac sarcoplasmic reticulum fragments. The effect of electrical stimulation started at different times during the Ca^{++} uptake phase of single incubations is shown in Fig. 5. In this experiment, aliquots from a single suspension were placed in five different Millipore syringes and filtered through the Millipore filter after the incubation with different experimental conditions as indicated in this figure. During the 3

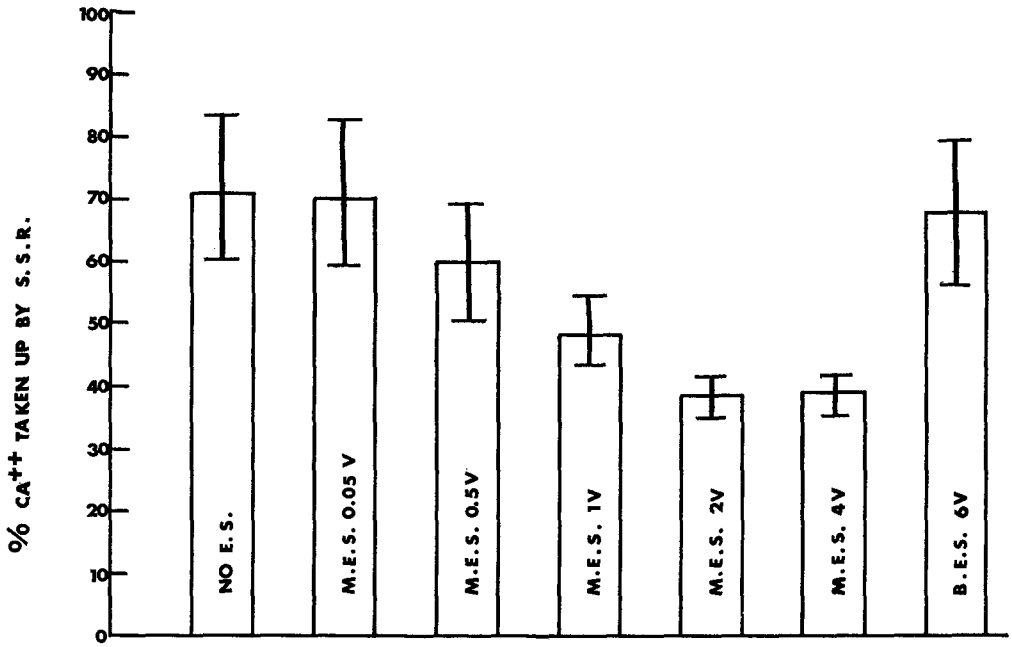

FIGURE 3. Effect of stimulation voltage on Ca^{++} uptake of S.S.R.F. All tubes contained; 0.02 M Tris-maleate buffer (pH 6.5), 0.1 M KCl, 2 mM $MgCl_2$, 3 mM ATP, S.S.R.F. 0.5 mg protein/ml, 1 mM CP, 0.1 mg/ml CPkinase and 30 μM total Ca^{++} ($CaCl_2$ + Ca^{45} Cl_2). Monophasic electrical stimulation (M.E.S.) was 10 msec in duration, 60/min. Biphasic electrical stimulation (B.E.S.) was 5 msec in duration, 60/min. Incubation, 3 min period. Each column is an average of 8 experiments. Vertical bars in this and following figures represent standard errors.

min incubation period, 67% of the Ca^{++} was taken up by S.S.R.F. when there was no electrical stimulation (first column) whereas only 43% of the Ca^{++} was taken up by S.S.R.F. when the suspension was stimulated (column 2). During the 6 min incubation period without stimulation, 72% of the Ca^{++} in the medium was taken up by S.S.R.F. (column 3). When the first 3 min incubation period without stimulation was followed by the second 3 min incubation period with stimulation, 48% of the Ca^{++} was taken up by S.S.R.F. (column 4). This indicated that a large portion of the Ca^{++} taken up during the first incubation period without stimulation was released during the sec-

ond period with stimulation. On the other hand, when the first 3 min incubation with stimulation was followed by the second 3 min period without stimulation, 68% of the Ca^{++} was taken up by S.S.R.F. (column 5). Since S.S.R.F took up only 43% of the Ca^{++} from the medium during the first 3 min incubation period with stimulation (column 2), this indicated that the Ca^{++} which was not taken up during the initial 3 min period with stimulation was subsequently taken up during the second period without stimulation.

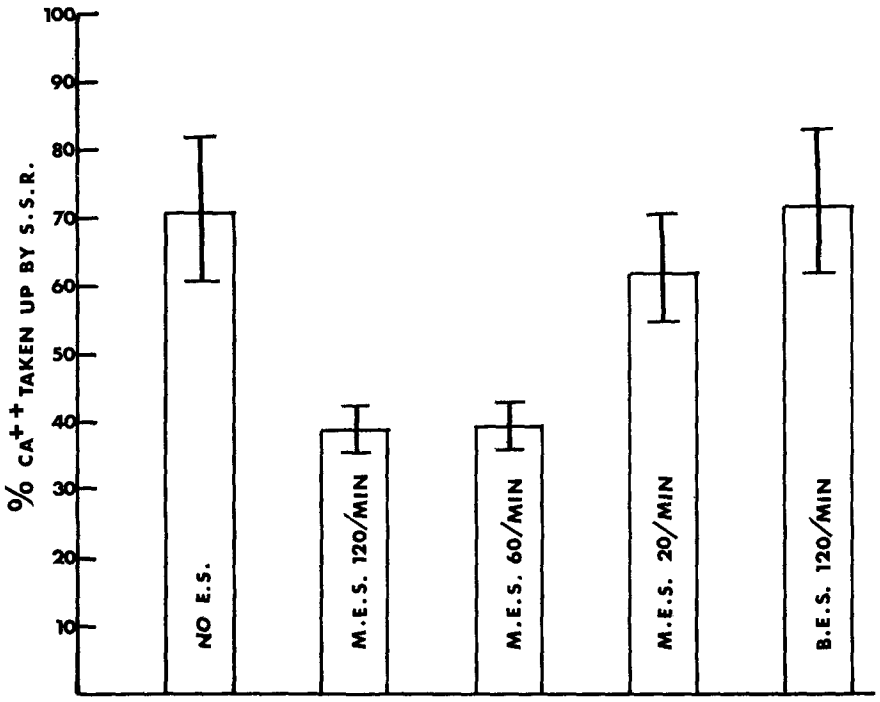

FIGURE 4. Effect of frequency of stimulation on Ca^{++} uptake of S.S.R.F. The composition of incubation medium; same as in Fig. 3. M.E.S.; monophasic square wave of 10 msec duration and 2 v. B.E.S.; biphasic, 5 msec duration and 6 v. 3 min incubation. Each column, average of 7 experiments.

In experiments described above, the concentration of CP used was 1 mM. When the amount of CP was increased to 6 mM, it was found that Ca^{++} uptake by S.S.R.F. was very much increased. Even when the amount of S.S.R.F. used was decreased from 0.5 mg protein S.S.R.F./ml (which was the case with 1 mM CP) to 0.2 mg/ml, over 90% of Ca^{++} was taken up from a solution containing 30 μM Ca^{++} in the presence of 6 mM CP. Consequently, the rest of the experiments were performed in a medium containing 6 mM CP and 0.2 mg protein S.S.R.F. per ml.

