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Mechanism of Calcium Release from Skeletal Sarcoplasmic Reticulum

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Summary. Ca^{2+}-induced Ca^{2+} release at the terminal cisternae of skeletal sarcoplasmic reticulum was demonstrated using heavy sarcoplasmic reticulum vesicles. Ca^{2+} release was observed at 10 μM Ca^{2+} in the presence of 1.25 mM free Mg^{2+} and was sensitive to low concentrations of ruthenium red and was partially inhibited by valinomycin. These results suggest that the Ca^{2+}-induced Ca^{2+} release is electrogenic and that an inside negative membrane potential created by the Ca^{2+} flux opens a second channel that releases Ca^{2+}. Results in support of this formulation were obtained by applying a Cl^- gradient or K^+ gradient to sarcoplasmic reticulum vesicles to initiate Ca^{2+} release. Based on experiments the following hypothesis for the excitation-contraction coupling of skeletal muscle was formulated. On excitation, small amounts of Ca^{2+} enter from the transverse tubule and interact with a Ca^{2+} receptor at the terminal cisternae and cause Ca^{2+} release (Ca^{2+}-induced Ca^{2+} release). This Ca^{2+} flux generates an inside negative membrane potential which opens voltage-gated Ca^{2+} channels (membrane potential-dependent Ca^{2+} release) in amounts sufficient for contraction.

Key words sarcoplasmic reticulum · Ca^{2+} release · excitation-contraction coupling · muscular contraction · valinomycin · ruthenium red

Introduction

The role of Ca^{2+} in the contraction mechanism of skeletal muscle is well understood [15, 58, 59]. The central role of the sarcoplasmic reticulum in the removal of Ca^{2+} from the cytoplasm is also well established [28, 31, 41, 57, 60]. However, the coupling of the action potential of the transverse tubule (T-tubule) membrane to the release of Ca^{2+} from the sarcoplasmic reticulum has remained a mystery [14, 17, 21, 42]. It has been proposed that the release of Ca^{2+} is induced by (*a*) depolarization, (*b*) Ca^{2+}, and (*c*) charge movements.

The proposition for the depolarization mechanism is based on observations that in a medium of less permeant anions such as proprionate or methanesulfonate, substitution with chloride ions results in the release of Ca^{2+} from sarcoplasmic reticulum of skinned muscle fibers [18, 19, 56] or from isolated sarcoplasmic reticulum [9, 34–36]. It is not clear, however, how such a change in chloride ion composition could be achieved under physiological conditions. Although other modes of depolarization are possible, there is no evidence for a direct electrical coupling between the T-system and the sarcoplasmic reticulum [15, 24, 25].

Experimental evidence in support of the Ca^{2+}-induced Ca^{2+} release was obtained with skinned fibers [20, 23] as well as with isolated sarcoplasmic reticulum [32, 37, 48]. It was proposed that small amounts of Ca^{2+} which cross the T-tubule membrane during the action potential trigger the massive release of Ca^{2+} from the sarcoplasmic reticulum. However, Ca^{2+}-induced Ca^{2+} release was observed only at relatively high Ca^{2+} and low Mg^{2+} concentrations which led Endo [17] to conclude that "the Ca^{2+}-induced Ca^{2+} release mechanism does not seem to play the primary role in the physiological activation mechanism in skeletal muscle," and that it "may play an important role under certain pharmacological or pathological conditions, however." In a previous communication [46] we have presented new data that allow us to reconsider Ca^{2+} as a transmitter in the excitation from the T-tubules to the terminal cisternae of sarcoplasmic reticulum.

The proposal for a charge-movement induced Ca^{2+} release is based on observations on electrical stimulation of muscle showing a correlation in the time course and threshold between charge movements and contractility [1, 12, 26, 51], but there is still some controversy about the correlation in stretched fibers [2, 30]. Basically it is a mechanical model in which a connecting rod is induced by charge movements to open (or close) a Ca^{2+} channel in SR.

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In this paper we present further evidence on the Ca^{2+}-induced Ca^{2+} release, using an isolated preparation of heavy sarcoplasmic reticulum, and the role of a membrane potential change in a secondary mechanism for Ca^{2+} release.

Materials and Methods

Sarcoplasmic reticulum (SR) vesicles were isolated from rabbit skeletal muscle and fractionated as described [46]. Fractions obtained from a 0.85–1.6 M linear sucrose gradient were used immediately or quickly frozen and stored in liquid nitrogen.

Treatment of SR Vesicles at High Salt Concentrations

Sarcoplasmic reticulum fractions were diluted with a equal volume of 0.6 M KCl, 5 mM Tris-maleate (pH 6.5) and centrifuged at 130,000 × *g* for 45 min. The pellets were suspended in 0.6 M KCl, 5 mM Tris-maleate pH 6.5 and incubated for 1 or 16 hr at 0°. The suspensions were centrifuged at 130,000 × *g* for 45 min. The pellets were suspended in 0.1 M KCl, 5 mM Tris-maleate (pH 6.5) and again centrifuged at 130,000 × g for 45 min. The resulting pellets were suspended in 0.1 M KCl, 5 mM Tris-maleate (pH 6.5) at about 20 mg/ml and stored at 0°.

Assay of Ca^{2+} Uptake

High salt-treated SR fractions were equilibrated prior to assay at 2 mg/ml for 2 hr at 0° with 0.3 M K methanesulfonate (KMS), 20 mM MES-Tris (pH 6.5). Ca^{2+} uptake was started at 23° by adding 50 µl of the equilibrated SR suspension to 1 ml of uptake solution containing 0.3 M KMS, 20 mM MES-Tris (pH 6.5), 2 mM ATP, 3 mM $MgSO_4$, 40 µM $^{45}CaCl_2$ (25 µCi/µmol), 0–10 mM EGTA without or with 5 µM ruthenium red. At the indicated times 150 µl aliquots were passed through Millipore filters and washed with 2 ml of 0.3 M KMS and 20 mM MES-Tris (pH 6.5). The filters were dried under an infrared lamp and counted in Liquiscint (National Diagnostics) in a Beckman scintillation counter. Free Ca^{2+} concentrations were calculated according to Harafuji and Ogawa [27].

Assay of Ca^{2+} or Caffeine-Induced Ca^{2+} Release

Sarcoplasmic reticulum fractions or high salt-treated SR fractions were equilibrated at 2 mg/ml prior to assay for 2 hr at 0° with either 0.1 M K glutamate (pH 6.9), or 0.3 M KMS, 20 mM MES-Tris (pH 6.5). Aliquots of 20 µl were loaded at 23° with $^{45}Ca^{2+}$ by addition of 200 µl of a solution containing 0.1 M K glutamate (pH 6.9) or 0.3 M KMS, 20 mM MES-Tris (pH 6.5) in addition to 2 mM ATP, 3 mM $MgSO_4$ and 10 µM $^{45}CaCl_2$. At 2 min 55 sec, when almost all Ca^{2+} in the medium was taken up by SR vesicles, caffeine or $CaCl_2$ was added at the indicated concentrations. At 3 min 2 sec an aliquot was passed through a Millipore filter (HAWP 025), washed with 2 ml of 0.1 M K glutamate (pH 6.9) or 0.3 M KMS and 20 mM MES-Tris (pH 6.5) and assayed as described above.

