Reexamination of Electrical Stimulation on Sarcoplasmic Reticulum Fragments In Vitro

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ABSTRACT The effect of direct electrical stimulation on suspensions of sarcoplasmic reticulum membrane fragments (SRF) was carefully re-examined using the method of Lee et al. (1966) *J. Gen. Physiol.* 49:689. Inhibition of Ca++ uptake or release by electrical stimulation was observed. When platinum electrodes were used as stimulating electrodes, the effect was dependent on the total current passed through the suspension. On the contrary, when silver-silver chloride electrodes were used, no effect was observed even if voltage and current were the same as in the case of the platinum electrodes. In addition, apparent re-uptake of Ca++ after cessation of electrical stimulation using platinum electrodes was shown to be due to a binding of Ca++ to denatured SRF which did not require an energy supply such as ATP, although such re-uptake had been taken as strong evidence of electrical response of SRF in Lee's paper. Finally, it was concluded that the effect of electrical stimulation on SRF was attributable to the irreversible denaturation of SRF due to the oxidation caused by the chlorine generated at the platinum electrode.

INTRODUCTION

The role of calcium as a mediator of excitation-contraction coupling in muscle and the identification of the calcium pump mechanism of the sarcoplasmic reticulum (SR) system as the cause of relaxation are well established (Ebashi and Endo, 1968; Ebashi and Lipmann, 1962; Hasselbach and Makinose, 1961; Jôbisis and O'Connor, 1966; Weber et al., 1966). According to the concept of excitation and contraction coupling, electrical excitation of muscle membrane releases Ca++ from the SR which initiates muscular contraction. After the contraction, the SR again takes up Ca++ from the cytoplasm causing relaxation of myofibrils.

Costantin and Podolsky (1967) have shown that depolarization of the SR membrane directly causes release of Ca++ in skinned skeletal muscle fibers of the frog. On the other hand, Lee et al. (1966) applied direct electrical...
stimulation to a sarcoplasmic reticulum fragments (SRF) suspension and obtained the following results. The SRF took up Ca++ very actively from media. When monophasic square waves were applied to the SRF suspension, the Ca++ uptake by SRF was decreased. When the suspension was stimulated electrically after the Ca++ was taken up by SRF, the initiation and cessation of the stimulation were followed by release and re-uptake of Ca++ by SRF, respectively. The degree of inhibition of the Ca++ uptake as well as the degree of the Ca++ release by electrical stimulation were dependent on the voltage and the frequency of stimulation. Interestingly when a biphasic electrical stimulation was applied, the effect was very weak. Very good parallelism of the effects of the electrical stimulation on SRF and glycogenated fibers was obtained.

The latter experiment was a typical one which had been attempted to directly demonstrate electrical responsiveness of SR by applying electrical stimulation in vitro. Many researchers followed this experiment and obtained similar results (Scales and McIntosh, 1968; Turina and Jenny, 1968). However, their interpretation was not necessarily accepted. Many questions were left unsettled. It was uncertain if the current sufficient to evoke electrical excitation of SRF passed through the membrane (Costantin and Podolsky, 1967). Because SRF is very heat labile, might SRF be thermally denatured by the Joule's heat generated locally in the solution although the temperature elevation of the solution during and after the electrical stimulation was negligible? Also, might SRF be affected by a local change of pH although pH of the whole solution during and after the electrical stimulation was constant? Other researchers applied high voltage alternating current to the SRF suspension, but they could not obtain effects such as those found by Lee et al. (Van der Kloot, 1966). It was not explained why a monophasic rectangular impulse was effective but biphasic impulses, either rectangular or sinusoidal, were not.

Above all, Turina and Jenny (1968) repeated Lee's experiment more quantitatively, directly measuring the electric current. On the whole they confirmed the results of Lee et al. (1966), but they found some differences. However, they could not clarify the meaning of the effect of direct electrical stimulation of SRF, although they suggested the possibility of oxidation of the membrane constituents by the electrical stimulation.