To demonstrate more clearly the Ca^{++} uptake by S.S.R.F. without electrical stimulation and the release of Ca^{++} from S.S.R.F. during electrical

stimulation, the following experiments were performed. Five ml of the suspension medium containing 30 μM Ca^{++} and S.S.R.F. were incubated with the electrodes in the medium, and samples were taken periodically for the determination of Ca^{++} in the medium before and after electrical stimulation. The results are shown in Fig. 6. As can be seen in this figure, the addition of S.S.R.F. into the medium was followed by the Ca^{++} uptake by S.S.R.F. and within 5 min after the incubation, approximately 90% of Ca^{++} in the medium

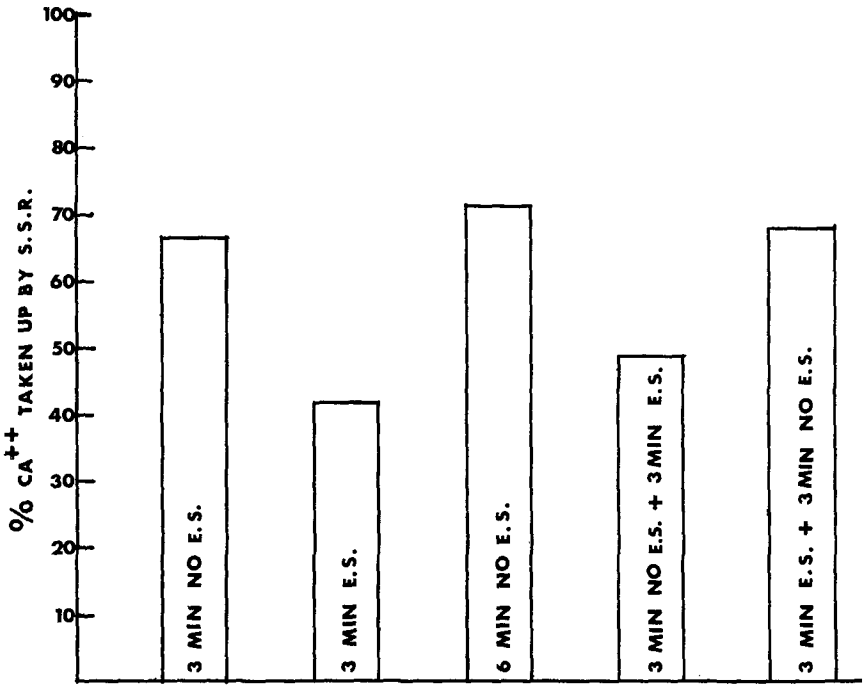

FIGURE 5. Effect of stimulation during half of incubation period on Ca^{++} uptake o S.S.R.F. Incubation medium has the same composition as that in Fig. 3. E.S.; electrical stimulation with monophasic square waves of 10 msec duration, 2 v at 60/min frequency.

was accumulated in S.S.R.F. When the monophasic square waves of 2 v and 10 msec duration were passed through the medium at a frequency of 60 per min there was a release of Ca^{++} from S.S.R.F. into the medium, and after 3 min of electrical stimulation about 30% of Ca^{++} taken up was released. The cessation of electrical stimulation was followed by the re-uptake of Ca^{++} by S.S.R.F. from the medium.

A similar experiment was performed in the presence of 10 mM inorganic phosphate (IP) or 2.5 mM oxalate and the results are shown in Fig. 7. It can be seen in Fig. 7 that the presence of 10 mM IP increased the rate of Ca^{++}

uptake by S.S.R.F. Following electrical stimulation, there was a very slight release of Ca^{++} but the degree of release was very much less than that observed in the absence of added IP (Fig. 6). Fig. 7 also shows that when oxalate was present in the system, Ca^{++} uptake was speeded up markedly and no detectable Ca^{++} was released from the system following the electrical stimulation.

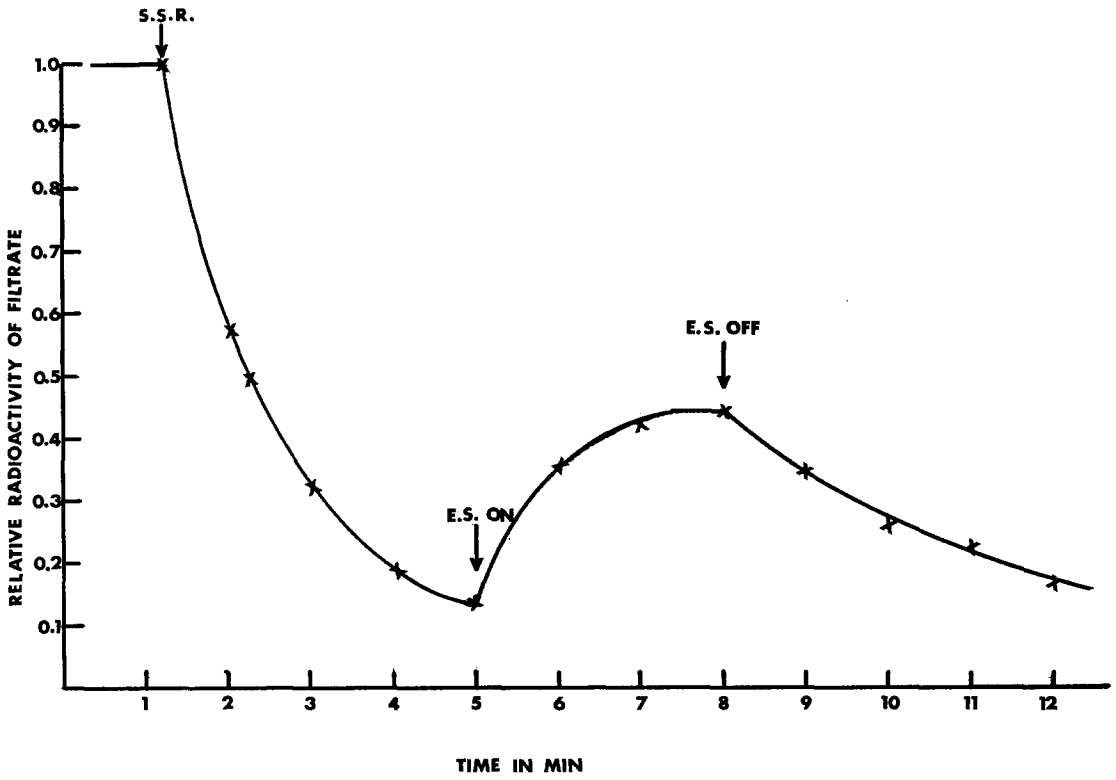

FIGURE 6. Effect of E.S. on release and uptake of Ca^{++}. Incubation medium contained; 0.02 M Tris-maleate (pH 6.5), 0.1 M KCl, 3 mM $MgCl_2$, 3 mM ATP, 6 mM CP, 0.1 mg/ml CPkinase and 30 μM total Ca^{++} ($CaCl_2$ + Ca^{45} Cl_2). S.S.R.F. 0.2 mg protein/ml was added. E.S.; monophasic, 2 v 10 msec duration at 60/min.

In view of the lack of response to stimulation in the case of IP or oxalate, the effect of desoxycholate on the Ca^{++} release from S.S.R.F. was studied in the presence and absence of IP or oxalate. As can be seen in Fig. 8, the addition of desoxycholate to the medium after most of the Ca^{++} had been accumulated by S.S.R.F. during a 10 min incubation period, released Ca^{++} immediately when oxalate or IP was absent, but released almost no Ca^{++} when IP or oxalate was present in the system.

To investigate the possibility that electrical stimulation may alter the com-

position of medium or denature S.S.R.F., the following experiments were performed. During stimulation of medium with monophasic square waves of 4 v and 10 msec duration at 60 per min, the pH of the solution was continuously measured using Beckman pH meter and was found to be unchanged during a 10 min stimulation period. Also the careful observation of platinum electrodes during the above period revealed no visible sign of gas production.