Assay of Membrane Potential Dependent Ca^{2+} Release

High salt-treated SR fractions at 2 mg/ml were equilibrated prior to assay for 2 hr at 0 °C with 0.3 M KMS, 20 mM MES-Tris (pH 6.5). Aliquots of 20 µl were loaded at 23° with $^{45}Ca^{2+}$ as described above. At 3 min an aliquot was passed though a Millipore filter and washed with 2 ml of washing solution and assayed. For Cl^- gradient experiments, the uptake solution contained 0–294 mM KMS, 6–300 mM KCl (at a total K concentration of 300 mM), 20 mM MES-Tris (pH 6.5), 2 mM ATP, 3 mM $MgSO_4$ and 10 µM $^{45}CaCl_2$. The washing solution contained 0.3 M KCl and 20 mM MES-Tris (pH 6.5). For the K-gradient experiments the uptake solution contained 0.3 M KMS, 20 mM MES-Tris (pH 6.5), 2 mM ATP, 3 mM $MgSO_4$, 10 µM $^{45}CaCl_2$, 1 µg/ml valinomycin, and 0.05 % hexamethylphosphorotriamide (HMPA). The washing solution contained 0–294 mM Tris-MS, 300–6 mM KMS, 20 mM MES-Tris (pH 6.5) and 1 µg/ml valinomycin.

Passive Loading of Ca^{2+} and Efflux Assay

HSR (4 mg/ml) was incubated in 0.3 M KMS, 20 mM MES-Tris (pH 6.5), and 1 mM $^{45}CaCl_2$ for 16 hr at 0°. Aliquots of 50 µl were diluted into 1 ml of 0.3 M KMS, 20 mM MES-Tris (pH 6.5) in the presence (free $Ca^{2+} = 0.5$ µM) or absence (free $Ca^{2+} = 50$ µM) of 0.5 mM EGTA and 1 mM $MgSO_4$. Caffeine when added was at 2 mM. At the indicated times 150 µl aliquots were passed through Millipore filters (HAWP 025) and washed with 2 ml of 0.3 M KMS and 20 mM MES-Tris (pH 6.5).

The standard error of the Ca^{2+} measurement was below 10 %.

Materials

Valinomycin and ruthenium red were obtained from Sigma; hexamethylphosphorotriamide from Pfaltz and Bauer; $^{45}CaCl_2$ from ICN. Other materials and assay procedures were as described previously [29].

Results

Calcium-Induced Calcium Release in Terminal Cisternae

It has been repeatedly stated that the heavy fraction of SR has less Ca^{2+} uptake capacity than the light fraction [9, 10, 40]. The conclusion was therefore drawn that the heavy fraction of SR contains less Ca^{2+} pump ATPase than the light fraction [40]. However, the heavy fraction of SR is mainly derived from terminal cisternae of SR [11, 43] which *in vivo* contain much more calcium than the longitudinal portion of SR [53]. The heavy fraction of SR therefore should have a higher Ca^{2+} loading capacity, and the distribution of calsequestrin is consistent with this notion.

We were able to demonstrate that the heavy fraction of SR has indeed a higher Ca^{2+}-uptake capacity than the light fraction, provided the Ca^{2+}-release pathway was blocked by ruthenium red (Fig. 1). Since ruthenium red is known to be an inhibitor of Ca^{2+}-induced Ca^{2+} release [46, 48], the lower Ca^{2+}-uptake capacity of HSR in the absence of ruthenium red may be due to the activation of the Ca^{2+}-induced Ca^{2+} release by residual Ca^{2+} in the medium. This possibility was further investigated by using Ca-EGTA buffer. The time course of Ca^{2+} uptake by HSR was measured at various free Ca^{2+}

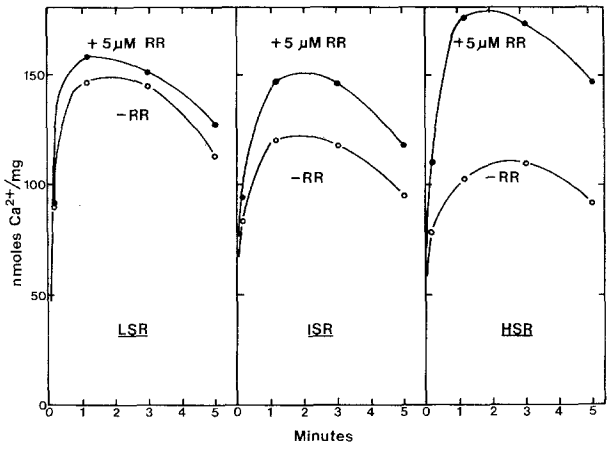

Fig. 1. Time course of Ca^{2+} uptake by sarcoplasmic reticulum fractions in the presence and absence of ruthenium red. Ca^{2+} uptake was measured as described under Materials and Methods in the presence (●) or absence (○) of 5 μM ruthenium red. The Ca^{2+} concentration in the medium was 40 μM and was reduced to about 25 μM when uptake was complete

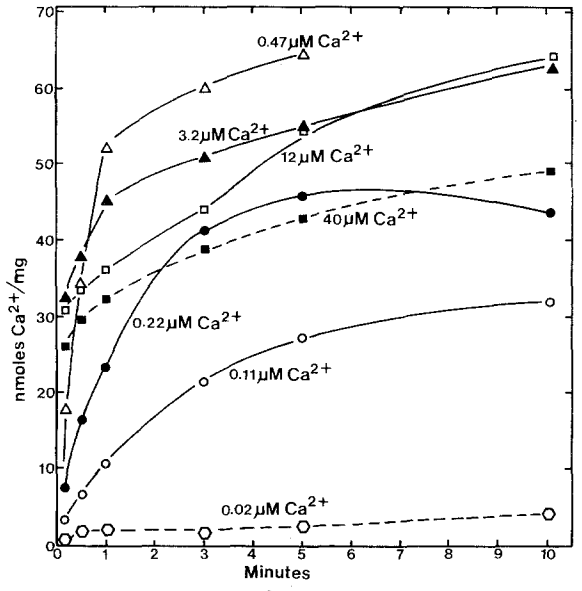

Fig. 2. Time course of Ca^{2+} uptake by HSR at various concentrations of free Ca^{2+}. Ca^{2+} uptake was measured as described under Materials and Methods. Total Ca^{2+} in the medium was 40 μM; free Ca^{2+} was adjusted by EGTA as follows: 10 mM EGTA: 0.02 μM Ca^{2+} (○), 2 mM EGTA: 0.11 μM Ca^{2+} (○), 1 mM EGTA: 0.22 μM Ca^{2+} (●), 500 μM EGTA: 0.47 μM Ca^{2+} (△), 100 μM EGTA: 3.2 μM Ca^{2+} (▲), 40 μM EGTA: 12 μM Ca^{2+} (□) and 0 μM EGTA: 40 μM Ca^{2+} (■)

concentrations varying from 0.02 to 40 μM. As shown in Fig. 2, both the initial rate of Ca^{2+} uptake as well as the level of Ca^{2+} uptake were increased with increasing the Ca^{2+} concentration from 0.02 to 0.47 μM. Above 3 μM Ca^{2+} the initial uptake rates were faster than at lower concentrations, but the Ca^{2+}-uptake levels were decreased. This decrease in the final level of Ca^{2+} was due to an increased Ca^{2+} efflux because the initial Ca^{2+}-uptake rate was

