Adenine Nucleotide Stimulation of Ca\(^{2+}\)-induced Ca\(^{2+}\) Release in Sarcoplasmic Reticulum*

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Rabbit skeletal muscle sarcoplasmic reticulum was fractionated into a “Ca\(^{2+}\)”-release and “control” fraction by differential and sucrose gradient centrifugation. External Ca\(^{2+}\) (2–20 \(\mu\)M) caused the release of 40 nmol of \(^{45}\)Ca/mg of protein/s from Ca\(^{2+}\)-release vesicles passively loaded at pH 6.8 with an internal half-saturation Ca\(^{2+}\) concentration of 10–20 mM. Ca\(^{2+}\)-induced Ca\(^{2+}\) release had an approximate pK value of 6.6 and was half-maximally inhibited at an external Ca\(^{2+}\) concentration of 7 \(\times\) 10^{-5} M. \(^{45}\)Ca\(^{2+}\) efflux from control vesicles was slightly inhibited at external Ca\(^{2+}\) concentrations that stimulated the rapid release of Ca\(^{2+}\) from Ca\(^{2+}\)-release vesicles.

Adenine, adenosine, and derived nucleotides caused stimulation of Ca\(^{2+}\)-induced Ca\(^{2+}\) release in media containing a “physiological” free Mg\(^{2+}\) concentration of 0.6 mM. At a concentration of 1 mM, the order of effectiveness was AMP-PCP > cAMP > AMP > ADP > adenine > adenosine. Other nucleoside triphosphates and caffeine were minimally effective in increasing \(^{45}\)Ca\(^{2+}\) efflux from passively loaded Ca\(^{2+}\)-release vesicles. La\(^{3+}\), ruthenium red, and procaine inhibited Ca\(^{2+}\)-induced Ca\(^{2+}\) release. Ca\(^{2+}\) flux studies with actively loaded vesicles also indicated that a subpopulation of sarcoplasmic reticulum vesicles contains a Ca\(^{2+}\) permeation system that is activated by adenine nucleotides.

Sarcoplasmic reticulum is a specialized intracellular membrane that regulates the contraction-relaxation cycle of skeletal muscle by releasing and reabsorbing Ca\(^{2+}\) (for reviews see Refs. 1–8). Muscle contracts when myoplasmic Ca\(^{2+}\) reaches 10^{-7} M, and relaxes as Ca\(^{2+}\) is pumped back into SR, thereby establishing a new steady state at below 10^{-7} M. Release of Ca\(^{2+}\) from SR is triggered by an action potential at the membrane that regulates the contraction-relaxation cycle of skeletal muscle. Furthermore, the release of Ca\(^{2+}\) is mediated by a Ca\(^{2+}\)-ATPase located in the SR membrane. The Ca\(^{2+}\)-ATPase is a membrane protein that uses ATP as an energy source to pump Ca\(^{2+}\) out of the cell against a concentration gradient.

More indirect approaches toward the study of Ca\(^{2+}\) release have made use of “skinned” muscle fibers or isolated SR vesicle fractions. In both systems, accumulated Ca\(^{2+}\) is released either by caffeine, ATP, an increase in external Ca\(^{2+}\), conditions that are thought to slow the Ca\(^{2+}\) pump of SR such as low Mg\(^{2+}\) or temperature, alteration in pH, or changes in the ionic environment that result in changes of surface membrane charge or a depolarized membrane (for reviews see Refs. 1, 2, 6, 8). On the basis of these observations, it has been proposed that physiological release of Ca\(^{2+}\) from SR is induced by Ca\(^{2+}\), “depolarization” of the SR membrane, changes in surface membrane charge, and/or a pH gradient.

In this study, we have made use of previous observations of a differential Ca\(^{2+}\) release activity of muscle membrane fractions (9–11) to isolate from rabbit skeletal muscle by differential and sucrose gradient centrifugation a “Ca\(^{2+}\)”-release fraction, which at external Ca\(^{2+}\) of 2–20 \(\mu\)M rapidly released \(^{45}\)Ca\(^{2+}\), Mg\(^{2+}\) was inhibitory, whereas adenine nucleotides potentiated Ca\(^{2+}\)-induced Ca\(^{2+}\) release. No or only little Ca\(^{2+}\) release activity was exhibited by another SR fraction which was utilized as a control (“control” fraction).

MATERIALS AND METHODS

Reagents—\(^{45}\)Ca\(^{2+}\) was purchased from ICN Pharmaceuticals, Irvine, CA. Nucleotides including the ATP analog AMP-PCP were obtained from Sigma. All other chemicals were of reagent grade.

Isolation of Membranes—Sarcoplasmic reticulum membrane fractions derived from rabbit skeletal muscle were prepared as follows: 50 g of back and white leg muscle were minced and homogenized in 250 ml of 0.1 M NaCl, 5 mM Tris maleate, pH 6.8, at 4 °C for 60 s in a Waring blender. The homogenate was centrifuged for 30 min at 4,000 rpm (2,600 \(g\)) in a GSA rotor in a Sorvall RC-2 centrifuge. Three crude membrane fractions were obtained from the supernatant by sequential centrifugation for 30 min at 10,000 \(g\), 30 min at 35,000 \(g\), and 60 min at 130,000 \(g\) using a DuPont AH641 rotor. The pellets were resuspended in 0.3 M sucrose containing 0.1 M KCl and 5 mM Tris/Mes buffer, pH 6.8, quickly frozen, and stored at -65 °C before use.