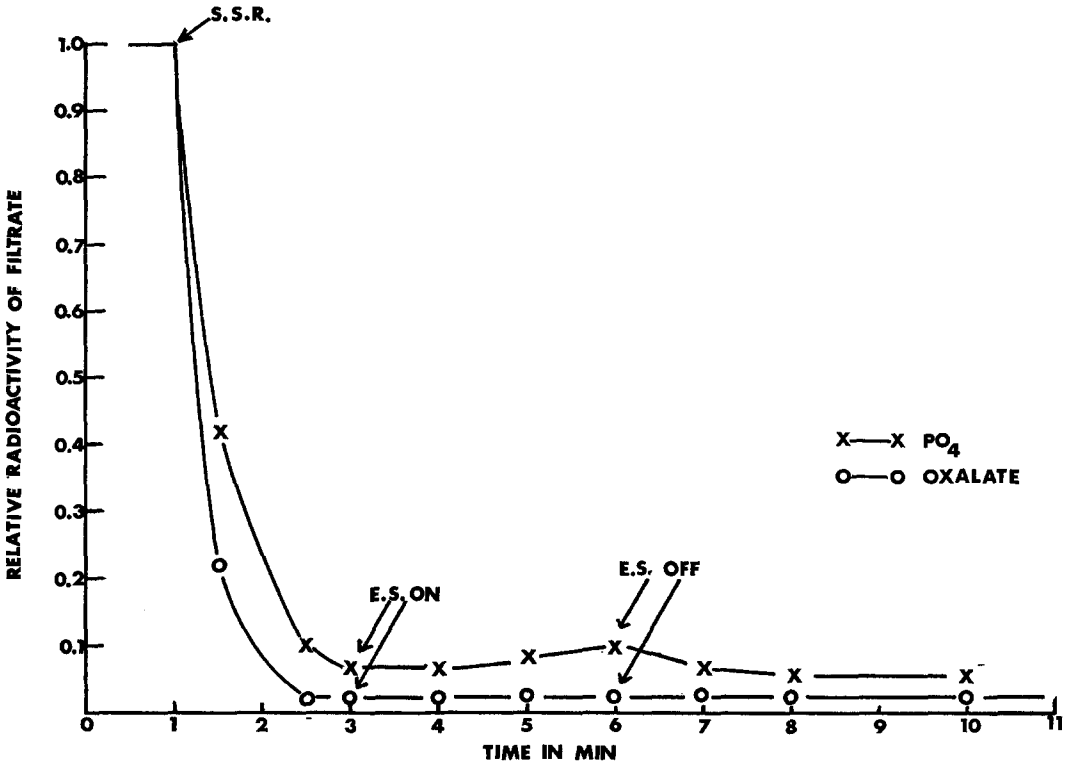

FIGURE 7. Effect of E.S. on release and uptake of Ca^{++} in the presence of phosphate or oxalate. Conditions of incubation, same as those in Fig. 6 except for the presence of 10 mM IP or 2.5 mM oxalate.

However, since the platinum electrodes are known to produce some physical and chemical changes of solution, experiments were performed with silver-silver chloride electrodes instead of platinum electrodes, and results are shown in Table I. It is apparent that results obtained with silver-silver chloride electrodes are essentially similar to those obtained with platinum electrodes. In previous experiments with platinum electrodes, biphasic stimulation did not have any effect up to 6 v. Stimulating voltages higher than 6 v were not used in fear of the possible chemical change induced by platinum electrodes. With silver-silver chloride electrodes, voltage of biphasic stimulation (10

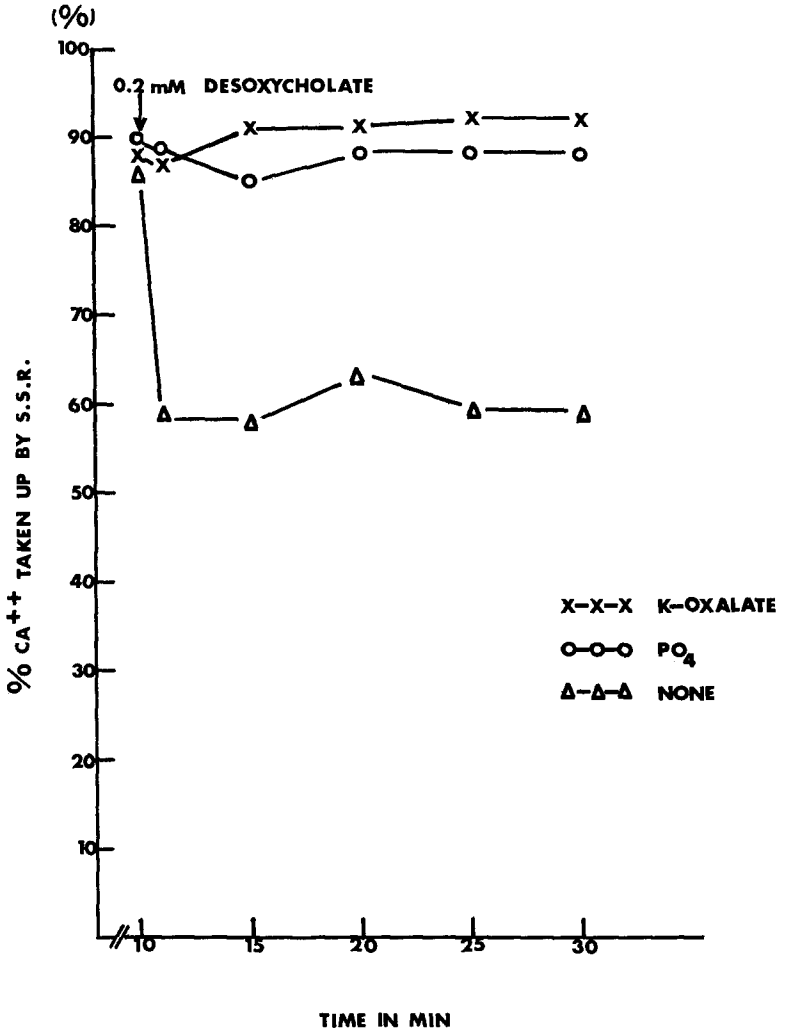

FIGURE 8. Effect of desoxycholate on S.S.R.F. preincubated with Ca^{++}. S.S.R.F. suspension, containing the same components as that in Fig. 6 in addition to that indicated in the figure, was incubated for 100 min, then desoxycholate was added into the medium. Concentrations of IP and oxalate were 10 mM and 2.5 mM, respectively.

msec duration, 60 per min) was raised up to 30 v and it was found that the inhibitory effect of stimulation on Ca^{++} uptake by S.S.R.F. became evident when the voltage was increased above 15 v.

CARDIAC SARCOPLASMIC RETICULUM FRAGMENTS (C.S.R.F.) When C.S.R.F. was prepared from dog hearts, it was found that the Ca^{++} uptake of C.S.R.F. in the presence of ATP alone was very weak even in the presence of oxalate (2.5 mM) or IP (10 mM) during a 3 min incubation period. How-

TABLE I

EFFECT OF ELECTRICAL STIMULATION ON CA^{++} UPTAKE

Condition	No stimulation	Electrical stimulation	
		Monophasic	Biphasic
	%	%	%
Ca^{++} uptake after 3 min incubation	83	53 (2 *v*)	82 (6 *v*) 70 (15 *v*) 61 (30 *v*)

Duration and frequency of stimulation were 10 msec and 60 per min, respectively. All stimulation by silver-silver chloride electrodes. Incubation medium; same as in Fig. 6.

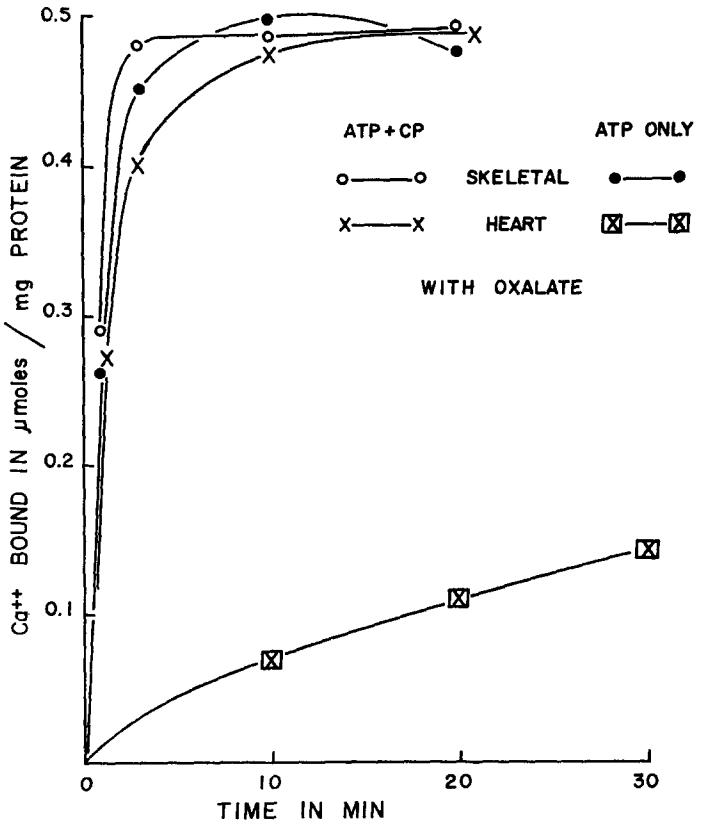

FIGURE 9. Comparison of Ca^{++} uptake by S.S.R.F. and C.S.R.F. Incubation medium of "ATP only" contained; 0.02 M Tris-maleate (pH 6.5), 0.1 M KCl, 2 mM $MgCl_2$, 0.1 mM total Ca^{++} ($CaCl_2$ + Ca^{45} Cl_2), 2.5 mM oxalate, 0.2 mg S.R.F. protein/ml and 3 mM ATP. Incubation medium of "ATP + CP" contained; all components in "ATP only" and in addition, 6 mM CP and 0.1 mg/ml CPkinase.