We have, therefore, repeated Lee's experiment as precisely and quantitatively as possible. It is shown that, under conditions similar to those of Lee et al. (1966), SRF may be denatured as a result of the oxidation caused by chlorine which was generated by electrolysis of potassium chloride at platinum electrodes when electric current was passed. By the denaturation of SRF, Ca++ is released from SRF irreversibly.
EXPERIMENTAL

Materials
The SRF were isolated as a microsomal fraction by the method of Weber et al. (1966) with slight modifications (Nakamura et al., 1972). Rabbit dorsal and hind leg muscles were homogenized with 4 vol of 0.1 M NaCl, 5 mM Tris-maleate at pH 7.0 by a Waring blender (Waring Products Div. [Dynamics Corp. of America], New Hartford, Conn.) for 2 min. The homogenate was centrifuged at 1,000 g for 20 min to remove myofibrils, nuclei, and other large fragments. The supernate was centrifuged at 8,000 g for 30 min to remove mitochondria. The supernatant was centrifuged at 53,000 g for 50 min. The precipitate was suspended in 20-30 vol of 0.6 M KCl, 5 mM Tris-maleate at pH 6.5, and centrifuged at 53,000 g for 50 min. The resulting precipitate was suspended in 0.1 M KCl, 5 mM Tris-maleate at pH 6.5, and the suspension was centrifuged again. The sediment was resuspended in the same buffer solution, and stored at 0°C in ice. The prepared SRF was used within 1 wk after isolation.

Creatine phospho transferase (CrP kinase) was prepared by the method described by Noda et al. (1955).

46Ca was purchased from International Chemical & Nuclear Corporation, Burbank, Calif. ATP and Creatine phosphate (CrP) were obtained from Sigma Chemical Co., St. Louis, Mo. Other reagents were commercial products of analytical grade.

Assay of Uptake and Release of Ca++ by SRF
Assay of uptake and release of Ca++ by SRF was made by the method described by Lee et al. (1966). SRF was suspended in a medium containing 46Ca and other components as indicated in the text and figures. In most experiments, 1 ml of the suspension was placed directly in a syringe, incubated for the desired period, and then filtered through a Millipore filter (Millipore Corp., Bedford, Mass.) by applying a negative pressure to the bottom flask. Millipore filters were of 0.45-μm pore size (HAWP 025). In most experiments, remaining free 46Ca was removed by passing 3 ml of a washing solution containing 0.1 M KCl, 2 mM MgCl₂, 20 mM Tris-maleate (pH 6.5) through the filter. The radioactivity of SRF that adhered to the Millipore filter was determined after drying under an infrared lamp and soaking in 10 ml of liquid scintillation counting medium of toluene. Sometimes the radioactivity of the filtrate was also determined. In this case, 0.1 ml of the filtrate collected in a tube was put into a flask containing 10 ml of liquid scintillation counting medium of dioxane and the radioactivity was determined. The total count of the suspension was also determined by the latter method. The calculation was made from these values for the amount of Ca++ taken up by or released from SRF. All reactions were performed at room temperature (about 23°C) and started by adding SRF last unless specified otherwise.

Electrical Stimulation of SRF
Electrical stimulation of SRF was also carried out carefully by the method described by Lee et al. (1966). For stimulation of the suspension medium containing SRF, a
pair of platinum electrodes (4-mm wide, 7-mm long, 3-mm apart) were inserted in the syringe tube. Monophasic square wave impulses of varying duration, voltages, and frequencies were passed through the suspension, by means of an electrical stimulator, Model MSE-3 (Nihon Koden, Tokyo, Japan). The voltage, current, frequency, and duration of the stimulation recorded in figures were measured at the same time by using an oscilloscope, Model SS-5050 (Iwatsu, Tokyo, Japan). In some experiments, a pair of silver-silver chloride electrodes of the same size as the platinum electrodes were used.

Other Measurements

ATPase activity was determined in the same solvent condition as Ca++ uptake by the method of Taussky and Shorr (1953).

The ultraviolet difference absorption spectrum was measured by using a Hitachi spectrophotometer Model 139 (Hitachi Ltd., Tokyo, Japan). In this case the experiment was done in 20 mM phosphate buffer (pH 6.5) instead of Tris-maleate, and without ATP.

The protein concentration was estimated by the biuret reaction calibrated by nitrogen determination. The amount of SRF is given as milligrams protein of SRF.