The three crude membrane fractions were further subfractionated on sucrose gradients. The thawed samples were diluted with an equal volume of 1.1 M KCl, 5 mM Tris/Mes, pH 6.8, incubated for 60 min at 0 °C, and sedimented by centrifugation for 45 min at 130,000 \(g\). The pellets were resuspended in 0.3 M sucrose, 0.4 M KCl, 20 \(\mu\)M CaCl\(_2\), and 5 mM Tris/Mes, pH 6.8, and placed on a discontinuous sucrose gradient consisting of 5 ml of 20% (w/v), 8 ml of 29%, 8 ml of 32%, 6 ml of 35%, and 6 ml of 38% sucrose. Sucrose gradient solutions contained 0.4 M KCl, 20 \(\mu\)M CaCl\(_2\), and 5 mM Tris/Mes, pH 6.8. After centrifugation for 4–5 h at 26,000 rpm (120,000 \(X\)g) in a Beckman SW27 rotor, membranes present at sucrose interfaces of 0.3 M/20% (Fraction 1), 20/29% (Fraction 2), 29/32% (Fraction 3), 32/35% (Fraction 4), and 35/38% (Fraction 5) were collected, diluted with 1.5 volumes of 0.4 M KCl, 20 \(\mu\)M CaCl\(_2\), 5 mM Tris/Mes, pH 6.8, and sedimented by centrifugation for 60 min at 130,000 \(X\)g. The pellets were resuspended in 0.3 M sucrose, 0.1 M KCl, 20 \(\mu\)M CaCl\(_2\), and 5 mM Tris/Mes, pH 6.8, quickly frozen, and stored at -65 °C before use. This fractionation scheme resulted in 15 membrane frac-
ions which varied in their content of SR, mitochondria, and surface membranes. Two SR fractions found to have high Ca\(^{2+}\) release activity (see "Results") were combined and designated Ca\(^{2+}\) release fraction (32/35\% and 35/38\% sucrose interfaces of 2,600–10,000 \(\times g\) membrane fraction), while two other fractions designated control had ATPase activity (29/32\% and 32/35\% sucrose interfaces of 35,000–130,000 \(\times g\) membrane fraction) (see Table II). Isotope Flux Measurements—Ca\(^{2+}\) efflux rates from vesicles passively loaded with Ca\(^{2+}\) were determined by Millipore filtration. Unless otherwise indicated, vesicles were equilibrated for 30 min at 0° C in a large volume (0.1–0.2 mg protein/ml) of incubation medium (0.1 M KCl, 20 mM CaCl\(_2\), and 10 mM K Pipes, pH 6.8), sedimented by centrifugation for 30 min at 100,000 \(\times g\) and resuspended in a small volume (5–10 mg of protein/ml) of incubation medium. Vesicles were passively loaded with Ca\(^{2+}\) for 2 h at 22° C in the presence of 5 mM Ca\(^{2+}\) (0.2 mM Ca\(^{2+}\)/ml). Vesicles were diluted 300–300-fold into an iso-osmolar, unlabeled release medium. Ca\(^{2+}\) efflux was terminated by placing 0.5-ml aliquots on 0.45-μm HAWP Millipore filters followed by rapid rinsing with unlabeled medium. The time required to execute filtration and rinsing was about 20 s. An Update System 1000 Chemical Quench apparatus was used in experiments where rapid Ca\(^{2+}\) efflux was inhibited at times of less than 5 s after vesicle dilution. One 10-ml glass syringe was filled with release medium and a second one with a quenching solution, and the sample (5 ml) was placed in a test tube. A first "push" delivered the release medium only. Rapid Ca\(^{2+}\) "release" was stopped 1–3 s later by delivering with a second push an equal volume of release and quenching solution. The radioactivity retained on the filters was counted in 4.5 ml of a scintillation liquid which completely dissolved the filters (12).

Biochemical Assays—Protein was estimated by the method of Lowry et al. (13) using bovine serum albumin as a standard. Unless otherwise indicated, "basic" and Mg\(^{2+}\)-dependent, Ca\(^{2+}\)-stimulated ATPase (Ca\(^{2+}\)-ATPase) activities were determined at 32°C. Basic ATPase activity was measured in 2 ml of a medium containing 10 mM K Hepes, pH 7.3, 0.1 M KCl, 2.5 mM ATP, 5 mM Mg\(^{2+}\), and 1 mM EGTA. Ca\(^{2+}\)-ATPase activity was calculated by subtracting basic ATPase activity from the ATPase activity measured in the absence or presence of the ionophore A23187 (2 μg/ml) in 10 mM K Hepes, pH 7.3, 0.1 M KCl, 2.5 mM ATP, 5 mM Mg\(^{2+}\), 100 μM Ca\(^{2+}\), and 100 μM EGTA. The reactions were stopped by the addition of 0.7 ml of 1.5 M HClO\(_4\). Inorganic phosphorus was determined on 1 ml of the protein-free supernatant (14) with elon as a reducing agent. The Ca\(^{2+}\)-free Mg\(^{2+}\) concentration of 5 mM Mg\(^{2+}\) was diluted 300-fold into release media containing either 5 mM Mg\(^{2+}\) plus 1 mM EGTA or 50 μM EGTA plus 50 μM Ca\(^{2+}\). The total amount of Ca\(^{2+}\) trapped by the vesicles as well as the amount slowly released, were obtained by back extrapolation of the two releases curves to the time of vesicle dilution (c.f. Fig. 1). The percent of Ca\(^{2+}\) release indicates the portion of trapped 45Ca\(^{2+}\) that was rapidly released in the Mg\(^{2+}\)-free medium containing about 20 μM free Ca\(^{2+}\). Data for the 2,600–10,000 \(\times g\) crude and derived sucrose gradient fractions 1a–5a are the average of four preparations ± S.E. Ca\(^{2+}\) release data of the remaining fractions are the average of three determinations of four pooled preparations. S.E. ± 25% or less.

RESULTS

Fractionation and Composition of Muscle Membrane Fractions—Table I compares the Ca\(^{2+}\) release activities of rabbit skeletal muscle membrane fractions obtained by differential and sucrose gradient centrifugation. The muscle homogenate was subjected to four sequential centrifugations of increasing speed to remove myofibrils and cell debris as well as to isolate three crude membrane fractions. After treatment with 0.6 M KCl, the crude fractions were further fractionated by sucrose density centrifugation. Among the three crude membrane fractions, the 2,600–10,000 \(\times g\) fraction displayed the highest Ca\(^{2+}\) release activity (Table I). About two-thirds of 45Ca\(^{2+}\) retained by the vesicles in a dilution medium containing 5 mM Mg\(^{2+}\) and 2 \(\times 10^{-8}\) M free Ca\(^{2+}\) was rapidly released on dilution into a medium containing 20 μM free Ca\(^{2+}\) (cf. Fig. 1). In the 10,000–35,000 \(\times g\) fraction of about half of the vesicles displayed Ca\(^{2+}\) release activity, whereas in the high speed 35,000–130,000 \(\times g\) fraction only a small portion of the trapped 45Ca\(^{2+}\) was lost on dilution into the medium containing 20 μM free Ca\(^{2+}\). On the sucrose gradients, in all three crude membrane fractions the highest Ca\(^{2+}\) release activity was recovered from the 35/38% sucrose interface. For the 2,600–10,000 \(\times g\) fraction, high Ca\(^{2+}\) release activity was also found in fractions sedimenting at the 32/35% and 29/32% sucrose interfaces.