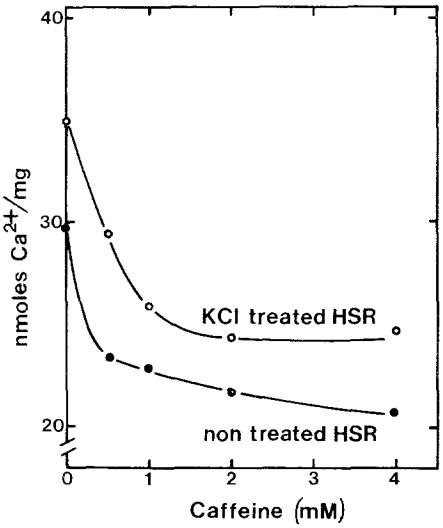

Fig. 3. Caffeine-induced Ca^{2+} release by HSR before and after treatment with high concentration of salt. HSR was treated with 0.6 M KCl as described under Materials and Methods. Treated and nontreated HSR were loaded with ^{45}Ca in 0.3 M KMS uptake solution as described under Materials and Methods. At 2 min 55 sec, 1 to 8 μl of 100 mM caffeine was added to the reaction mixture, and at 3 min 2 sec, a 150 μl aliquot was passed through a Millipore filter and counted as described under Materials and Methods. As a control, a 150 μl aliquot of the reaction mixture without caffeine was passed at 3 min through the filter. KCl treated HSR: ○; nontreated HSR: ●

much faster at the higher Ca^{2+} concentrations. The Ca^{2+}-uptake level of LSR was never decreased by increasing the Ca^{2+} concentration up to 40 μM. These results are consistent with the previous conclusion that the Ca^{2+}-induced Ca^{2+} release is operating in terminal cisternae of SR under physiological conditions [46]. The free Ca^{2+} concentration of the media was calculated as described under "Materials and Methods."

Effect of Treatment of HSR at High Salt Concentrations on Ca^{2+}- or Caffeine-Induced Ca^{2+} Release

According to the report by Campbell et al. [8], HSR vesicles retain their feet projections during the isolation of SR vesicles but they are removed by treatment of HSR at high concentrations of salt. The feet structure has been considered to play a crucial role in the excitation-contraction coupling of skeletal muscle because they connect the T-tubule membrane with the terminal cisternae of SR membrane. In order to explore a possible role of the feet in the Ca^{2+}-induced Ca^{2+} release mechanism, the HSR was treated with salt as described [8].

HSR and LSR were exposed to 0.6 M KCl as described under "Materials and Methods." As shown in Fig. 3 the caffeine-induced Ca^{2+} release

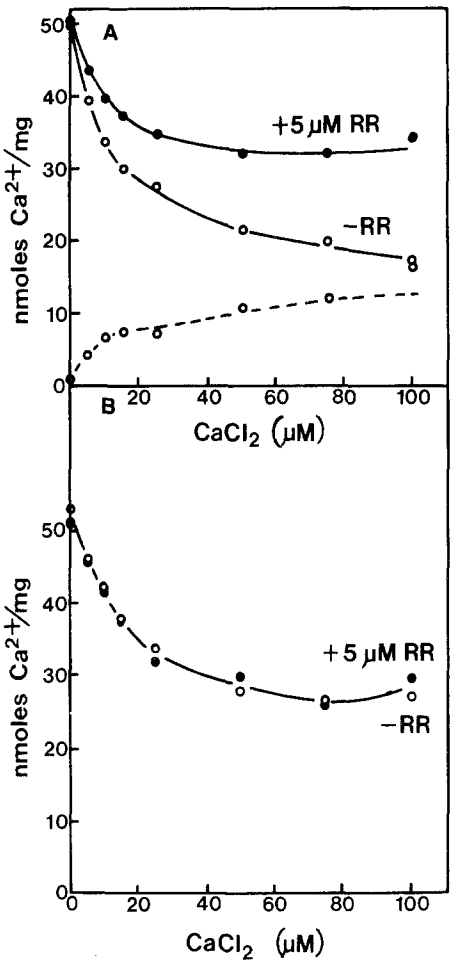

Fig. 4. Ca^{2+}-induced Ca^{2+} release by HSR and LSR after salt treatment. HSR (*A*) and LSR (*B*) were treated with 0.6 M KCl and were loaded with $^{45}Ca^{2+}$ in 0.3 M KMS uptake solution as described under Materials and Methods in the absence (○) or presence (●) of 5 μM ruthenium red. At 2 min 55 sec when almost all the Ca^{2+} in the medium was taken up by the SR vesicles, 2 μl of 0.5 to 10 mM $CaCl_2$ was added to the reaction mixture and at 3 min 2 sec a 150-μl aliquot was passed through a Millipore filter and assayed as described under Materials and Methods. As a control the same amount of reaction mixture was passed through the filter at 3 min without adding $CaCl_2$. Amount of released Ca^{2+}: ---○---; *RR*: ruthenium red

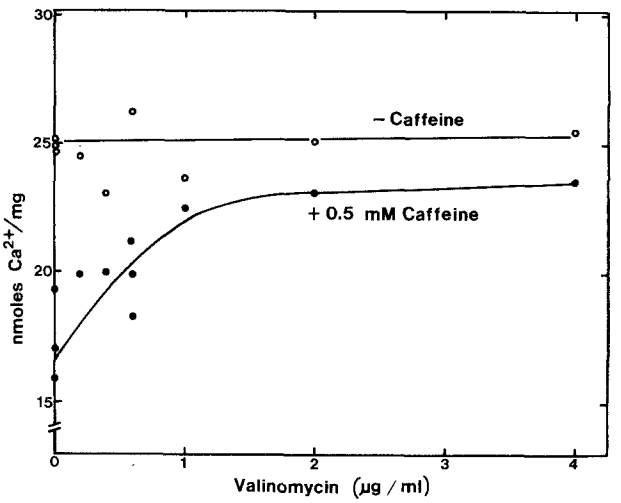

Fig. 5. Partial inhibition of caffeine-induced Ca^{2+} release by valinomycin. HSR was loaded with $^{45}Ca^{2+}$ in 0.1 M K glutamate uptake solution and in the presence of indicated amounts of valinomycin as described under Materials and Methods. At 2 min 55 sec, 1 μl of 100 mM caffeine was added to the reaction mixture (●), and at 3 min 2 sec, a 150-μl aliquot was passed through a Millipore filter and assayed. As a control the same amount of the reaction mixture was passed at 3 min through the filter without adding caffeine (○)