ever, when 6 mM CP and 0.1 mg/ml creatine-phosphokinase (CP kinase) were present in addition to ATP and oxalate (or IP), the Ca^{++} uptake of C.S.R.F. was just as good as that observed with S.S.R.F. (Fig. 9). The effect of electrical stimulation on the Ca^{++} uptake of C.S.R.F. in the presence of oxalate is shown in Figs. 10 and 11. In the presence of 2.5 mM oxalate, the passage of monophasic square waves of 2 v and 10 msec duration during an

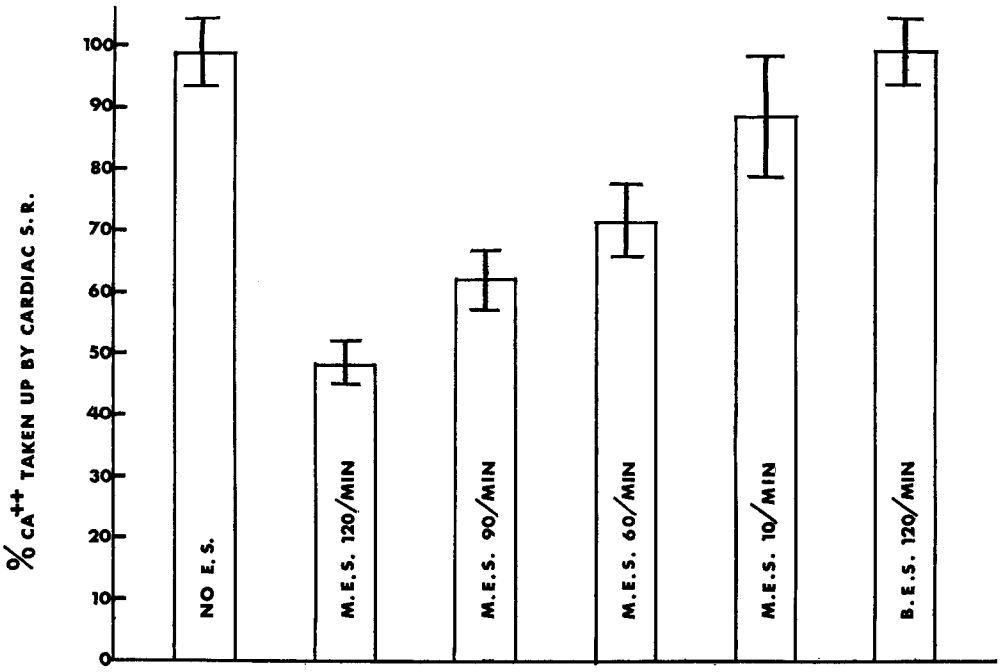

FIGURE 10. Effect of frequency of stimulation on Ca^{++} uptake by C.S.R.F. Incubation medium contained; 0.02 M Tris-maleate (pH 6.5), 0.1 M KCl, 3 mM $MgCl_2$, 30 μM total Ca^{++} ($CaCl_2$ + Ca^{45} Cl_2), 0.2 mg protein C.S.R.F./ml, 2.5 mM oxalate, 3 mM ATP, 6 mM CP, and 0.1 mg/ml CPkinase. M.E.S.; monophasic square waves of 2 v, and 10 msec duration. B.E.S.; biphasic, 2 v and 5 msec duration. Each column, average of 9 experiments.

incubation period of 3 min inhibited the Ca^{++} uptake of C.S.R.F. and the degree of inhibition was dependent on the frequency of stimulation as can be seen in Fig. 10. Similar results were obtained when 10 mM IP was present instead of oxalate. When the frequency of stimulation was kept constant (60 per min), the degree of inhibition of Ca^{++} uptake by stimulation was dependent on the voltage of stimulation as shown in Fig. 11.

It should be noted that very active preparations of C.S.R.F. were also required, as in the case of S.S.R.F., for the demonstration of the inhibitory effect of stimulation on the Ca^{++} uptake or the release of Ca^{++} by stimulation

following a period of uptake. In some preparations which showed only about 50% Ca^{++} uptake from the medium, it was difficult to see the stimulation effect even though the tendency was noticed. Thus it can be seen in Fig. 12, that when ATP alone was used, the uptake of Ca^{++} by C.S.R.F. was poor and the release of Ca^{++} following electrical stimulation was not well demonstrated. It should be noted, however, that in most cases the slight release of Ca^{++} could be detected. When both ATP + CP system (CP and CP kinase) and IP 10 mM were present and Ca^{++} was taken up by C.S.R.F. very actively

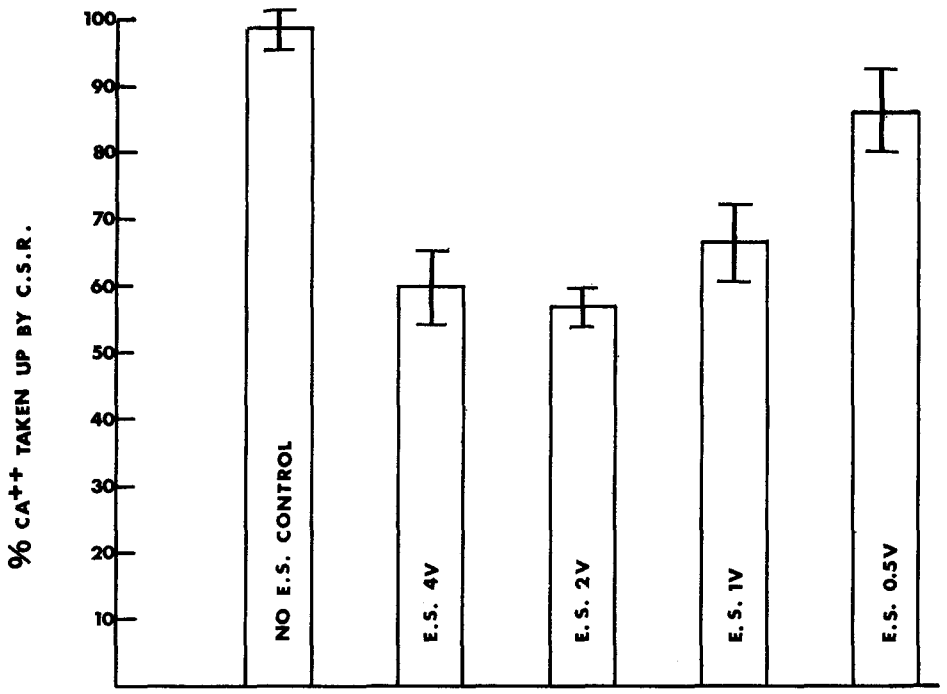

FIGURE 11. Effect of stimulation voltage on Ca^{++} uptake by C.S.R.F. Incubation medium, same as in Fig. 10. E.S.; monophasic square waves of 10 msec duration, at a frequency of 60/min. Each column, average of 8 experiments.

during a preliminary incubation period of 10 min, the passage of monophasic square waves of 2 v and 10 msec duration with a frequency of 60 per min (E.S.) for 3 min released part of the Ca^{++} taken up; and a cessation of E.S. was followed again by a re-uptake of the Ca^{++} released by electrical stimulation. However, when oxalate was present no Ca^{++} was released during the electrical stimulation.