Table II compares the enzymatic properties of two SR fractions displaying high or low Ca\(^{2+}\) release activity, and which are designated Ca\(^{2+}\)-release fraction and control fraction, respectively. Both membrane fractions displayed properties characteristic of sarcoplasmic reticulum, i.e. accumulation of Ca\(^{2+}\) in the presence of the Ca\(^{2+}\) precipitating agent oxalate, and Ca\(^{2+}\)-stimulated Mg\(^{2+}\)-dependent ATPase (Ca\(^{2+}\)-ATPase) activity. Comparison of the levels of Ca\(^{2+}\)-ATPase activity in the presence of the ionophore A23187 indicated that the Ca\(^{2+}\)-ATPase content of the Ca\(^{2+}\) release fraction was about two-thirds of that of the control vesicles. Initial Ca\(^{2+}\) loading rates of the two SR fractions differed by a factor of 5. As shown below (Table IV), the low loading rate of the Ca\(^{2+}\)-release fraction is likely due to the fact that an appre-

| Fraction | Sucrose | Yield | Rapid | Ca\(^{2+}\)  |
|----------|---------|-------|-------|------------|
| 2,600–10,000 \(\times g\) crude | 160 ± 50 | 60 ± 15 | 70 ± 5 |
| 1a | 10/20 | 2.5 ± 0.5 | 50 ± 20 | 19 ± 9 |
| 2a | 20/25 | 2.5 ± 0.5 | 50 ± 20 | 19 ± 9 |
| 3a | 29/32 | 4 ± 0.5 | 65 ± 7 | 55 ± 9 |
| 4a | 32/35 | 27 ± 5 | 84 ± 6 | 78 ± 8 |
| 5a | 35/38 | 10 ± 2 | 70 ± 10 | 82 ± 5 |
| 10,000–35,000 \(\times g\) crude | 80 ± 10 | 70 ± 10 | 50 ± 5 |
| 1b | 10/20 | 0.25 | 80 ± 10 | |
| 2b | 20/29 | 2.5 | 64 ± 25 | |
| 3b | 29/32 | 6.5 | 51 ± 30 | |
| 4b | 32/35 | 15 | 53 ± 30 | |
| 5b | 35/38 | 20 | 96 ± 75 | |
| 35,000–130,000 \(\times g\) crude | 70 ± 20 | 25 ± 5 | 20 ± 10 |
| 1c | 10/20 | 0.3 | 33 ± 15 | |
| 2c | 20/29 | 2.5 | 42 ± 5 | |
| 3c | 29/32 | 10 | 50 ± 20 | |
| 4c | 32/35 | 35 | 38 ± 15 | |
| 5c | 35/38 | 15 | 53 ± 40 | |
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![Graph](image_url)

**Fig. 1. Measurement of \textsuperscript{45}Ca\textsuperscript{2+} efflux rates.** Ca\textsuperscript{2+}-release (A) and control (B) fractions of Table II were incubated for 2 h at 22 °C in a medium containing 10 mM K Pipes, pH 6.8, 0.1 mM KCl, and 5 mM \textsuperscript{45}Ca\textsuperscript{2+}. Vesicles were diluted at 22 °C 300-fold into unlabeled release media containing 10 mM K Pipes, pH 6.8, 0.1 mM KCl, and 5 mM Mg\textsuperscript{2+} plus 1 mM EDTA (\textbullet{}), 1 mM EGTA (\texttriangle{}), or 50 \mu M EGTA plus 50 \mu M Ca\textsuperscript{2+} (○). Final free Ca\textsuperscript{2+} concentrations indicated in the figure were calculated using a binding constant of \(10^6\) M\textsuperscript{-1}\) (17) for the Ca\textsuperscript{2+}-EGTA complex. In a second type of experiment, \textsuperscript{45}Ca\textsuperscript{2+} efflux from vesicles present in the two Mg\textsuperscript{2+}-free release media was slowed down by adding 1 volume of release medium and another one containing 10 mM K Pipes, pH 6.8, 0.1 mM KCl, and amounts of Mg\textsuperscript{2+} and EGTA to yield final concentrations of 5 and 1 mM, respectively (cf. "Materials and Methods"). Aliquots of 0.5 ml were placed on Millipore filters and rinsed for 10 s with the final vesicle suspension medium, and amounts of radioactivity remaining with the vesicles on the filters were determined. Amounts of \textsuperscript{45}Ca\textsuperscript{2+} initially trapped by all of the vesicles (80 nmol/mg of protein for the Ca\textsuperscript{2+}-release fraction) as well as amounts not readily released from a subpopulation of vesicles (8 nmol/mg of protein for the Ca\textsuperscript{2+}-release fraction) were obtained by back extrapolation to the time of vesicle dilution. In the inset in A, the time course of \textsuperscript{45}Ca\textsuperscript{2+} efflux from the vesicle population capable of rapid release was obtained by subtracting the amount of \textsuperscript{45}Ca\textsuperscript{2+} not readily released (8 nmol/mg of protein).

**TABLE II**

| Activity                      | Ca\textsuperscript{2+} release fraction | Control fraction |
|-------------------------------|----------------------------------------|-----------------|
| Rapid \textsuperscript{45}Ca\textsuperscript{2+} release (%) | 80 ± 10 | 15 ± 10 |
| ATPase (\mu mol/mg protein/min) | 0.15 ± 0.06 | 0.10 ± 0.03 |
| "Basic"                      | 4.5 ± 1.3 | 6.5 ± 1.0 |
| Ca\textsuperscript{2+} loading rate | 0.5 ± 0.15 | 4.0 ± 1.0 |
| (\mu mol Ca\textsuperscript{2+}/mg protein/min) | 0.15 ± 0.03 | 0.01 ± 0.003 |
| Succinate cytochrome c reductase (\mu mol/mg protein/min) | | |

Specific activity of basic ATPase, an enzyme associated with the plasmalemma and T-system of skeletal muscle (18), was low in both SR fractions. Since the surface membrane structures of rabbit skeletal muscle have been found in our laboratory to have a specific activity of about 5 \mu mol/mg of protein/min (19), surface membrane content of the two fractions of Table II was estimated to be less than 5%. The Ca\textsuperscript{2+}-release fraction was contaminated with inner mitochondrial membranes, as indicated by a succinate cytochrome c reductase activity of 0.15 \mu mol/mg of protein/min. Inner mitochondrial membrane content of the control fraction was low.