RESULTS

Ca++ Uptake and Effect of Electrical Stimulation

Although one purpose of this work was to follow precisely the experimental conditions described by Lee et al. (1966), the preparation of SRF differed slightly from Lee's. Our SRF was found to take up Ca++ from the medium much faster than the SRF of Lee, in the presence of 3 mM ATP, 1 mM CrP, 0.1 mg/ml CrP kinase, 30 μM total Ca++ (CaCl₂ + ⁴⁶CaCl₂), 0.1 M KCl, 2 mM MgCl₂, 0.02 M Tris-maleate (pH 6.5), i.e., the same solvent condition described by Lee et al. Over 95% of ⁴⁶Ca was taken up into SRF from a medium containing 30 μM CaCl₂ within 1 min when no electric current was passed through the medium and this level remained constant for more than 10 min (see Fig. 4). When electrical stimulation was applied, Ca++ uptake was inhibited. In the absence of CrP and CrP kinase almost the same results were obtained for Ca++ uptake and for the effect of the electrical stimulation. Therefore, all experiments shown below in this paper were done in the absence of the ATP regenerating system.

In the last 2.5 min of the incubation period, monophasic square wave pulses of 10-ms duration were passed through the medium at varying frequencies and voltages. Then, the amount of Ca++ taken up by SRF during the incubation period fell markedly as shown in Fig. 1. In this figure, the voltage values recorded by the oscilloscope were used. These voltage values were very much different from those of the stimulator because of a large voltage drop due to the low resistance of the sample solution. The degree of inhibition of Ca++ uptake by the electrical stimulation was dependent on
Figure 1. Effect of stimulation voltage and frequency on \( \text{Ca}^{++} \) uptake of SRF. All tubes contained: 20 mM Tris-maleate buffer (pH 6.5), 0.1 M KCl, 2 mM MgCl\(_2\), 3 mM ATP, 0.2 mg/ml SRF, and 30 \( \mu \text{M} \) total Ca\(^{++}\) (\( \text{CaCl}_2 + ^{45}\text{CaCl}_2 \)). Monophasic electrical stimulation was 10 ms in duration. Voltage was measured at the electrodes by using an oscilloscope. Electrical stimulation was applied in the last 2.5 min of the incubation period. Abscissa shows radioactivity of \( \text{Ca}^{++} \) taken up by SRF. Over 95\% of total Ca\(^{++}\) was taken up by the control sample.

the stimulation voltage and frequency. Results similar to those described by Lee et al. (1966) were observed but there were some differences. In their experiments, saturation effects of voltages and frequencies were reported; that is, even at the highest voltages and frequencies only half of the Ca\(^{++}\) uptake was inhibited. In our experiments, however, complete inhibition was observed at higher voltages or frequencies. Such complete inhibition was also observed by Turina and Jenny (1968). Moreover, in Lee's experiments, a very low voltage such as 0.5 V was effective on Ca\(^{++}\) uptake, but in our study no effect was observed below 1.5 V.

During and after stimulation no change was detected by Lee in pH and temperature. In addition, Lee detected no gas production, whereas in our study gas production was observed at the platinum electrodes when the voltage exceeded 2 V. Furthermore, after strong electrical stimulation, it became difficult for the sample solution to pass through the Millipore filter.

Electric current passing through the electrodes was measured by the oscilloscope and at the same time voltage values were recorded (Fig. 1). Below about 1.5 V, no current passed, and with elevation of the applied voltage, the current increased linearly with an inflection point at about 1.5 V. This value corresponds roughly to the so-called decomposition voltage. When
the applied voltage was 5 V, electric current was about 40 mA in our solvent condition. The data in Fig. 1 were replotted in Fig. 2 against the total current which passed during the stimulation. All the data obtained by changing frequencies and voltages lie on one curve. Furthermore, when the duration of impulse was changed, the data obtained lie on the same curve.

From this observation it became clear that the effect of the electrical stimulation was dependent only on the total current which passed through the solution. Next the shape of the impulse was changed, i.e. the voltage and duration of impulse were changed, the frequency and the total current remaining constant. The result is shown in Fig. 3. The effect of the electrical stimulation was independent of the applied voltage when the total current was kept constant.