Since Ca^{++} was not released by electrical stimulation when Ca^{++} had been already taken up by C.S.R.F. in the presence of oxalate, experiments were carried out to see the difference of degree of Ca^{++} release following the addition of desoxycholate in the presence and absence of oxalate. Desoxy-

cholate in a concentration of 0.2 mM was found, in experiments not shown here, to inhibit the Ca^{++} uptake by C.S.R.F. almost completely when this agent was present in the medium from the beginning of incubation. In experiments shown in Fig. 13, C.S.R.F. was incubated without desoxycholate for 10 min during which period about 97% of Ca^{++} in the medium was taken up by C.S.R.F. and then desoxycholate was added in the medium. It was

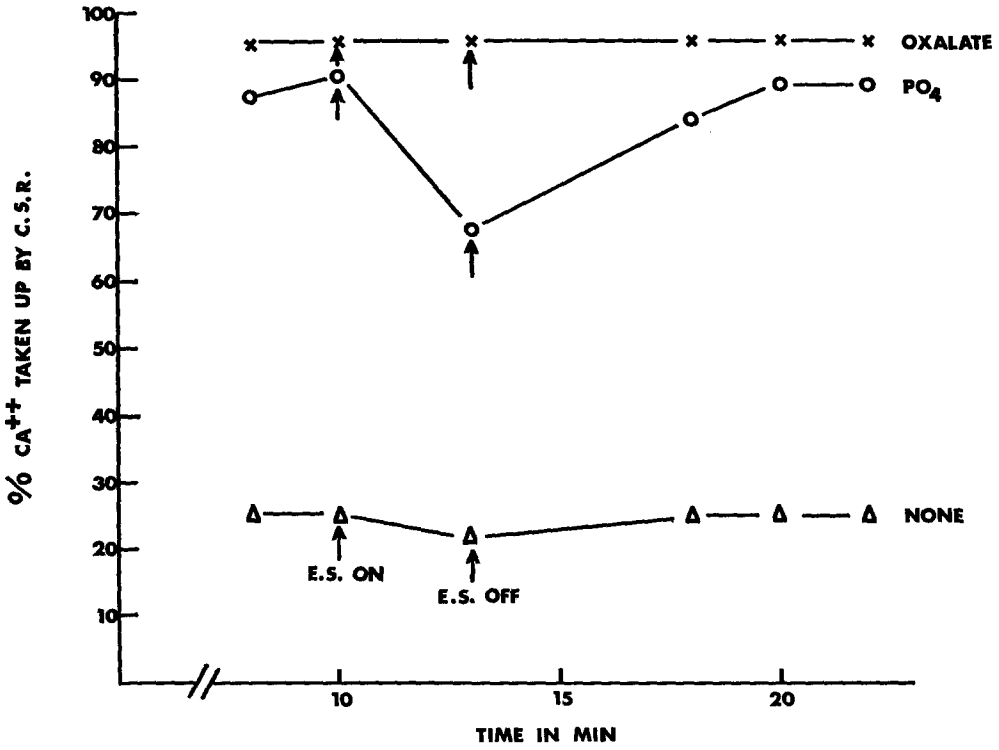

FIGURE 12. Effect of electrical stimulation on Ca^{++} release from C.S.R.F. Incubation medium of "oxalate," same as that in Fig. 10. Medium of "PO_4," same as that in Fig. 10 except that 10 mM IP replaced oxalate. Medium of "None," same as that in Fig. 10 but without oxalate. E.S.; monophasic square waves of 2 v, 10 msec duration at a frequency of 60/min.

found, as can be seen in the figure, that desoxycholate released Ca^{++} rapidly when no oxalate was present but no significant amount of Ca^{++} was released from C.S.R.F. when oxalate was present.

Experiments Using the Glycerol-Extracted Fibers

Since the experiments described above showed the release of Ca^{++} from S.R.F. by electrical stimulation in vitro, attempts were made to observe the release of Ca^{++} by electrical stimulation from unfractionated sarcoplasmic

reticulum remaining in myofibers, and the interaction of the released Ca^{++} with myofibrils in vitro. For this purpose, the glycerol-extracted fiber was selected as a muscle model, since it contains both sarcoplasmic reticulum

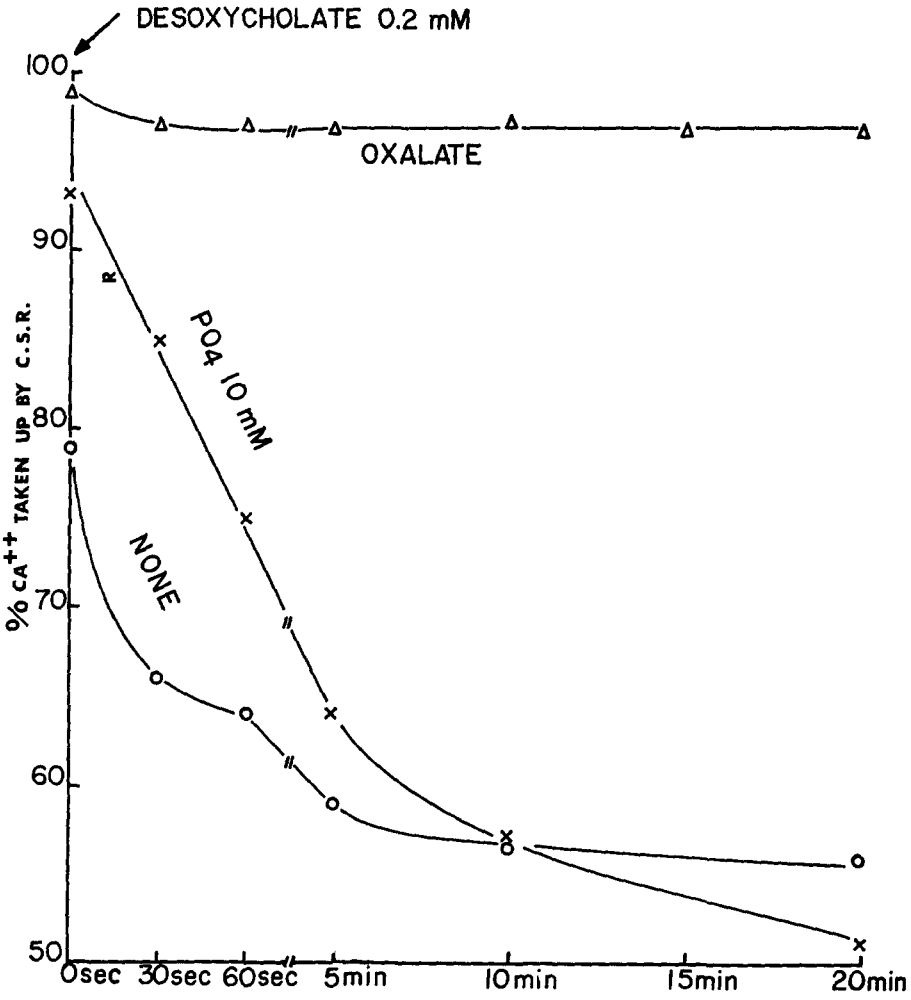

FIGURE 13. Effect of desoxycholate on Ca^{++} release from C.S.R.F. C.S.R.F. suspensions having same components as that in Fig. 12 were incubated for 10 min, then desoxycholate 0.2 mM was added into the medium.

and myofibrils and yet is permeable to various agents such as ATP and CP. Muscle fibers were extracted in 50% glycerol for a short time, usually 3 to 7 days, to preserve the functional S.R., as indicated by the induction of relaxation following the addition of CP subsequent to a contraction with ATP (Lee, 1961). Fibers which did not relax with CP were discarded.

Fibers were placed in the muscle chamber containing the incubation medium and tension development was recorded as shown in Fig. 2. The incubation medium consisted of 0.02 M Tris-maleate buffer, 3 mM $MgCl_2$, 0.1 M KCl. The results of a typical experiment are shown in Fig. 14.