was not changed by this treatment either with respect to the sensitivity to caffeine or the amount of Ca^{2+} that was released. Actually, a small increase in Ca^{2+} uptake level was noted which was probably due to the removal of some contaminated proteins from the HSR. Since the effect of caffeine to release Ca^{2+} from SR has been attributed to the activation of the Ca^{2+}-induced Ca^{2+} release [17], high salt treatment was not expected to change the Ca^{2+}-induced Ca^{2+} release. As shown in Fig. 4*A* HSR maintained its high Ca^{2+}-induced Ca^{2+} release sensitivity after exposure to high salt concentrations. Increasing the amount of added $CaCl_2$, after loading of HSR with ^{45}Ca as indicated, released more Ca^{2+} and about 50% of this release was inhibited by 5 μM ruthenium red. Higher ruthenium red concentrations did not give further inhibition. The Ca^{2+} release in the presence of ruthenium red was considered to be caused by a Ca^{2+}/Ca^{2+} exchange catalyzed by the Ca^{2+} pump ATPase. The difference in the presence or absence of ruthenium red was used to calculate the Ca^{2+}-induced Ca^{2+} release as previously described [46]. As shown in Fig. 4*B* there was no ruthenium-red sensitive Ca^{2+} efflux in LSR either before or after treatment with high salt. These results indicate that the feet do not seem to play a significant role in the mechanism of the Ca^{2+}-induced Ca^{2+} release. The function of the feet is more likely to be concerned with the conduction of a signal from the T-tubule to the terminal cisternae, as will be discussed later.

Partial Inhibition of Calcium- or Caffeine-Induced Ca^{2+} Release by Dissipation of the Membrane Potential

The Ca^{2+}-induced Ca^{2+} release could generate an inside-negative membrane potential and should then be affected by a change in the membrane potential. When valinomycin was included in the assay medium, caffeine-induced Ca^{2+} release was indeed inhibited. However, in contrast to the inhibition by ruthenium red [46], the inhibition by valinomycin was only partial. As shown in Fig. 5, the Ca^{2+} re-

leased by 0.5 mM caffeine was inhibited by about 75% when the vesicles were equilibrated in 0.1 M K-glutamate and 1 μg valinomycin per ml. Higher concentrations of valinomycin did not give any further inhibition. Inhibition by valinomycin was also seen when the Ca^{2+}-induced Ca^{2+} release was measured in the presence of 1 μM ruthenium red (Fig. 6). The Ca^{2+}-induced Ca^{2+} release was inhibited completely at 20 μM ruthenium red. Although Ca^{2+} release was reduced in the presence of 1 μM ruthenium red, significant release was still observed on addition of 10 μM $CaCl_2$. This Ca^{2+} release was again partially inhibited by valinomycin in the medium. If caffeine or Ca^{2+} induce an electrogenic Ca^{2+} movement, dissipation of membrane potential by valinomycin would be expected to increase the flux. The opposite effect was observed. We propose that the Ca^{2+}- or caffeine-induced Ca^{2+} release creates an inside negative membrane potential which opens voltage-sensitive Ca^{2+} channels. This explanation is consistent with the Cl^- or "depolarization"-induced Ca^{2+} release observed in either skinned fiber or fragmented SR [9, 18, 19, 34–36, 56].

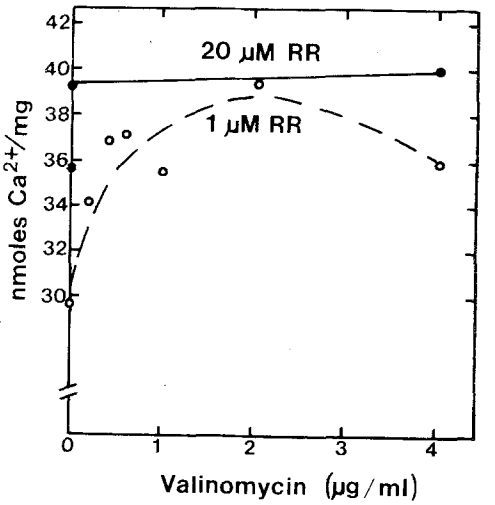

Fig. 6. Partial inhibition of Ca^{2+}-induced Ca^{2+} release by valinomycin. HSR was loaded with $^{45}Ca^{2+}$ in 0.1 M K glutamate, 2 mM ATP, 3 mM $MgSO_4$, 10 μM $^{45}CaCl_2$, the indicated amounts of valinomycin, and 1 or 20 μM ruthenium red. At 2 min 55 sec, 4 μl of 0.5 mM $CaCl_2$ was added to the reaction mixture, and at 3 min 2 sec, a 150-μl aliquot was passed through a Millipore filter and assayed as described before. ○: 1 μM ruthenium red; ●: 20 μM ruthenium red

Membrane Potential-Dependent Ca^{2+} Release

Sarcoplasmic reticulum vesicles were equilibrated at various concentrations of Cl^- and loaded with ^{45}Ca in the presence of ATP. After 3 min the SR suspension was passed through a Millipore filter and was washed with 300 mM KCl. Osmolarity was maintained constant by K methanesulfonate. Since the SR membrane is 50 times more permeable for Cl^- than for MS^- or for K^+ [38], the Cl^- gradient formed in the experiments generates an inside negative membrane potential. As shown in Fig. 7 Ca^{2+} release by the Cl^- gradient is sigmoidal and half of maximal stimulation at −20 mV. This result was obtained with HSR as well as with LSR. This kind of Ca^{2+} release has been reported previously [9, 34–36]. Objections have been raised such as the possibility of osmotic swelling [6, 44]. It should be pointed out that osmotic swelling could not have taken place, for example, when 0.3 M KMS was exchanged by 0.5 M KCl or even 0.88 M KCl. The evidence against a role of swelling is based on the fact that the rate of Ca^{2+} released by Cl^-, measured by absorbance change of arsenazo III, was faster than the Cl^- flux with a permeation time $T_{1/2}$ of 400 msec [38] and even faster than the water flux ($t_{1/2}$ = 100 ms) [33] across the SR membrane. It took only 25 msec for the efflux of half of maximal Ca^{2+} release induced by Cl^- (data not shown). This indicates that the Cl^--induced Ca^{2+} release is completed before Cl^- influx might cause SR vesicles to swell.

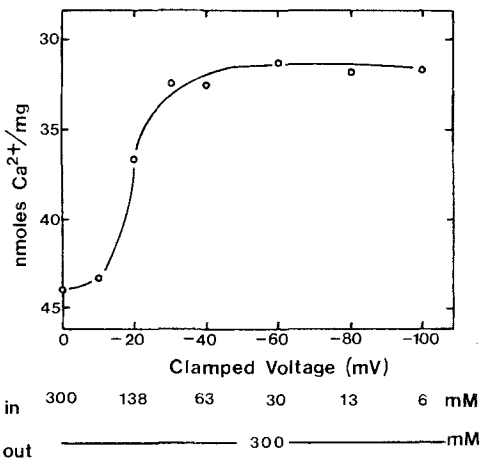

Fig. 7. Ca^{2+} release from sarcoplasmic reticulum by a Cl^- gradient. LSR was loaded with ^{45}Ca as described under Materials and Methods. At 3 min a 150-μl aliquot was passed through a Millipore filter and washed with 2 ml of 0.3 M KCl, 20 mM MES-Tris (pH 6.5). Clamped voltage values were calculated according to the Nernst equation

Ca^{2+} release also cannot be attributed to a shrinkage of the SR vesicles because exchange of 0.3 M KMS to 0.5 of 0.88 M KMS had no effect. It is of interest to note that the objections raised against a membrane potential-induced Ca^{2+} release were based on experiments [6, 44] in which loading of Ca^{2+} was achieved without ATP.