Ca\textsuperscript{2+} Efflux and Influx Rates of Ca\textsuperscript{2+}-Release Fraction—In Fig. 1, the Ca\textsuperscript{2+}-release fraction of Table II was incubated in 5 mM \textsuperscript{45}Ca\textsuperscript{2+} for 2 h at 22 °C. SR vesicles were diluted 300-fold into an unlabeled release medium, were collected on Millipore filters at time intervals ranging from 1/2 to 5 min, and the radioactivity remaining with the vesicles on the filters was determined. The amount of \textsuperscript{45}Ca\textsuperscript{2+} remaining with the vesicles depended on the composition of the release medium. Vesicles diluted into a medium containing 5 mM Mg\textsuperscript{2+} and 2 \times 10^{-8} M free Ca\textsuperscript{2+} (1 mM EGTA and ~20 \mu M Ca\textsuperscript{2+}) retained 80 nmol of Ca\textsuperscript{2+}/mg of protein, that was slowly released with time (\(t_{1/2} = 5\)–10 min). In the presence of the ionophore A23187 (2 \mu g/ml), greater than 95% of \textsuperscript{45}Ca\textsuperscript{2+} was released within 30 s, indicating that the retained \textsuperscript{45}Ca\textsuperscript{2+} was trapped inside the vesicles (not shown). Omission of Mg\textsuperscript{2+} from the release medium containing 2 \times 10^{-8} M free Ca\textsuperscript{2+} greatly accelerated the rate of \textsuperscript{45}Ca\textsuperscript{2+} efflux (\(t_{1/2} \sim 30\) s). A further dramatic increase in the initial \textsuperscript{45}Ca\textsuperscript{2+} efflux rate was observed, as the free Ca\textsuperscript{2+} concentration in the Mg\textsuperscript{2+}-free release medium was increased to 20 \mu M. By comparison, an increase in external Ca\textsuperscript{2+} from 2 \times 10^{-8} M to 2 \times 10^{-5} M slightly decreased \textsuperscript{45}Ca\textsuperscript{2+} efflux from control vesicles (\(t_{1/2} \sim 150\) s versus \(t_{1/2} \sim 300\) s (Fig. 1B)). Addition of 5 mM Mg\textsuperscript{2+} to the low Ca\textsuperscript{2+} medium did not appreciably affect \textsuperscript{45}Ca\textsuperscript{2+} efflux.

Data of Fig. 1 are in accord with previous suggestions (1, 2,
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6) that sarcoplasmic reticulum contains a permeation system for Ca$^{2+}$ which is activated by external Ca$^{2+}$ and inhibited by Mg$^{2+}$. Release of greater than 90% of $^{45}$Ca$^{2+}$ within 1-2 min suggested that a majority of the vesicles of the Ca$^{2+}$-release fraction of Table II were capable of Ca$^{2+}$-induced Ca$^{2+}$ release. A small fraction of the vesicles seemed to lack this permeation system as indicated by slow release of Ca$^{2+}$ that remained with the vesicles 1-2 min after dilution. Ca$^{2+}$-release vesicles accounted for about half of the vesicles recovered in the 15 sucrose gradient fractions of Table I.

$^{45}$Ca$^{2+}$ and EGTA were found to effectively block further $^{45}$Ca$^{2+}$ efflux when added to vesicles diluted into the Ca$^{2+}$-release medium containing 20 $\mu$M free Ca$^{2+}$ but use of these two "quenching" agents therefore permitted us to stop Ca$^{2+}$ release at time intervals ranging from 1-5 s (inset of Fig. 1A). A straight line was obtained from the Ca$^{2+}$-permeable vesicle fraction suggesting that Ca$^{2+}$ release followed first order kinetics. In the presence of 20 $\mu$M free Ca$^{2+}$, the vesicles released Ca$^{2+}$ with an initial rate of about 20 nmol/mg of protein/s.

$^{45}$Ca$^{2+}$ influx was measured by the time of vesicle preincubation. Ca$^{2+}$-releasing vesicles were preincubated from 3 min to 4 h at 22 °C in the presence of 5 mM $^{45}$Ca$^{2+}$ before being diluted into the two media of Fig. 1 that blocked or promoted rapid Ca$^{2+}$ release. Dilution into the Mg$^{2+}$ medium that inhibited Ca$^{2+}$ release showed a rapid increase in the amount of $^{45}$Ca$^{2+}$ retained, indicating that initial $^{45}$Ca$^{2+}$ inward movement was fast (Fig. 2). After a preincubation time of 2 to 4 h, $^{45}$Ca$^{2+}$ had nearly equilibrated across the vesicle membranes. Vesicles were therefore routinely preincubated for 2 h at 22 °C prior to the Ca$^{2+}$-release studies. The ability of the vesicles to release most of their $^{45}$Ca$^{2+}$ within 10 s in the Ca$^{2+}$-release promoting medium was independent of the time of preincubation, suggesting that Ca$^{2+}$ release was not altered by preincubation for 2 h at 22 °C. Isolation of the membrane fractions or preincubation of the vesicles in the presence of the potent serine protease inhibitor, diisopropyl fluorophosphate at a concentration of 1.5 mM, also showed no effect (not shown).

**Coupling of Ca$^{2+}$ Movement to Other Ions**—The presence of an obligate Ca$^{2+}$-Ca$^{2+}$ exchange reaction was assessed by diluting $^{46}$Ca$^{2+}$ loaded vesicles into release media containing 50 $\mu$M unlabeled Ca$^{2+}$ or 50 $\mu$M $^{45}$Ca$^{2+}$ with a radioisotope specificity identical with the one used in the incubation medium. Ca$^{2+}$ release was stopped after 5 and 10 s by the addition of an equal volume of buffer containing 10 mM Mg$^{2+}$ and 2 mM EGTA (cf. Fig. 1). An identical rapid $^{46}$Ca$^{2+}$ efflux rate was observed in both release media suggesting that outward movement of $^{45}$Ca$^{2+}$ was not coupled to inward movement of Ca$^{2+}$.