When 3 mM ATP was added to the medium, fibers developed tension immediately. The addition of 6 mM CP was followed by the relaxation indicating a functioning sarcoplasmic reticulum (Lee, 1961). Following CP, electrical stimulation of muscle fibers with monophasic square waves of 15 v and 10 msec duration at a frequency of 5/sec for only 30 sec was followed by a slow

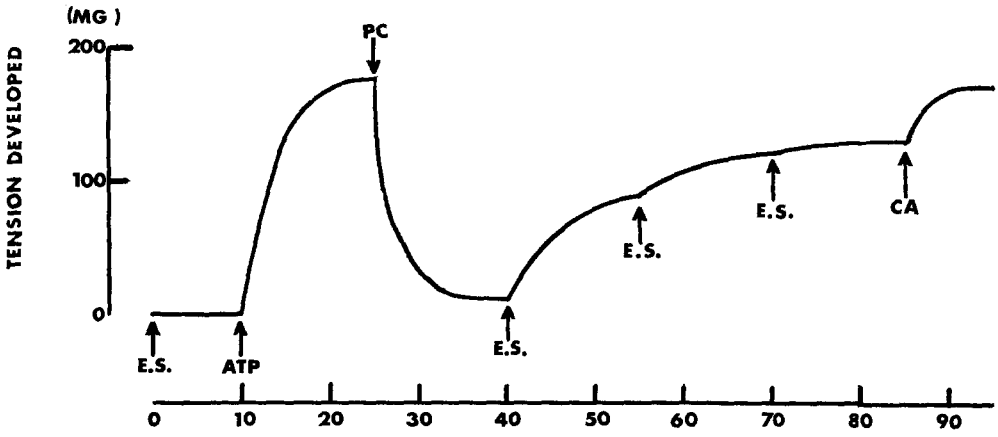

FIGURE 14. Effects of ATP, CP and stimulation on tension development of skeletal glycerol-extracted fiber. Medium contained; 0.1 M KCl, 0.02 M Tris-maleate (pH 6.8) and 3 mM $MgCl_2$. Concentrations of ATP, Ca^{++} and CP were 3 mM, 1 mM, and 6 mM, respectively. E.S.; monophasic square waves of 15 v, 10 msec duration at 5 per sec for 30 sec period. PC = CP.

and gradual development of contractile tension which continued 15 min or longer. Successive stimulations of the same nature were followed by stepwise increments of tension development. Electrical stimulation before addition of ATP had no effect, as seen in Fig. 14.

The degree of tension development by the electrical stimulation was a function of the voltages and frequencies employed. Thus, at a fixed voltage of 15 v, it was observed that the higher the frequency of stimulation the greater the tension developed (Fig. 15). Also decreasing the voltage below 15 v was followed by a decrease of tension developed, and at 10 v no significant tension was caused by stimulation under the experimental conditions employed. However, further increase beyond 15 v was not accompanied by

any further increment in tension developed. When the medium was stimulated electrically by biphasic waves (20 v, 5 msec duration, 10/sec) no change was observed. To ascertain the possible influence of temperature and chemical changes of solution which may have been brought about by stimulation on the fiber tension, following experiments were performed. After CP resulted in the relaxation of ATP-induced tension, the muscle chamber was perfused continuously with the solution containing the same composition (ATP, PC,

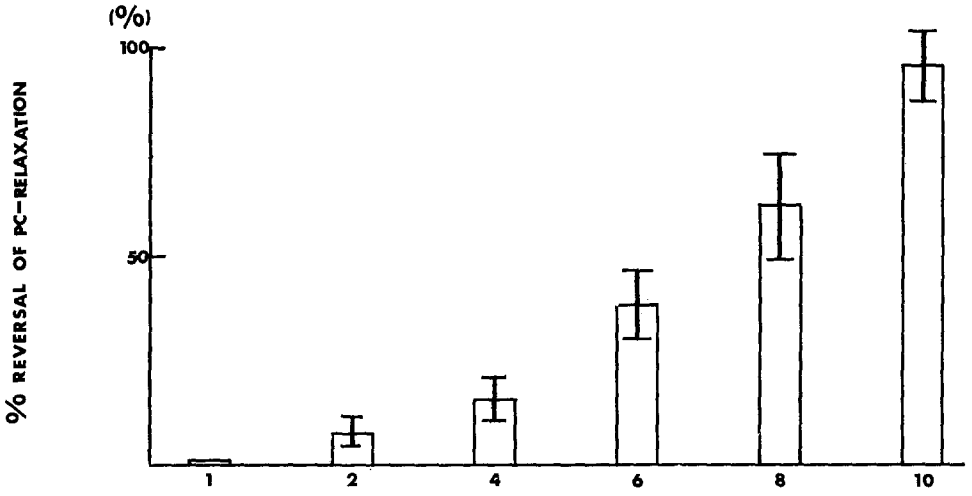

FIGURE 15. Effect of stimulation frequency on stimulation-induced tension development of skeletal glycerol-extracted fiber. Muscle fibers were incubated in media containing; 0.02 M Tris-maleate (pH 6.8), 3 mM $MgCl_2$ and 0.1 M KCl. Initial addition of 3 mM ATP caused contraction. Addition of 6 mM CP after ATP produced relaxation. Then electrical stimulation (E.S.) by monophasic square waves of 15 v, 10 msec duration at various frequencies caused contraction. The degree of contraction caused by E.S. is expressed in per cent of reversal of CP induced relaxation. PC = CP. Each column represents an average of 9 experiments.

and other components) at a speed which replaced all solutions in the chamber every 30 sec. Then fibers were stimulated electrically in the same manner as in Fig. 14. The effect of stimulation under this condition of perfusion was same as that under the nonperfused condition.

As noted previously, when fibers were extracted for a prolonged period (4 to 5 weeks) in glycerol, they responded to CP, added after ATP, with a further contraction rather than relaxation presumably because of loss of functioning S.R. Electrical stimulation of the same nature as Fig. 14 had no significant effect on these fibers.

In another experiment, influence of EGTA (ethylene glycol bis (β-amino-ethylether)-N,N′-tetraacetic acid) and oxalate on the effect of electrical stimulation was studied. In these experiments, EGTA (1 mM) or oxalate (2.5 mM) was added in the muscle chamber after the muscle was relaxed by CP following the ATP-induced contraction. Then the relaxed muscle fibers were stimulated with the monophasic square waves of 20 v, 10 per sec, and 10 msec duration. It was found that the presence of EGTA or oxalate com-

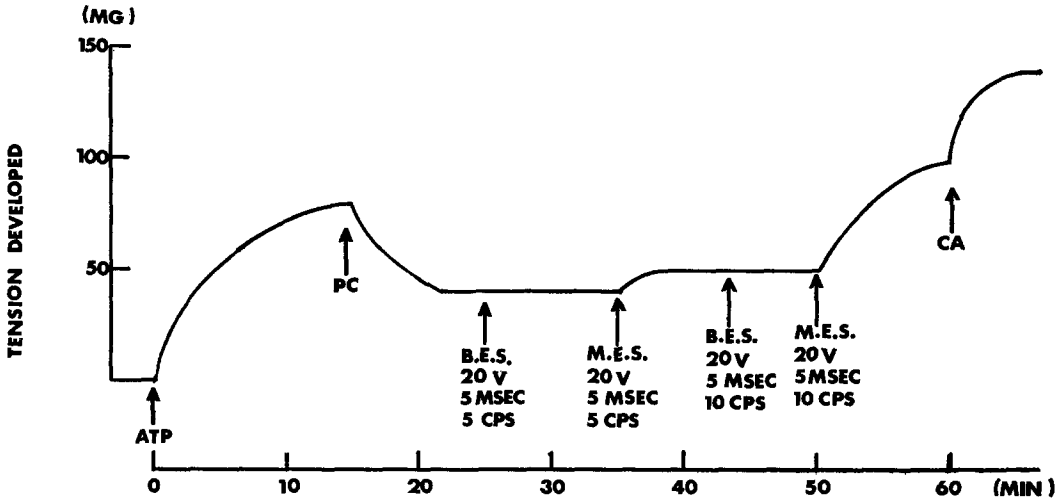

FIGURE 16. Effects of ATP, CP and stimulation on tension of cardiac glycerol-extracted fibers. Experimental conditions are same as those in Fig. 14. Duration of each E.S. was 30 sec. Concentration of Ca^{++}, 1 mM.

pletely abolished the response of muscle fibers to electrical stimulation. After the washout of EGTA or oxalate, the same monophasic stimulation as in Fig. 14 induced the contractile tensions similar to those seen in Fig. 14. However, the response to electrical stimulation after EGTA washout was less than that observed in Fig. 14. With EGTA in the medium, it is likely that some of Ca^{++} bound to sarcoplasmic reticulum are removed as Ca-EGTA during the washout period. In spite of this, electrical stimulation after washout still showed similar although smaller responses. This was probably due to the fact that the contaminated Ca^{++} in the solution used for washout was taken up by the sarcoplasmic reticulum during the washout period and was released following stimulation.