In order to change the membrane potential of SR vesicles, a K^+ gradient was applied together with valinomycin. Sarcoplasmic reticulum vesicles

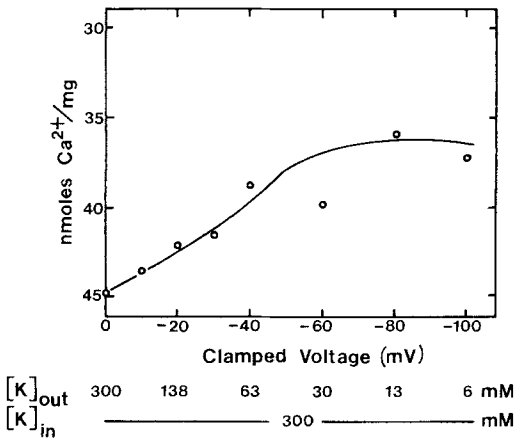

Fig. 8. Ca^{2+} release from HSR by a K^+ gradient. HSR was loaded with ^{45}Ca as described under Materials and Methods. At 3 min a 150 µl aliquot was passed through a Millipore filter and washed with 2 ml of washing solution containing various amounts of K^+

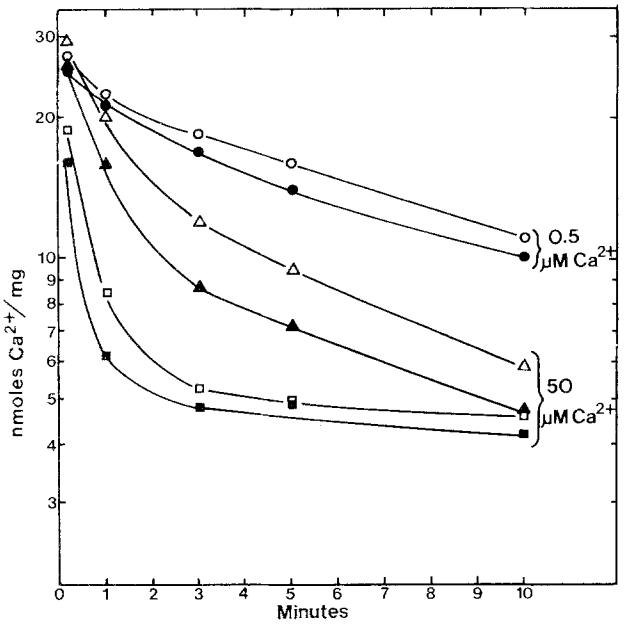

Fig. 9. Ca^{2+} efflux from HSR in the absence of ATP. HSR was passively loaded with 1 mM ^{45}Ca as described under Materials and Methods. An aliquot of 50 µl was diluted into 1 ml 0.3 M KMS, 20 mM MES-Tris (pH 6.5) in the presence (free Ca^{2+} = 0.5 µM ○, ●) or absence (free Ca^{2+} = 50 µM △, ▲, □, ■) of 0.5 mM EGTA and 1 mM $MgSO_4$ (○, ●, △, ▲), 2 mM caffeine (●, ▲, ■). The assays were performed as described under Materials and Methods

were equilibrated with high K^+ (0.3 M) in the presence of valinomycin and changed to low K^+, maintaining constant osmolarity with Tris. Under these conditions osmotic swelling should not occur while an inside negative membrane potential should be generated by K^+ efflux. As can be seen in Fig. 8, Ca^{2+} was released from HSR, provided 0.05% hexamethylphosphorotriamide was included in the assay medium. Similar experiments with LSR have not thus far been successful. Although it is conceivable

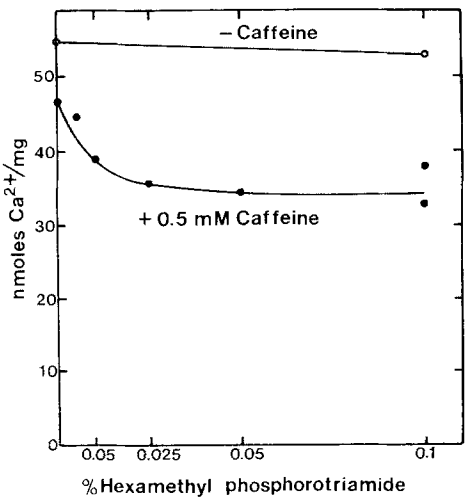

Fig. 10. Effect of hexamethylphosphorotriamide on caffeine-induced Ca^{2+} release. HSR was loaded with ^{45}Ca in 0.3 M KMS uptake solution in the presence of the indicated amounts of HMPA. At 2 min 55 sec, 1 µl of 100 mM caffeine was added to the reaction mixture (●), and at 3 min 2 sec, a 150-µl aliquot was passed through a Millipore filter and assayed. As a control, the same amount of the reaction mixture was passed at 3 min through the filter without adding caffeine (○)

that valinomycin acts by a mechanism other than as a K^+ ionophore, the most likely explanation is that it changes the membrane potential.

The Role of ATP on Caffeine or Ca^{2+}-Induced Ca^{2+} Release

Ca^{2+}-induced Ca^{2+} release in skinned fiber or SR vesicles was markedly increased on addition of ATP [18]. Similar observations were made on caffeine-induced Ca^{2+} release.

HSR vesicles were passively loaded with $^{45}CaCl_2$ by incubating the vesicles with 1 mM $^{45}CaCl_2$ for 16 hr at 0°. The HSR suspension was diluted with 20 volumes of a solution containing the same salt, as well as caffeine. When the free Ca^{2+} was low (0.5 µM), caffeine had almost no effect in the presence of 1 mM Mg^{2+}. In the presence of 50 µM Ca^{2+}, caffeine stimulated Ca^{2+} efflux under these conditions. When Mg^{2+} was removed Ca^{2+} efflux was much faster (Fig. 9). It should be noted that the sensitivity to Ca^{2+} was lower and the efflux rate much slower than when Ca^{2+} loading was achieved with ATP. These results again suggest a role of ATP in the Ca^{2+}-induced Ca^{2+} release phenomenon.