Next, the platinum electrodes were replaced by silver-silver chloride electrodes. When electrodes of the same size and shape were used, the current which passed through the suspension was almost the same as when platinum electrodes were used. The results obtained with changing voltage are shown by the upper curve in Fig. 2. Although almost the same current and voltage were applied to the SRF suspension as when platinum electrodes were used, Ca++ uptake was maintained in its fully active level in spite of the electrical stimulation. No gas production was observed even at a high voltage such as

![Figure 2](image-url)

**Figure 2.** Effect of electrical stimulation on Ca++ uptake of SRF. All data were replotted from Fig. 1 against total electric current passed through the suspension. In the lower curve the same symbols as those in Fig. 1 are used. In the upper curve (+—+), silver-silver chloride electrodes were used. In this case the same solvent condition shown in Fig. 1 was used. The stimulation was 10 ms in duration and 2 Hz.
5 or 10 V. When small electrodes of silver-silver chloride wire (1 mm diameter, 7-mm long, 3-mm apart) similar to Lee's were used, Ca++ uptake was inhibited at high voltages. In this case a small amount of gas was produced; moreover the extent of inhibition was smaller than that seen when platinum electrodes were used.

This difference, dependent on the kind of the electrodes, can be explained as follows. When using platinum electrodes electrolysis of potassium chloride occurs in the solvent. Chlorine is generated at the anode and hydrogen is generated at the cathode. On the other hand, when using silver-silver chloride electrodes, such a chemical reaction does not occur, unless the current density is too great. The reaction $\text{Ag} + \text{Cl}^- \rightarrow \text{AgCl} + e^-$ occurs at the anode and the reverse reaction at the cathode; as a result, no electrolysis occurs in the solution. Thus the occurrence of the electrolysis must have a close relation to the inhibition of Ca++ uptake of SRF.

However, if the surface of the electrode is too small in relation to the current passed, then even when silver-silver chloride electrodes are used, some electrolysis of potassium chloride may occur; that is, some generation of chlorine and hydrogen occurs at the electrodes. These products act upon SRF in the same way as they do when platinum electrodes are used. Our final experiment and Lee's experiment which both used small electrodes of silver-silver chloride wire, probably reflect the effects of the production of chlorine.

**Ca++ Rebinding to SRF after Cessation of the Electrical Stimulation**

In Lee's paper, re-uptake of Ca++ after electrical stimulation was reported. This supported the belief that the release of Ca++ must be due to the electrical response of the SRF membrane in vitro. We repeated Lee's experiment;
our result is shown in Fig. 4. Release of Ca\(^{++}\) during electrical stimulation and re-uptake of Ca\(^{++}\) after cessation of electrical stimulation were observed. Overall behavior of Ca\(^{++}\) uptake and release was similar to that described by Lee. However, in our study the rate of Ca\(^{++}\) uptake in the initial phase is very fast. The final level of Ca\(^{++}\) content was decreased to nearly zero by the electrical stimulation, and the rate of re-uptake was slow. In Lee's study the rates of initial uptake and re-uptake were both slow and the Ca\(^{++}\) level stayed at about 50% after electrical stimulation, as noted before.

Data in Fig. 4 suggest that the mechanism of Ca\(^{++}\) uptake in the initial phase and that of re-uptake after electrical stimulation are different. We tried to separate the Ca\(^{++}\)-release process from energy supplies such as ATP. The effect of electrical stimulation was therefore examined using SRF loaded with Ca\(^{++}\) by incubation without active transport. Concentrated SRF was incubated with radioactive Ca\(^{++}\) for more than 10 h at 0°C. The SRF was diluted about 100 times into a medium containing only buffer without ATP and Ca\(^{++}\). The release of radioactive Ca\(^{++}\) was followed after the dilution. The diluted suspension was taken into the syringe tube and stimulation was applied by the method described in the previous section. In the other case electrical stimulation was stopped at an appropriate time and the re-uptake was followed. The result is shown in Fig. 5. The release of Ca\(^{++}\) without stimulation was very slow and the effect of electrical stimulation was very similar to the case in Fig. 4. Electrical stimulation is effective in releasing Ca\(^{++}\) from
Figure 5. Effect of the electrical stimulation on release and rebinding of Ca++ by SRF loaded with Ca++ by incubation. SRF was incubated for 15 h in 1 mM CaCl₂ (CaCl₂ + ⁴⁰CaCl₂), 0.1 M KCl, 2 mM MgCl₂, 20 mM Tris-maleate (pH 6.5), 20 mg/ml SRF. 10 µl of the incubated SRF was taken into 1 ml of 0.1 M KCl, 2 mM MgCl₂, 20 mM Tris-maleate (pH 6.5) and the diluted suspension was put into the syringe. The application of electrical stimulation was initiated 30 s after dilution under the same conditions shown in Fig. 4. O—O, continued stimulation; •—•, stimulation stopped after 1.5 min; X—X, Ca++ content without stimulation.