In agreement with this suggestion, vesicles loaded with 5 mM unlabeled Ca$^{2+}$ and then diluted into a release medium containing $^{45}$Ca$^{2+}$ did not accumulate the radioisotope. Ca$^{2+}$ release also appeared to be not directly coupled to any specific monovalent cation since rapid release was observed in KCl, NaCl, or choline Cl media. Native SR vesicles are permeable to H$^{+}$ (20), so it was not possible to determine whether Ca$^{2+}$ efflux was coupled to H$^{+}$.

Addition of 5 $\mu$M valinomycin to the KCl release medium did not appreciably affect $^{46}$Ca$^{2+}$ release, suggesting that Ca$^{2+}$ efflux did not result in the formation of a large membrane potential, which in turn might have limited the extent of Ca$^{2+}$ release. Probably, the intrinsic permeability of the SR vesicles to K$^+$, Cl$^-$, and H$^+$ (7, 8) was sufficiently high to dissipate any potential that might have formed during electrogenic Ca$^{2+}$ efflux.

**Effect of External Ca$^{2+}$ and Mg$^{2+}$ on $^{45}$Ca$^{2+}$ Release**—The effect of varying external Ca$^{2+}$ and Mg$^{2+}$ concentrations on the initial Ca$^{2+}$ efflux rate from SR vesicles capable of rapid Ca$^{2+}$ release and equilibrated with 5 mM $^{45}$Ca$^{2+}$ is shown in Fig. 3. A maximal initial Ca$^{2+}$ efflux rate of 18 nmol of Ca$^{2+}$/mg of protein/s was observed in Mg$^{2+}$-free media containing between 2 and 20 $\mu$M free Ca$^{2+}$. Both a decrease in free Ca$^{2+}$ concentration to 2 × 10$^{-3}$ M or an increase to 10$^{-2}$ M decreased the initial Ca$^{2+}$ release rate to less than 2 nmol/mg of protein/s. Addition of Mg$^{2+}$ to the release medium slowed down $^{45}$Ca$^{2+}$ efflux. The initial Ca$^{2+}$ release rate was approximately half-maximal at 7 × 10$^{-3}$ M Mg$^{2+}$ in a medium containing 20 $\mu$M free Ca$^{2+}$. The intracellular concentration of free Mg$^{2+}$ has been estimated to be 0.6 mM in frog skeletal muscle (21). Fig. 3 shows that Ca$^{2+}$-induced Ca$^{2+}$ release was nearly fully abol-

![Fig. 2. Effect of incubation time on amounts of $^{45}$Ca$^{2+}$ retained by the vesicles. Vesicles of Ca$^{2+}$-release fraction of Table II were incubated at 22 °C for the indicated time in 10 mM K Pipes, pH 6.8, 0.1 M KCl, and 5 mM $^{45}$Ca$^{2+}$. Vesicles were then diluted 300-fold into media containing 10 mM K Pipes, pH 6.8, 0.1 M KCl, and 5 mM Mg$^{2+}$ plus 1 mM EGTA (●), or 50 $\mu$M EGTA plus 50 $\mu$M Ca$^{2+}$ (○). At 10 s after the initial vesicle dilution, $^{45}$Ca$^{2+}$ efflux from vesicles present in the Mg$^{2+}$-free release medium was inhibited by adding an equal volume of medium containing 10 mM K Pipes, pH 6.8, 0.1 M KCl, 10 mM Mg$^{2+}$, and 2 mM EGTA. The amount of $^{45}$Ca$^{2+}$ remaining with the vesicles was obtained by back extrapolation to the time of vesicle dilution (●) or addition of the quenching medium (○) (cf. Fig 1). The difference between the upper and lower curves indicates the amounts of $^{45}$Ca$^{2+}$ released within 10 s in the Mg$^{2+}$-free release medium (○).](image1)

![Fig. 3. Effect of external Ca$^{2+}$ and Mg$^{2+}$ concentration on the initial $^{45}$Ca$^{2+}$ efflux rates. The Ca$^{2+}$-release fraction of Table II was incubated for 2 h at 22 °C in a medium containing 20 mM K Pipes, pH 6.8, 0.1 M KCl, and 5 mM $^{45}$Ca$^{2+}$. Vesicles were then diluted into Mg$^{2+}$-free media containing the indicated concentrations of free Ca$^{2+}$ (●) or 20 $\mu$M free Ca$^{2+}$ plus the indicated concentrations of Mg$^{2+}$ (○). The final free Ca$^{2+}$ concentration in the release media was obtained by using varying concentrations of EGTA and Ca$^{2+}$. In media promoting rapid release of $^{46}$Ca$^{2+}$, initial efflux rates were determined by inhibiting $^{46}$Ca$^{2+}$ efflux by the addition of 5 mM Mg$^{2+}$ to the release medium at 1, 2, 3, 5, or 10 s after the initial vesicle dilution (cf. inset of Fig. 1A).](image2)
ished at this physiological concentration of 0.6 mM Mg2+.

La3+, ruthenium red, and procaine have been reported to inhibit Ca2+-induced Ca2+ release from SR (1, 2, 6, 22-24). At a concentration of 10 μM, La3+ and ruthenium red reduced the initial Ca2+ release rate to less than 0.5 nmol/mg of protein/s. Procaine at a concentration of 10 mM lowered the initial Ca2+ release rate from 18 to about 5 nmol/mg of protein/s (not shown).

Effect of Nucleotides on 45Ca2+ Efflux—Inhibition of Ca2+-induced Ca2+ release by physiological concentrations of Mg2+ raised the possibility that a regulatory factor might have been removed during purification resulting in increased Mg2+-sensitivity of the Ca2+-release system of sarcoplasmic reticulum. Data of Fig. 4 and Table III suggest that adenine nucleotides may play such a role. Ca2+-release vesicles equilibrated with 5 mM 45Ca2+ rapidly released all of their Ca2+ when diluted into a medium containing about 10 μM free Ca2+, 1.5 mM Mg2+, and 1 mM of the nonhydrolyzable ATP analog, AMP-PCP. The free Mg2+ concentration of the medium determined with an ion electrode was 0.7 ± 0.1 mM. Ca2+ releasing action of AMP-PCP was also observed when vesicles were first diluted into a medium containing 1.5 mM Mg2+ and 10 μM free Ca2+ followed by the addition of the nucleotide to a final concentration of 1 mM. The stimulatory effect of AMP-PCP was less pronounced when the free Ca2+ concentration was lowered to about 10-8 M by the addition of an excess of EGTA (Table III). An increase in Mg2+ concentration to 10 mM nullified the stimulatory effect of AMP-PCP. Vesicles did not actively take up 45Ca2+ when incubated in a medium containing 1.5 mM Mg2+, 1 mM AMP-PCP, and 20 μM free Ca2+, suggesting that the nucleotide did not exert its effect by activating the Ca2+-transport system of sarcoplasmic reticulum. In another control experiment, we found that AMP-PCP did not significantly increase 45Ca2+ efflux from control vesicles.