The mechanism of action of ATP on the Ca^{2+}-induced Ca^{2+} release is not known. Similarly, when 0.025% HMPA was included in the assay medium, the caffeine-induced Ca^{2+} release was enhanced (Fig. 10), and we have no explanation for this observation either. When SR vesicles were incubated without ATP before loading, Ca^{2+} uptake was al-

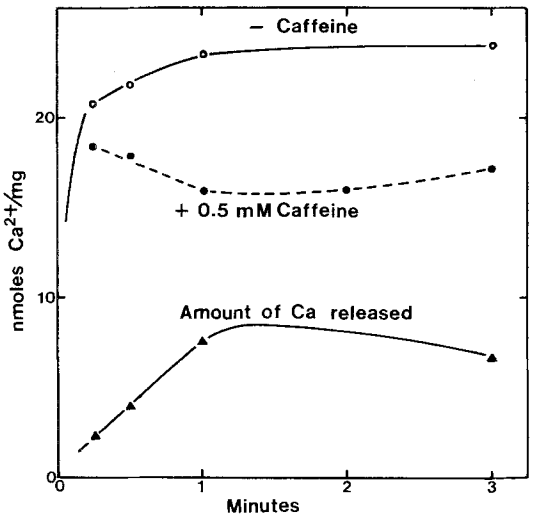

Fig. 11. Dependency of caffeine-induced Ca^{2+} release on time of Ca^{2+} uptake. HSR (2 mg/ml) was incubated in 0.1 M K glutamate (pH 6.9) for 2 hr at 0°. An aliquot of 20 µl was added to 200 µl of uptake solution containing 0.1 M K glutamate (pH 6.9), 2 mM ATP, 3 mM $MgSO_4$ and 10 µM $^{45}CaCl_2$ at 23 °C. At the indicated time 1 µl of 100 mM caffeine was added to the reaction mixture and a 150 µl aliquot was passed through a Millipore filter within 7 sec (●). As a control the same amount of the reaction mixture was passed through the filter at indicated times without adding caffeine (○). ▲: amount of Ca^{2+} released

most optimal 15 sec after addition of 2 mM ATP. On the other hand, under these conditions the caffeine-induced Ca^{2+} release was very small at 15 sec. It took about 1 min to reach the optimum effect of caffeine. During this time span the amount of released Ca^{2+} was much greater than expected from the slight increase in Ca^{2+} loading and may possibly have been caused by a secondary change due to the presence of ATP (Fig. 11).

Discussion

The observations on Ca^{2+}-induced Ca^{2+} release reported in this paper lead us to reconsider Ca^{2+} as a possible transmitter of a signal from the T-tubule membrane to the sarcoplasmic reticulum. A particular feature is the high sensitivity of HSR to Ca^{2+} even in the presence of 1.25 mM free Mg^{2+}. Where is the Ca^{2+} coming from to trigger Ca^{2+} release? Armstrong et al. [3] and Sandow [50] have presented evidences against Ca^{2+} as a transmitter of excitation-contraction coupling of skeletal muscle. They showed that skeletal muscle fiber bathed in 1 mM EGTA medium (which should have reduced the free Ca^{2+} to below 10^{-8} M) maintained contractility for at least 20 min. This was distinctly different from cardiac muscle which did not maintain contractility in a Ca^{2+}-free medium [4]. However, skeletal muscle has a much narrower T-tubular system than cardiac muscle [14] and the T-tubules have a Ca^{2+} ATPase which pumps Ca^{2+} out [7]. The removal of external Ca^{2+} [3, 50] may have stimulated the Ca^{2+} pump along the T-tubules and supplied sufficient Ca^{2+} to trigger the Ca^{2+} release from SR even in the presence of 1 mM EGTA. Evidence in favor of this explanation was provided by Putney and Bianchi [49] who observed a rapid decrease in isometric tension by the addition of 2.5 mM EGTA after treatment of the muscle with 8.3 µM Na dantrolene. Additional evidence for the role of Ca^{2+} in the medium was presented by Barrett and Barrett [5] who observed in skeletal muscle fibers at high concentration EGTA and citrate (80 to 90 mM) a reversible inhibition of contraction. Thus, the different requirements for Ca^{2+} in the medium between skeletal muscle and cardiac muscle is more likely due to structural differences of the T-system [55] than to a fundamentally different mechanism.

Why is the isolated HSR more sensitive to Ca^{2+} than the skinned fiber? One possibility is that the Ca^{2+} receptor site of SR is located in a compartment not readily accessible either from the myoplasm or from outside and that during isolation this barrier is destroyed. A good candidate for such a structure is the interior of feet projections. The space between the feet projections is accessible from the myoplasm [25] and cannot therefore be the pathway for Ca^{2+} movement. Recent EM observations of the feet structure showed that there may be an internal vacant space. According to Nunzi et al. [47] the most prominent profile of the feet structure is "a less dense core with two dense lines on either side, crossing the gap." A similar electron lucent core inside of the feet spanning the junctional gap was observed by Somlyo [52].

Based on the above considerations, the following hypothesis was formulated (Fig. 12).

1. The arrival of an action potential of the T-system opens a voltage-gated Ca^{2+} channel which allows Ca^{2+} influx from the T-tubule membrane to the compartment inside the feet structure.

2. This Ca^{2+} interacts with a Ca^{2+} receptor of the terminal cisternae membrane and opens a Ca^{2+} ruthenium red-sensitive channel (Ca^{2+}-induced Ca^{2+} release).

3. The Ca^{2+}-induced Ca^{2+} release generates an inside negative membrane potential which is propagated to the entire SR membrane and opens voltage-gated Ca^{2+} channels (membrane potential-dependent Ca^{2+} release).

After excitation the Ca^{2+} ATPase in the T-tubule membrane pumps the Ca^{2+} from the feet structure compartment, thereby closing the Ca^{2+} channel which is responsible for the Ca^{2+}-induced Ca^{2+} release. The membrane potential-dependent Ca^{2+} channel may be closed by K^+ fluxes via the voltage-