Interestingly, Fig. 5 shows clearly that re-uptake of Ca++ after cessation of electrical stimulation proceeds in the same way whether ATP is present or absent. This phenomenon is caused by rebinding of Ca++ which does not require an energy supply; i.e., it is independent of active transport. The re-uptake of Ca++ after the cessation of the stimulation described in Lee's paper thus seems to be an artifact.

Release and rebinding of radioactive Ca++ were also studied after dilution of SRF into a solvent containing 0.5 mM EDTA or 1 mM of nonradioactive CaCl₂ in addition to the same buffer used to obtain the results shown in Fig. 5. After cessation of electrical stimulation, rebinding of radioactive Ca++ similar to the previous case was observed. In the next experiment, SRF incubated with nonradioactive Ca++ of 1 mM was diluted 100 times in buffer containing 10 µM of radioactive Ca++, and the binding of radioactive Ca++ followed after cessation of the electrical stimulation. No binding of radioactive Ca++ was observed. From these observations it can be concluded that only Ca++ released from SRF can rebind to SRF after cessation of electrical stimulation.

Other interesting phenomena were observed in this rebinding process. As shown in Fig. 6, when SRF was simply diluted after cessation of electrical stimulation, rapid rebinding was observed. This rapid rebinding activity continued for a few minutes just after cessation of stimulation. This process seems to be due to dilution in the solution of chlorine which was generated
FIGURE 6. Effect of dilution on rebinding of Ca\(^{++}\) and of reapplication of electrical stimulation. Experimental conditions were the same as shown in Fig. 5. Curve 0, rebinding of Ca\(^{++}\) after the cessation of 1.5 min electrical stimulation (control). Curve 1, SRF was further diluted with 3 ml of the same solvent just after cessation of electrical stimulation. Curve 2, SRF was further diluted with 3 ml of the solvent 15 s after cessation of the stimulation. Curve 3, electrical stimulation was reapplied 30 s after cessation of the initial stimulation. Thin line shows the Ca\(^{++}\) content when the electrical stimulation was continued (data from Fig. 5).

at the electrode. After the rebinding of Ca\(^{++}\), electrical stimulation was applied again, and a very rapid decrease of Ca\(^{++}\) content was observed as shown in Fig. 6. However the Ca\(^{++}\) level after this rapid decrease was the same as the level just after cessation of electrical stimulation. All of these observations show that the mechanism of rebinding of Ca\(^{++}\) is different from that of the initial rapid uptake since the initial rapid uptake requires the energy of ATP.

The observations described above were obtained when platinum electrodes were used. When silver-silver chloride electrodes were used, increase of the rate of Ca\(^{++}\) release was not observed. The release of Ca\(^{++}\) from SRF by electrical stimulation using platinum electrodes is essentially irreversible and the rebinding of Ca\(^{++}\) after the cessation of electrical stimulation is caused by a denaturation of SRF. The mechanism of this artifact cannot be explained at present.

Some Changes of the Properties of SRF

The effect of electrical stimulation on SRF can thus be considered to result from the products of electrolysis at the electrodes, especially chlorine. Chlorine is a powerful oxidant. To compare this with other chemical oxidation reactions, the effect of known oxidants was studied.

Initially the effect of chlorine itself was studied. SRF suspension was ex-
posed to sodium hypochlorite during the uptake of Ca++. The results are shown in Fig. 7. A small amount of chlorine strongly inhibited Ca++ uptake. The effect of hydrogen peroxide was also examined. During the uptake of Ca++ the suspension was exposed to 40 mM of hydrogen peroxide for 3 min, and Ca++ uptake was inhibited about 35%.