Other nucleotide triphosphates did not significantly affect the Ca2+-release system of sarcoplasmic reticulum. No increase in the release rate was seen when GTP, ITP or UTP, or the two nucleotide analogs GMP-PCP or UMP-PCP were added at a concentration of 1 mM to media containing 1.5 mM Mg2+ and 10 μM free Ca2+ (Table III). ADP, AMP, cAMP, adenine and, to a lesser extent adenosine behaved like AMP-PCP in that they accelerated Ca2+ release in media containing about 0.6 mM free Mg2+. By contrast, when caffeine, reported to augment Ca2+-induced Ca2+ release (1, 2, 6), was added to a medium containing 0.6 mM Mg2+, 45Ca2+ efflux was only minimally stimulated. GMP at a concentration of 5 mM accelerated 45Ca2+ efflux 1.5-fold, suggesting that other nucleotides may activate the Ca2+-release system of SR when present at a high concentration.

Both AMP-PCP and ADP exerted their effect on the Ca2+-release system of SR in a dose-dependent manner, with AMP-PCP being the more effective of the two nucleotides (Fig. 5). In a medium containing 1 mM AMP-PCP, vesicles released about 90% of the rapidly releasable 45Ca2+ at 5 s postdilution. Taken together, the data of Table III and Figs. 4 and 5 suggest that the Ca2+-release system of SR may be regulated by nucleotides. Adenosine nucleotides seem to be the preferred substrate since the ATP analog AMP-PCP was the only one of the various nucleoside triphosphates tested that significantly activated Ca2+-release.

Dependence of 45Ca2+ Efflux on Internal Ca2+ Concentration and pH—A vesicle fraction shown to release rapidly Ca2+ was incubated for 2 h at 22 °C in media containing trace amounts of 45Ca2+ and between 2 and 62 mM Ca2+. As the Ca2+ concentration of the incubation medium increased, vesicles retained increasing amounts of Ca2+ upon dilution into a medium containing 6 mM Mg2+ and low concentrations of free Ca2+ (Fig. 6). 45Ca2+ efflux was slow in media containing 0.6 mM Mg2+ and between 5 and 10 μM free Ca2+. In accordance with data of Fig. 1 and Table III, omission of Mg2+ or addition of AMP-PCP caused a dramatic increase in the initial 45Ca2+ efflux rates. A half-maximal initial release rate of about 100 nmol of Ca2+/mg of protein was obtained for vesicles filled with a Ca2+ concentration of 10-20 mM and subsequently

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**Table III**

Effect of various nucleotides on 45Ca2+ efflux

| Additions to release medium | Free Ca2+ | Mg2+ | Nucleotide | 45Ca2+ efflux rate |
|----------------------------|----------|------|------------|-------------------|
|                           | μM       | mM   |            | protein/s         |
| Free Ca2+                 | 0.01     | 6    | AMP-PCP    | 0.15              |
|                           | 0.6      | 0.2  | AMP-PCP    | 0.8               |
|                           | 1.5      | 1.5  | AMP-PCP    | 1.5               |
|                           | 0.6      | 1.5  | AMP-PCP    | 1.2               |
|                           | 1.5      | 1.5  | AMP-PCP    | 1.3               |
|                           | 0.6      | 0.6  | AMP-PCP    | 0.8               |
|                           | 0.6      | AMP  | 7.0        |
|                           | 0.6      | cAMP | 9.5        |
|                           | 0.6      | Adenine | 4.5       |
|                           | 0.6      | Adenine | 6.0       |
|                           | 0.6      | Caffeine | 2.0      |

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**Fig. 4.** Effect of AMP-PCP on 45Ca2+ efflux. Ca2+-release fraction of Table II, preincubated for 2 h at 22 °C in the presence of 5 mM 45Ca2+, was diluted 300-fold into 20 mM K Pipes, pH 6.8, 0.1 M KCl, approximately 10-8 M free Ca2+ (1 mM EGTA and Ca2+ at a final concentration of 125 μM) or 10-3 M free Ca2+ (50 μM EGTA and Ca2+ at a final concentration of 55 μM), the indicated amounts of Mg2+, and nucleotides at a concentration of 1 mM. Initial 45Ca2+ efflux rates were determined by adding at 5, 10, and 30 s (in the case of AMP-PCP at 1, 2, and 3 s) after vesicle dilution a quenching solution containing amounts of Mg2+ and EGTA to yield final concentrations of 6 and 2 mM, respectively (cf. Fig. 1). Data are the average of four determinations. S.E. ± 20% or less.
was inhibited by the addition of an equal volume of 0.1 M KCl, 20 mM Pipes, and a free Mg$^2+$ concentration of 0.7 mM. The graph shows the amounts of Ca$^{2+}$ that were released by the vesicles within the Ca$^{2+}$-release fraction of Table II, was diluted 150-fold into release media containing 20 mM K Pipes, pH 6.8, 0.1 M KCl, 100 mM EGTA, 80 μM Ca$^{2+}$, the indicated amounts of AMP-PCP (□) or ADP (○), and a free Mg$^2+$ concentration of 0.7 ± 0.1 mM as measured with a divalent cation electrode. At 5 s after vesicle dilution, Ca$^{2+}$ efflux was inhibited by the addition of an equal volume of 0.1 mM KCl, 20 mM K Pipes, pH 6.8, buffer containing 10 mM Mg$^2+$ and 2 mM EGTA. The graph shows the amounts of Ca$^{2+}$ that were released by the vesicles within 5 s after dilution in media containing the nucleotides at a free Mg$^2+$ concentration of about 0.7 mM. Total amount of Ca$^{2+}$ rapidly released by the vesicles (cf. Fig. 1) corresponded to 57 ± 5 nmol of Ca$^{2+}$/mg of protein.