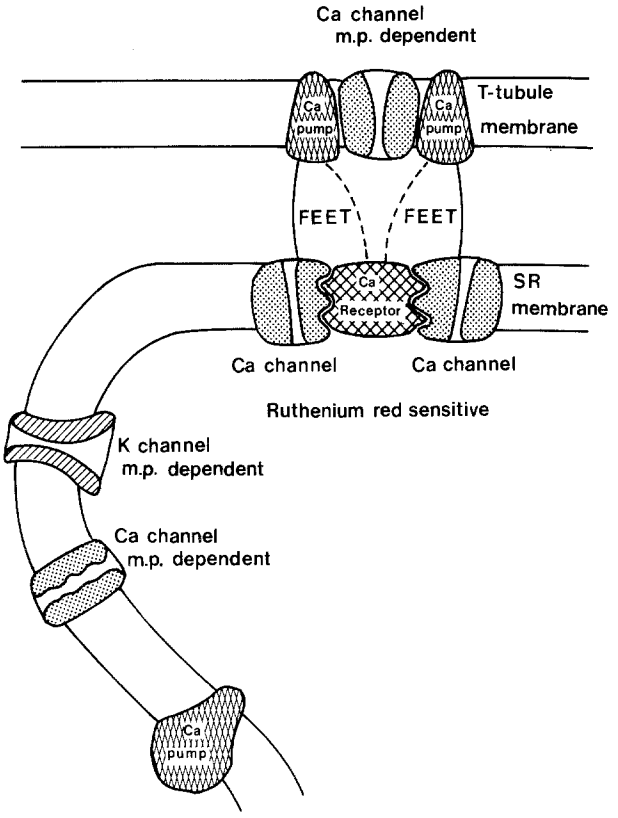

Fig. 12. Schematic model of excitation-contraction coupling. When the action potential generated at the endplate is propagated to the T-tubule, the Ca^{2+} channel in the membrane opens. It allows Ca^{2+} in the T-system to get inside of the feet projections which connect the T-tubule membrane with SR terminal cisternae. The Ca^{2+} binding to Ca^{2+} receptor causes ruthenium red-sensitive Ca^{2+} channel in the SR to open. Ca^{2+} efflux through this channel (Ca^{2+}-induced Ca^{2+} release) render the SR membrane inside negative. This membrane potential shift opens another Ca^{2+} channel of SR, and with the aid of K^+ movement in the opposite direction via the K^+ channel, SR release enough Ca^{2+} for contraction

gated K^+ channel of SR [13, 39, 45]. This K^+ channel was also proposed to serve as a charge compensation mechanism which enables SR membranes to release Ca^{2+} very rapidly. This proposal was supported by the observation that K^+ concentration in the terminal cisternae was increased during Ca^{2+} release as revealed by electron probe analysis [54]. The hypothesis described above is therefore consistent with the data presented in this paper and with observations on changes of the membrane potential in skinned fiber of skeletal muscle during caffeine or Ca^{2+}-induced Ca^{2+} release [22].

Sodium dantrolene was proposed by Putney and Bianchi [49] to act at a membrane potential-dependent Ca^{2+} channel of the T-tubule membrane. No inhibition of Ca^{2+} release by Na dantrolene has been observed in skinned fiber [17]. In our system neither Ca^{2+}-induced Ca^{2+} release nor membrane potential-dependent Ca^{2+} release was inhibited by Na dantrolene. It is therefore possible that Na dantrolene acts at the initial stage of excitation [51] which is no longer operative after disruption of the muscle structure.

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References

1. Adrian, R.H., Almers, W. 1976. Charge movement in the membrane of striated muscle. *J. Physiol. (London)* **254**:339–360
2. Adrian, R.H., Capato, C., Huang, C.L.-H. 1978. Effect of stretch on intramembrane charge movement in striated muscle. *J. Physiol. (London)* **284**:151 P
3. Armstrong, C.M., Bezanilla, F.M., Horowicz, P. 1972. Twitches in the presence of ethylene glycol bis (β-aminoethyl ether)-N, N'-tetraacetic acid. *Biochim. Biophys. Acta* **267**:605–608
4. Bailey, L.E., Dresel, P.E. 1968. Correlation of contractile force with a calcium pool in the isolated cat heart. *J. Gen. Physiol.* **52**:969–982
5. Barrett, J.N., Barrett, E.F. 1978. Excitation-contraction coupling in skeletal muscle: Blockade by high extracellular concentrations of calcium buffers. *Science* **200**:1270–1272
6. Beeler, T., Martonosi, A. 1979. The relationship between membrane potential and Ca^{2+} fluxes in isolated sarcoplasmic reticulum vesicles. *FEBS Lett.* **98**:173–176
7. Brandt, N.R., Caswell, A.H., Brunschwig, J.-P. 1980. ATP-energized Ca^{2+} pump in isolated transverse tubules of skeletal muscle. *J. Biol. Chem.* **255**:6290–6298
8. Campbell, K.P., Franzini-Armstrong, C., Shamoo, A.E. 1980. Further characterization of light and heavy sarcoplasmic reticulum vesicles: Identification of the 'sarcoplasmic reticulum feet' associated with heavy sarcoplasmic reticulum vesicles. *Biochim. Biophys. Acta* **602**:97–116
9. Campbell, K.P., Shamoo, A.E. 1980. Chloride-induced release of actively loaded calcium from light and heavy sarcoplasmic reticulum vesicles. *J. Membrane Biol.* **54**:73–80
10. Caswell, A.H., Brandt, N.R. 1981. Ion-induced release of calcium from isolated sarcoplasmic reticulum. *J. Membrane Biol.* **58**:21–33
11. Caswell, A.H., Lau, Y.H., Brunschwig, J.-P. 1976. Ouabain-binding vesicles from skeletal muscle. *Arch. Biochem. Biophys.* **176**:417–430
12. Chandler, W.K., Rakowski, R.F., Schneider, M.F. 1976. Effects of glycerol treatment and maintained depolarization on charge movement in skeletal muscle. *J. Physiol. (London)* **254**:285–316
13. Coronado, R., Rosenberg, R.L., Miller, C. 1980. Ionic selectivity, saturation and block in a K^+-selective channel from sarcoplasmic reticulum. *J. Gen. Physiol.* **76**:425–446
14. Ebashi, S. 1976. Excitation-contraction coupling. *Annu. Rev. Physiol.* **38**:293–313
15. Ebashi, S., Endo, M. 1968. Calcium ion and muscle contraction. *Prog. Biophys. Mol. Biol.* **18**:123–183
16. Endo, M. 1975. Mechanism of action of caffeine on the sarcoplasmic reticulum of skeletal muscle. *Proc. Jpn. Acad.* **51**:479–484
17. Endo, M. 1977. Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.* **57**:71–108
18. Endo, M., Kitazawa, T. 1976. The effect of ATP on calcium