SRF is expected to be denatured by the oxidants. After exposure to the oxidants in the solvent condition shown in Fig. 8, the absorption spectrum of SRF in the ultraviolet region was measured and compared with that of non-oxidized SRF. In this experiment only phosphate buffer was used to avoid the high absorbance of maleate. Oxidation by sodium hypochlorite changed markedly the ultraviolet absorption spectrum, as shown in Fig. 8. In the case of oxidation by hydrogen peroxide the difference spectrum could not be measured because of the high absorbance of hydrogen peroxide.

When a sample solution of the same solvent was exposed to electrical stimulation, the difference absorption spectrum was also recorded. As shown in Fig. 8, the spectrum after electrical stimulation is very similar to that found after oxidation by chlorine. In both cases characteristic peaks of the difference spectrum appeared at about 240 nm and 300 nm.

The activity of ATPase after exposure to electrical stimulation was measured by applying electrical stimulation without ATP and then adding ATP. The final solvent condition was the same as in Fig. 1. When the electrical stimulation was weak, the ATPase increased slightly. With further increase of stimulation, the ATPase decreased gradually.

![Figure 7. Effect of chlorine oxidation on the uptake of Ca++. Sodium hypochlorite was used as chlorine source. Ca++ uptake was measured in the presence of chlorine. Other conditions are the same as shown in Fig. 1. pH of the solution increased to 6.7 in the presence of 3 mM chlorine.](image-url)
DISCUSSION

As described in the Results section, the effect of electrical stimulation on SRF using platinum electrodes depended completely on the total current passed through the suspension but not directly on the voltage, the frequency, or the duration of impulses. However when silver-silver chloride electrodes were used instead of platinum electrodes, Ca\(^{++}\) uptake remained essentially unchanged. After exposure to electrical stimulation by use of platinum electrodes, the absorption spectrum of SRF suspension was changed. This change was almost the same as that of SRF oxidized by chlorine.

The difference spectrum after electrical stimulation had characteristic peaks at about 240 nm and 300 nm. In the case of photosensitized oxidation of SRF by xanthene dyes a very similar change of the absorption spectrum was obtained (Kondo and Kasai, 1973). This change of SRF was accompanied
by a decrease of Ca\textsuperscript{++} uptake and ATPase activity. Therefore the change of the absorption spectrum of SRF caused by electrical stimulation might be caused by the oxidation of tryptophane or histidine residues of the protein component of SRF.

The chlorine generated at the anode can be calculated to be about 1.5 mM with a total current of 0.15 C; about 50\% inhibition of Ca\textsuperscript{++} uptake was observed. Direct comparison is difficult between this value and that of chlorine concentration which worked as the oxidant shown in Fig. 7. In the case of the electrolysis, not all chlorine generated on the electrode affects the SRF, because most of chlorine is lost as gas.

Re-uptake of Ca\textsuperscript{++} after cessation of electrical stimulation as observed in Lee's study was not due to the original uptake activity of SRF. Thus, the observed effect of electrical stimulation was essentially irreversible.

It has been reported that when an alternating current is passed directly through the suspensions, the effect of stimulation is not observed even if a very high voltage is applied (Van der Kloot, 1966). This result can be explained by a fact well known in the field of electrochemistry: When current density at the electrode surface is small, alternating current cannot cause electrolysis of the solution (Shipley and Goodeve, 1927). When platinum electrodes were used it was reported that the generation of chlorine does not occur at 60 Hz even at currents as high as 0.7 mA/cm\textsuperscript{2}. This is also the reason why in Lee's study biphasic stimulation was only slightly effective.

From these results, it can be concluded that the decrease of Ca\textsuperscript{++} uptake and the release of Ca\textsuperscript{++} from SRF by electrical stimulation using platinum electrodes results from irreversible denaturation of SRF secondary to oxidation caused by chlorine generated at the platinum electrodes by electrolysis of potassium chloride in solution. However, our experiment does not exclude the possibility of true electrical excitation by electrical stimulation.

In this paper direct evidence of the electrical responsiveness of the SRF membrane in vitro was not obtained. However, recently we succeeded in demonstrating electrical responsiveness of purified SRF in vitro (Kasai and Miyamoto, 1973). In that experiment SRF incubated in less permeable anions such as sulfate or methanesulfonate was diluted in the medium containing a permeable anion such as chloride. A transient increase of Ca\textsuperscript{++} efflux from SRF was observed.

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