HEART MUSCLE FIBERS Glycerol-extracted fibers from dog heart were prepared as mentioned in the Methods section and similar experiments to those with skeletal muscle were performed. The results of a typical experiment are shown in Fig. 16. In those fibers which were extracted for short periods in glycerol, CP added after ATP-induced contraction resulted in the relaxation as can be seen in Fig. 16. Electrical stimulation for only 30 sec caused the slow and prolonged contraction of heart fibers and each successive stimulation was followed by further contraction. No effect was observed with

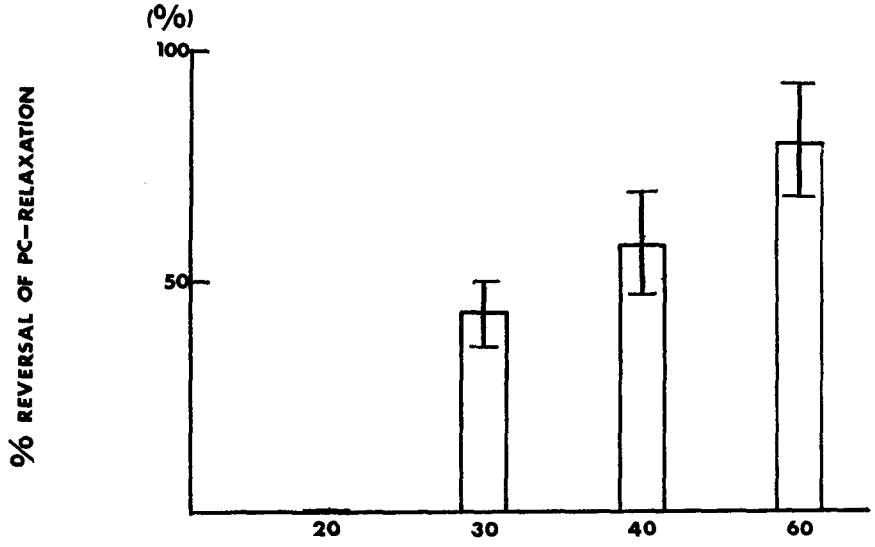

FIGURE 17. Effect of stimulation frequency on tension of cardiac glycerol-extracted fibers. Experimental conditions same as those in Fig. 15. Each column, an average of 11 experiments.

biphasic electrical stimulation. In another experiment, EGTA (1 mM) was added to the muscle chamber after the relaxation following CP. Similar electrical stimulation with monophasic square waves as in Fig. 16 did not have any effect when EGTA was present. However, after washout of the EGTA, the effect of E.S. could then be seen again. The effect of stimulation on heart fibers was also dependent on the frequency of stimulation. This is shown in Fig. 17. It appeared that heart fibers required a higher frequency of stimulation than skeletal muscle fibers for the same degree of tension to be developed. In another experiment, the solution in the muscle chamber was continuously perfused with a solution of a same composition during stimulation after CP relaxation. Under these experimental conditions, stimulation

with monophasic square waves (same as in Fig. 16) produced the same response as those seen in Fig. 16. This suggested that physical or chemical change of solution induced by electrical stimulation was not the cause of tension development following electrical stimulation.

DISCUSSION

Many recent findings have led to the following concept about the process of excitation-contraction coupling in muscle; the spread of the electrical excitation wave along the muscle membrane releases the Ca^{++} from the sarcoplasmic reticulum (S.R.) which initiates contraction of the myofibrils; then after termination of excitation relaxation is brought about by the re-uptake of Ca^{++} by the S.R. (Adrian, 1963; Bennett, 1965; Ebashi, 1961*b*; Porter, 1961; Porter and Armstrong, 1965; Rusk et al., 1958). In the present work we have shown that electrical stimulation of suspensions of fragments of sarcoplasmic reticulum (both from cardiac muscle, C.S.R.F., and skeletal muscle, S.S.R.F.) releases Ca^{++} previously taken up by the S.R.F.; and, furthermore, that the cessation of electrical stimulation is followed by a re-uptake of Ca^{++} by the S.R.F. The results may be considered to be an in vitro demonstration of the two key steps in the current concept of excitation-contraction coupling.

In our previous studies on the influence of "relaxing factor" on the syneresis of myofibrils, it was found that the electrical stimulation through the suspension caused the syneresis of myofibrils in spite of the presence of the relaxing factor sufficient to inhibit completely syneresis of myofibrils (Lee et al., 1965*d*). The relaxing factor employed in the earlier study consisted of microsomal granules from skeletal or cardiac muscle, and corresponded to the S.R.F. used in the present study. These previous findings suggested that electrical stimulation of the relaxing factor caused an increase of Ca^{++} concentration in the medium, thereby causing the syneresis of myofibrils. The demonstration in this study that electrical stimulation releases Ca^{++} from both S.S.R.F. and C.S.R.F. provides direct evidence in support of this suggested mechanism and confirms the previous findings (Lee, 1965*a* and 1965*b*; Lee et al., 1965*c*).

The Effect of Electrical Stimulation on Ca^{++} Release and Ca^{++} Uptake by S.R.F.

As shown in Figs. 3, 4, 10, and 11, both C.S.R.F. and S.S.R.F. took up Ca^{++} very actively from the medium when no electrical stimulation was applied. In a sufficiently long incubation the final Ca^{++} content of the S.R.F. (which is equal to total Ca^{++} content minus that left in the medium) is the reflection of the steady state in which Ca^{++} efflux equals Ca^{++} influx. After the 10 min incubation of a suspension containing C.S.R.F. (PO_4 curve in Fig. 12) or S.S.R.F. (Fig. 6), a steady state was obtained in which the concentration gradient between the S.R.F. and the medium was over 1000:1 (calculated on the basis that 15 mg S.R.F. protein occupied 0.1 ml of S.R.F. precipitate). This extremely high concentration gradient requires a vigorous inward

transport mechanism to maintain the steady state. The energy for this mechanism is supplied by hydrolysis of ATP (Ebashi and Lipmann, 1962; Hasselbach and Makinose, 1961, 1962, and 1963).

When electrical stimulation in the form of monophasic square waves of short duration was passed through the S.R.F. suspension during an incubation period, the amount of Ca^{++} taken up by the S.R.F. was markedly decreased compared with the amount taken up by an unstimulated control. This inhibitory effect of stimulation on Ca^{++} uptake was dependent on the voltage strength and the frequency of stimulation. When electrical stimulation was passed through the S.R.F. suspension after the Ca^{++} in media was taken up by S.R.F., there was a release of Ca^{++} from S.R.F. into the medium and the cessation of electrical stimulation was followed by the re-uptake of Ca^{++} by S.R.F. from the medium. The fact described above that electrical stimulation can cause the release of Ca^{++} or interfere with the uptake of Ca^{++} could mean; (*a*) stimulation inhibits the rate of influx, namely uptake of Ca^{++}, (*b*) stimulation increases the rate of efflux of Ca^{++}, or (*c*) stimulation does both. More detailed studies on the kinetics of Ca^{++} movement are needed to settle which process is influenced by stimulation and further studies are in progress at present.