![Graph showing Ca$^{2+}$ efflux](image)

**Fig. 5.** Effect of nucleotide concentration on 45Ca$^{2+}$ efflux. Ca$^{2+}$-release fraction of Table II, passively loaded with 5 mM 45Ca$^{2+}$ as described in the legend to Fig. 1, was diluted 150-fold into release media containing 20 mM K Pipes, pH 6.8, 0.1 M KCl, 100 mM EGTA, 80 μM Ca$^{2+}$, the indicated amounts of AMP-PCP (□) or ADP (○), and a free Mg$^2+$ concentration of 0.7 ± 0.1 mM as measured with a divalent cation electrode. At 5 s after vesicle dilution, Ca$^{2+}$ efflux was inhibited by the addition of an equal volume of 0.1 mM KCl, 20 mM K Pipes, pH 6.8, buffer containing 10 mM Mg$^2+$ and 2 mM EGTA. The graph shows the amounts of Ca$^{2+}$ that were released by the vesicles within 5 s after dilution in media containing the nucleotides at a free Mg$^2+$ concentration of about 0.7 mM. Total amount of Ca$^{2+}$ rapidly released by the vesicles (cf. Fig. 1) corresponded to 57 ± 5 nmol of Ca$^{2+}$/mg of protein.

![Graph showing Ca$^{2+}$ efflux](image)

**Fig. 6.** Dependence of 45Ca$^{2+}$ efflux on intravesicular Ca$^{2+}$ concentration. The Ca$^{2+}$-release fraction of Table II was incubated for 2 h at 22 °C in 0.1 M KCl, 20 mM K Pipes, pH 6.8, buffer containing trace amounts of 45Ca$^{2+}$ and the indicated concentrations of Ca$^{2+}$. Vesicles were diluted 250-fold into media containing 20 mM K Pipes, pH 6.8, 0.1 M KCl, and 6 mM Mg$^2+$ plus 2 mM EGTA (Δ), or Ca$^{2+}$ at a final free concentration of 5–10 μM (1 mM Ca$^{2+}$) and 1.15 mM EGTA in the absence (○) or presence of 0.6 mM Mg$^2+$ (●) or 1.5 mM Mg$^2+$ plus 1 mM AMP-PCP (□). Initial 45Ca$^{2+}$ efflux in the two media containing no Mg$^2+$ or containing Mg$^2+$ plus nucleotide were determined by blocking rapid 45Ca$^{2+}$ efflux at 1, 2, and 3 s by the addition of 1 volume of release medium and 1 volume containing 18 mM Mg$^2+$ and 6 mM EGTA. Amounts of 45Ca$^{2+}$ trapped by the vesicles at zero time (Δ) or remaining with the vesicles at the time of addition of the quenching solution (○) were determined by back extrapolation as indicated in Fig. 1. Efflux rate of 45Ca$^{2+}$ in the 0.6 mM Mg$^2+$ (—AMP-PCP) medium was directly obtained from the slope of the efflux curves using the ½, 1½, and 2-min time points.

![Graph showing Ca$^{2+}$ efflux](image)

**Fig. 7.** Dependence of 45Ca$^{2+}$ efflux on pH. Ca$^{2+}$-release vesicle fraction of Table II was passively loaded for 2 h at 22 °C with 5 mM 45Ca$^{2+}$ in a 0.1 M KCl, 20 mM K Pipes medium at the indicated pH. Vesicles were then diluted 150-fold into release media containing 20 mM K Pipes at the indicated pH, 0.1 M KCl, and 5 mM Mg$^2+$ plus 1 mM EGTA (Δ), or 50 μM EGTA and 50 μM Ca$^{2+}$ in the absence (○) or presence of 0.6 mM Mg$^2+$ (●) or 1.5 mM Mg$^2+$ plus 1 mM AMP-PCP (□). In the three release media, at 5-s postdilution, 45Ca$^{2+}$ efflux was inhibited by the addition of an equal volume of 0.1 M KCl, 20 mM K Pipes medium at the indicated pH and containing 10 mM Mg$^2+$ and 2 mM EGTA. Amounts of 45Ca$^{2+}$ trapped by the vesicles at zero time (Δ) and released at 5 s after vesicle dilution (○, ●, □) were determined by back extrapolation as described in the legend to Fig. 1.
Ca\textsuperscript{2+} in the various uptake and washing solutions tested (Table IV). By contrast, the amount of \textsuperscript{45}Ca\textsuperscript{2+} retained by the Ca\textsuperscript{2+}-release fraction depended on the composition of the two media. Maximal amounts of \textsuperscript{45}Ca\textsuperscript{2+} were retained when vesicles were loaded and washed in media that were shown above that contain a free Ca\textsuperscript{2+} concentration of about 16 \textmu M, or containing in addition of 0.6 mM free Mg\textsuperscript{2+}, and for activity measured in the low Mg\textsuperscript{2+} medium was only minimally stimulated when all vesicles were rendered permeable to Ca\textsuperscript{2+} with the use of the ionophore A23187.

By comparison, the Ca\textsuperscript{2+}-ATPase activity of control vesicles was only slightly influenced by the Mg\textsuperscript{2+} concentration of the assay medium, increasing by a factor of less than 1.5 when the free Mg\textsuperscript{2+} concentration was lowered to 0.1 mM. In both media, addition of the ionophore A23187 increased the Ca\textsuperscript{2+}-ATPase activity by a factor of 3-4, suggesting that actively transporting control vesicles, unlike Ca\textsuperscript{2+}-release vesicles, maintained a similar permeability barrier for Ca\textsuperscript{2+} in high or low Mg\textsuperscript{2+} media. Taken together, Ca\textsuperscript{2+} flux and ATP hydrolysis data of Table IV support our contention that a subpopulation of sarcoplasmic reticulum vesicles contains a Ca\textsuperscript{2+}-permeation system that is controlled by Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, and ATP.