release mechanisms in the sarcoplasmic reticulum of skinned muscle fibers. *Proc. Jpn. Acad.* **52**:595–598
19. Endo, M., Nakajima, Y. 1973. Release of calcium induced by "depolarization" of the sarcoplasmic reticulum membrane. *Nature New Biol.* **246**:216–218
20. Endo, M., Tanaka, M., Ogawa, Y. 1970. Calcium induced release of calcium from the sarcoplasmic reticulum of skinned skeletal muscle fibres. *Nature (London)* **228**:34–36
21. Fabiato, A., Fabiato, F. 1977*a*. Calcium release from the sarcoplasmic reticulum. *Circulation Res.* **40**:119–129
22. Fabiato, A., Fabiato, F. 1977*b*. Variations of the membrane potential of the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscle detected with a potential-sensitive dye. *J. Gen. Physiol.* **70**:6*a*
23. Ford, L.E., Podolsky, R.J. 1968. Force development and calcium movements in skinned muscle fibers. *Fed. Proc.* **27**:375
24. Franzini-Armstrong, C. 1970. Studies of the triad. I. Structure of the junction in frog twitch fibers. *J. Cell Biol.* **47**:488–499
25. Franzini-Armstrong, C. 1971. Studies of the triad. II. Penetration of tracers into junctional gap. *J. Cell Biol.* **49**:196–203
26. Gilly, W.F., Hui, C.S. 1980. Voltage-dependent charge movement in frog slow muscle fibers. *J. Physiol. (London)* **301**:175–190
27. Harafuji, H., Ogawa, Y. 1980. Re-examination of the apparent binding constant of ethylene glycol bis(β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid with calcium around neutral pH. *J. Biochem.* **87**:1305–1312
28. Hasselbach, W. 1964. Relaxing factor and the relaxation of muscle. *Prog. Biophys. Mol. Biol.* **14**:167–222
29. Hinnen, R., Miyamoto, H., Racker, E. 1979. Ca^{2+} translocation in Ehrlich ascites tumor cells. *J. Membrane Biol.* **49**:309–324
30. Hui, C.S., Gilly, W.F. 1979. Mechanical activation and voltage-dependent charge movement in stretched muscle fibres. *Nature (London)* **281**:223–225
31. Inesi, G. 1972. Active transport of calcium ion in sarcoplasmic membranes. *Annu. Rev. Biophys. Bioeng.* **1**:191–210
32. Inesi, G., Malan, N. 1976. Mechanisms of calcium release in sarcoplasmic reticulum. *Life Sci.* **18**:773–779
33. Kasai, M., Kanemasa, T., Fukumoto, S. 1979. Determination of reflection coefficients for various ions and neutral molecules in sarcoplasmic reticulum vesicles through osmotic volume change studied by stopped flow technique. *J. Membrane Biol.* **51**:311–324
34. Kasai, M., Miyamoto, H. 1973. Depolarization induced calcium release from sarcoplasmic reticulum membrane fragments by changing ionic environment. *FEBS Lett.* **34**:299–301
35. Kasai, M., Miyamoto, H. 1976*a*. Depolarization-induced calcium release from sarcoplasmic reticulum fragments. I. Release of calcium taken up upon using ATP. *J. Biochem.* **79**:1053–1066
36. Kasai, M., Miyamoto, H. 1976*b*. Depolarization induced calcium release from sarcoplasmic reticulum fragments. II. Release of calcium incorporated without ATP. *J. Biochem.* **79**:1067–1076
37. Katz, A.M., Repke, D.I., Fudyma, G., Shigekawa, M. 1977. Control of calcium efflux from sarcoplasmic reticulum vesicles by external calcium. *J. Biol. Chem.* **252**:4210–4214
38. Kometani, T., Kasai, M. 1978. Ionic permeability of sarcoplasmic reticulum vesicles measured by light scattering method. *J. Membrane Biol.* **41**:295–308
39. Labarca, P., Coronado, R., Miller, C. 1980. Thermodynamic and kinetic studies of the gating behavior of a K^+-selective channel from the sarcoplasmic reticulum membrane. *J. Gen. Physiol.* **76**:397–424
40. Louis, C.F., Nash-Adler, P.A., Fudyma, G., Shigekawa, M., Akowitz, A., Katz, A.M. 1980. A comparison of vesicles derived from terminal cisternae and longitudinal tubules of sarcoplasmic reticulum isolated from rabbit skeletal muscle. *Eur. J. Biochem.* **111**:1–9
41. MacLennan, D.H., Holland, P.C. 1975. Calcium transport in sarcoplasmic reticulum. *Annu. Rev. Biophys. Bioeng.* **4**:377–404
42. MacLennan, D.H., Klip, A. 1979. Calcium transport and release by sarcoplasmic reticulum. A mini-review. *In:* Membrane Transduction Mechanisms. R.A. Cone and J.E. Dowling, editors. pp. 61–75. Raven Press, New York
43. Meissner, G. 1975. Isolation and characterization of two types of sarcoplasmic reticulum vesicles. *Biochim. Biophys. Acta* **389**:51–68
44. Meissner, G., McKinley, D. 1976. Permeability of sarcoplasmic reticulum membrane. The effect of changed ionic environments on Ca^{2+} release. *J. Membrane Biol.* **30**:79–98
45. Miller, C. 1978. Voltage-gated cation conductance channel from fragmented sarcoplasmic reticulum: Steady-state electrical properties. *J. Membrane Biol.* **40**:1–23
46. Miyamoto, H., Racker, E. 1981. Calcium-induced calcium release at terminal cisternae. *FEBS Lett.* **133**:235–238
47. Nunzi, G., Franzini-Armstrong, C., Peachey, L.D. 1979. Intramembranous particles and feet in triads of a fast striated muscle. *J. Cell Biol.* **83**:381*a*
48. Ohnishi, S.T. 1979. Calcium-induced calcium release from fragmented sarcoplasmic reticulum. *J. Biochem.* **86**:1147–1150
49. Putney, J.W. Jr., Bianchi, C.P. 1974. Site of action of dantrolene in frog sartorius muscle. *J. Pharmacol. Exp. Therap.* **189**:202–212
50. Sandow, A., Krishna, M., Pagala, D., Sphicas, E.C. 1975. Excitation-contraction coupling: Effects of "zero"-Ca^{2+} medium. *Biochim. Biophys. Acta* **404**:157–163
51. Schneider, M.F., Chandler, W.K. 1973. Voltage dependent charge movement in skeletal muscle: A possible step in excitation-contraction coupling. *Nature (London)* **242**:244–246
52. Somlyo, A.V. 1979. Bridging structures spanning the junctional gap at the triad of skeletal muscle. *J. Cell Biol.* **80**:743–750
53. Somlyo, A.V., Gonzalez-Serratos, H., Shuman, H., McClellan, G., Somlyo, A.P. 1981. Calcium release and ionic changes in the sarcoplasmic reticulum of tetanized muscle: An electron probe study. *J. Cell Biol.* **90**:577–594
54. Somlyo, A.P., Somlyo, A.V., Gonzalez-Serratos, H., Shuman, H., McClellan, G. 1980. The sarcoplasmic reticulum and its composition in resting and in contracting muscle. *In:* Muscle Contraction: Its Regulatory Mechanisms. S. Ebashi et al., editors. pp. 421–433. Japan Science Society Press, Tokyo, and Springer-Verlag, Berlin
55. Sommer, J.R., Waugh, R.A. 1976. The ultrastructure of the mammalian cardiac muscle cell – with special emphasis on the tubular membrane systems. *Am. J. Pathol.* **82**:192–221
56. Stephenson, E.W. 1978. Properties of chloride-stimulated ^{45}Ca flux in skinned muscle fibers. *J. Gen. Physiol.* **71**:411–430
57. Tada, M., Yamamoto, T., Tonomura, Y. 1978. Molecular mechanism of active calcium transport by sarcoplasmic reticulum. *Physiol. Rev.* **58**:1–79
58. Tonomura, Y., Inoue, A. 1975. Energy transducing mechanisms in muscle. *In:* MTP International Review of Science, Biochemistry Series One. Vol. 3. Energy Transducing Mechanisms. E. Racker, editor. pp. 121–161. Butterworths, London, and University Park Press, Baltimore
59. Weber, A. 1966. Energized calcium transport and relaxing factors. *In:* Current Topics in Bioenergetics. D.R. Sanadi, editor. pp. 203–254. Academic, New York
60. Weber, A., Herz, R., Reiss, I. 1966. Study of the kinetics of calcium transport by isolated fragmented sarcoplasmic reticulum. *Biochem. Z.* **345**:329–369

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