A Possible Basis for Release of Ca^{++} from S.R.F. by Electrical Stimulation

A number of possibilities may be considered for this mechanism. First, a reasonable basis for the release of Ca^{++} from S.R.F. by stimulation can be provided if a change of polarization of the vesicular membrane of S.R.F. occurs in the suspension during stimulation. Morphological studies during the past decade have provided convincing evidence that the sarcoplasmic reticulum is a membranous system separating different intracellular phases and ion concentrations (Bennett, 1956; Edwards et al., 1956; Fawcett and Revel, 1961; Muscatello et al., 1962; Palade, 1956; Porter, 1956 and 1961; Porter and Palade, 1957; Revel, 1964). Those and other findings have provided the concept that the sarcoplasmic reticulum may possess a transmembrane potential and serves in impulse conduction of membrane depolarization in muscle (Adrian, 1963; Huxley and Taylor, 1958; Porter, 1961; Porter and Armstrong, 1965; Ruska et al., 1958). If this concept is accepted, then the passage of square wave impulses through the S.R.F. suspension in the present experiments might be expected to cause the S.R.F. membrane to undergo a change of polarization, which somehow results in the release of Ca^{++} from the S.R. vesicles. Secondly, the passage of electrical current through the medium or the change of electrical field may alter the membrane permeability of sarcoplasmic reticulum in such a way to release Ca^{++} from S.R.F. There is evidence which suggests that, in living muscle, electrical stimulus cause T system depolarization (Freygang et al., 1964; Huxley, 1964; Huxley and

Taylor, 1958; Porter and Armstrong, 1965). Since S.R.F. used in the present study contain T system, the elctrical stimulus employed here may cause membrane permeability change of sarcoplasmic reticulum through the depolarization of T system and release Ca^{++} from S.R.F. If this is the case, then some difference of S.S.R.F. and C.S.R.F. observed in this study with regard to Ca^{++} release following electrical stimulation may be partly due to the difference of association of T system with sarcoplasmic membrane in skeletal and cardiac preparation. However, no sufficient electron microscopic data are available in our present study to support above possibility. In this connection, the remote possibility may be mentioned that local changes in the ATP and Ca^{++} concentrations of the medium due to the electric field might have an indirect effect on the Ca^{++} uptake of sarcoplasmic reticulum fragments in an unstirred solution. Thirdly, there is a possibility that some changes such as the disruption of S.R.F. resulting from the passage of the electrical current in the system was responsible for the Ca^{++} release. However, following findings are not in favor of this possibility. The Ca^{++} release by electrical stimulation was a reversible process and the cessation of stimulation was accompanied by the re-uptake of Ca^{++}. The monophasic square waves of as low as 1 v released Ca^{++} from S.R.F. and stimulation with impulses of 2 v was often more effective than that with impulses of 4 v. Lastly, the Ca^{++}-releasing effect of stimulation was observed with both platinum and silver-silver chloride electrodes and biphasic electrical stimulation was also effective when the stimulating voltage was sufficiently high enough. However, these findings do not rule out the above mentioned possibility that some S.R.F. may be altered by the electrical current and further studies are being conducted to examine this problem.

The Effect of Oxalate and IP on Ca^{++} Release by Stimulation

It has been shown that oxalate and IP increase the Ca^{++} uptake by S.R.F. (Hasselbach and Makinose, 1961; Lorand, 1964; Martonosi and Feretos, 1964). This also was found to be the case with S.S.R.F. and C.S.R.F. in this study. With C.S.R.F. the presence of oxalate increased the Ca^{++} uptake markedly and the inhibitory effect of stimulation with various frequencies on the Ca^{++} uptake by C.S.R.F. was best demonstrated when oxalate was present (see Fig. 10). However once Ca^{++} was taken up by C.S.R.F. in the presence of oxalate, the Ca^{++}-releasing effect of stimulation could not be demonstrated. The inability of stimulation to release Ca^{++} once it had already been accumulated to a very high concentration in the S.R. vesicles in the presence of oxalate may be due to the precipitation of calcium oxalate inside the vesicles under these circumstances, as found by Hasselbach (1965). When S.S.R.F. were used in the presence of oxalate, both the Ca^{++}-releasing effect and the inhibitory effect on the Ca^{++} uptake by stimulation could not be demon-

strated. This is probably due to the fact that the potency of Ca^{++} uptake is so much stronger with S.S.R.F. than with C.S.R.F. that the potentiating effect of oxalate overcomes the inhibitory effect of stimulation on Ca^{++} uptake by S.S.R.F.

Effect of Electrical Stimulation on Glycerol-Extracted Fibers

In glycerol-extracted fibers, under appropriate experimental conditions, electrical stimulation produced a rise in contractile tension as can be seen in Figs. 14 and 16. This effect of stimulation was clearly observed with fibers extracted for short periods which were initially contracted with ATP and then relaxed with CP before application of electrical stimulation. This effect of stimulation was not observed with fibers extracted for long periods which do not relax with CP following ATP contraction. The ability to relax with CP following ATP-induced contraction was taken to indicate the presence of still functional sarcoplasmic reticulum in fibers (Lee, 1961). Thus, it appears that the functional integrity of sarcoplasmic reticulum is required for the demonstration of clear-cut effect of electrical stimulation on the contractile tension of glycerol-extracted fibers. The failure to obtain the significant response of glycerol extracted fibers to electrical stimulation by the previous workers (Fujino et al., 1960) was probably due to the fact that the experimental conditions (described above) necessary for the demonstration of the effect of stimulation were not provided in those experiments. In this connection, it should be mentioned that Nayler and Merrillees (1964) reported the existence of an electrical potential difference between the glycerol-extracted fiber and the immersing medium. However, this potential difference was unaltered by replacing the immersing medium by 0.1 M KCl solution (Weiss, 1965) and therefore it is probably not equivalent to the resting potential across cell membranes.

The effect of stimulation on glycerol-extracted fibers closely resembles those observations in the syneresis study (Lee et al., 1965*c* and 1965*d*). It was found previously that electrical stimulation of a suspension containing myofibrils and S.R.F. (both from skeletal and heart muscle) in the amount sufficient to prevent syneresis resulted in the syneresis, thus stimulation reversing the relaxing effect of S.R.F. The suggestion was made, then, that electrical stimulation releases Ca^{++} (from S.R.F.) which in turn initiates syneresis of myofibrils.

In the present study, the following parallel relationships are noted between the effect of electrical stimulation on the movement of Ca^{++} in isolated S.R.F. and that on the tension development of glycerol-extracted fibers. First, the higher the frequency of stimulation, the more was the release of Ca^{++} from S.R.F. and the more was the tension developed in fibers. Second, the presence of oxalate which prevented electrical stimulation from releasing Ca^{++} pre-

viously taken up by S.R.F. also prevented stimulation from inducing the tension development in fibers. Third, electrical stimulation by biphasic impulses with the same voltages as those used in monophasic stimulation was ineffectual both in isolated S.R.F. and in glycerol-extracted fibers. These parallelisms, together with the finding that the presence of functioning S.R. in glycerol-extracted fibers is essential for the manifestation of the effect of stimulation lead to the conclusion that the stimulation-induced contraction of glycerol-extracted fibers is due to the release of Ca^{++} from the sarcoplasmic reticulum in fibers by stimulation. This conclusion is further supported by the finding that the presence of EGTA antagonized the effect of stimulation on fibers. The chelation of the Ca^{++} released from S.R. by stimulation with EGTA would prevent the interaction of Ca^{++} and myofibrils, thus, resulting in the abolition of stimulation effect by EGTA. Also, it should be noted that sarcoplasmic reticulum in glycerol-extracted fibers was found to accumulate Ca^{++} very actively (Hasselbach, 1965; Pease et al., 1965). However, another possibility exists that non response of glycerol fibers to electrical stimulation in the presence of EGTA may be due to the alteration of some membrane property in a calcium free medium as reported by Frank (1960 and 1964).

With regard to the reversibility of stimulation-induced contraction of fibers, it was noted that the relaxation of fiber tension after the cessation of electrical stimulation was unpredictable. It ranged from the complete return to the prestimulation level 20 min after the cessation of stimulation to the maintenance of total stimulation-induced tension even 60 min after the cessation of stimulation. The reason for this variability is not clear at present.

From the numerous studies mentioned above and the data presented here, the following processes can be visualized in excitation-contraction coupling in vivo. The excitation wave traveling along the muscle membrane alters the membrane potential of the transverse tubular system which then induces alteration of the polarization or the permeability of sarcoplasmic reticular membrane through the junction of triade. This change of the sarcoplasmic reticular membrane results in the liberation of Ca^{++} which in turn initiates the contraction of myofibrils. The return of the sarcoplasmic reticular membrane to the state prior to the electrical stimulation following the passing of the excitation wave is accompanied by the transfer of Ca^{++} from myofibrils back to sarcoplasmic reticulum by active Ca^{++} uptake process, and this would result in the relaxation of muscle.

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