DISCUSSION

This study has shown that rabbit skeletal muscle homogenates contain sarcoplasmic reticulum vesicles which differ in their permeability to Ca\textsuperscript{2+}. Ca\textsuperscript{2+}-release vesicles, which accounted for about half of the isolated SR vesicles, appear to contain a Ca\textsuperscript{2+}-permeation system that allows the rapid efflux of Ca\textsuperscript{2+}. The Ca\textsuperscript{2+} efflux rate is regulated by external Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, and adenine nucleotides. Control membrane vesicles, by definition, seem to lack the permeation system that mediates rapid Ca\textsuperscript{2+} release under the experimental conditions of this study. Absence of the Ca\textsuperscript{2+}-release system from a population of SR vesicles suggests that rapid Ca\textsuperscript{2+} efflux required a membrane component specific to Ca\textsuperscript{2+}-release vesicles.

Longitudinal sections and terminal cisternae of sarcoplasmic reticulum have been distinguished in electron micrographs of skeletal muscle (25). A structure known as a triad is formed where the terminal cisternae of SR abut on invaginations of the surface, the transverse tubule (T-system). Isolation of vesicle fractions enriched it: the different segments of the SR structure has been described (26-28). Vesicle fractions displaying a high Ca\textsuperscript{2+}-release activity (cf. Table I) exhibited a sedimentation behavior similar to that previously observed for "heavy" free terminal cisternae vesicles (26) as well as vesicles attached to transverse tubule in the form of diads or triads (27, 28). Control membranes were preferentially recovered from the low density region of the sucrose gradients, similar to "light" SR vesicles (26) derived from the longitudinal sections of SR.

Ca\textsuperscript{2+} is thought to be released from the terminal cisternae of SR in response to a T-system action potential (29, 30). It would be expected then that disruption of SR during homog-
Ca\(^{2+}\) Release in Sarcoplasmic Reticulum

...entization might result in the formation of some vesicles that contain, and others which lack, the Ca\(^{2+}\)-release system. Weber and Herz (9) first reported a differential Ca\(^{2+}\) release sensitivity of muscle membrane fractions. Caffeine released 25–50% of the accumulated Ca\(^{2+}\) when a 2,000–8,000 \(\times\) g membrane fraction was used, whereas high speed fractions released no more than 12%. In two recent studies, the majority of vesicles obtained by centrifugation between 2,400 and 8,600 \(\times\) g (10) or 4,000 and 10,000 \(\times\) g (11) possessed a "Ca\(^{2+}\)-gated" cation channel, whereas only a small portion of the vesicles sedimenting at higher speeds exhibited Ca\(^{2+}\)-induced Ca\(^{2+}\) release activity. Miyamoto and Racker (23) observed Ca\(^{2+}\)-induced and membrane potential-dependent Ca\(^{2+}\) release from actively loaded, terminal cisternae fractions, but not from light SR vesicle fractions. In addition in some (31, 32) but not all (12, 33) studies, alterations of surface charge distribution or transmembrane potential, induced by a change in ionic composition, have been reported to cause Ca\(^{2+}\) release from terminal cisternae and triad-encirclced vesicle fractions.

One popular hypothesis of Ca\(^{2+}\) release from SR in vivo is the Ca\(^{2+}\)-induced Ca\(^{2+}\) release or Ca\(^{2+}\)-triggering hypothesis which states that small amounts of Ca\(^{2+}\) entering the sarcomplasm during an action potential induce release of additional amounts of Ca\(^{2+}\) sufficient to activate muscle contraction (1, 2, 6). Two proteins generally considered to be capable of mediating rapid Ca\(^{2+}\) release from SR are the Ca\(^{2+}\) transport ATPase and the putative Ca\(^{2+}\) channel active during in vivo Ca\(^{2+}\) release. A phosphorylated intermediate of the Ca\(^{2+}\)-ATPase (34, 35) as well as the nonphosphorylated Ca\(^{2+}\)-ATPase (36) have been reported to mediate rapid release of Ca\(^{2+}\) from SR vesicles. On the other hand, a difference in the Ca\(^{2+}\) release activity of light and heavy vesicle fractions (Refs. 10, 11, 23, 37; Table I) strongly supports the existence of a unique Ca\(^{2+}\)-release structure or channel in SR.

Ca\(^{2+}\)-release vesicles diluted into a medium containing 2 \(\times\) 10\(^{-8}\) M free Ca\(^{2+}\) released \(^{45}\)Ca\(^{2+}\) with a \(t_{1/2}\) of about 30 s (Fig. 1), which corresponds to a passive Ca\(^{2+}\) permeability of about 4 \(\times\) 10\(^{-8}\) cm/s, assuming an average vesicle diameter of 0.1 \(\mu\)m (26). By comparison, previous studies with passively and actively loaded SR vesicles have indicated an initial passive Ca\(^{2+}\) permeability of 1.5–10 \(\times\) 10\(^{-8}\) cm/s for vesicles present in media containing 10\(^{-4}\) M (38) or less than 10\(^{-7}\) M free Ca\(^{2+}\) (12, 39–43). Varying effects of external Ca\(^{2+}\) upon the initial Ca\(^{2+}\) efflux rate have been reported. An increase of extravascular free Ca\(^{2+}\) from about 10\(^{-7}\) M or less to about 10\(^{-5}\) M either decreased (44), showed no effect (43), or as observed in this study, dramatically increased (41) the initial \(^{45}\)Ca\(^{2+}\) efflux rate. Further increase of external Ca\(^{2+}\) to 10\(^{-3}\)–10\(^{-2}\) M was without effect in one study (43), but otherwise reduced \(^{45}\)Ca\(^{2+}\) efflux to low values. Using a light-scattering method, Yamamoto and Kasai (24) found that choline influx into SR vesicles via the Ca\(^{2+}\)-release channel is controlled by Ca\(^{2+}\) and Mg\(^{2+}\). Measurement of choline influx rates indicated an apparent activation constant of 3 \(\times\) 10\(^{-6}\) M for Ca\(^{2+}\) and apparent inhibition constants of 1.4 \(\times\) 10\(^{-5}\) M for Mg\(^{2+}\) and 2.2 \(\times\) 10\(^{-6}\) M for Ca\(^{2+}\). In this study, \(^{45}\)Ca\(^{2+}\) efflux was half-maximally stimulated at 6 \(\times\) 10\(^{-7}\) M Mg\(^{2+}\), indicating an activating site with a high affinity to Ca\(^{2+}\) (see Fig. 3). The inhibiting site(s) had an apparent lower affinity, \(^{45}\)Ca\(^{2+}\) efflux being half-maximally inhibited at 7 \(\times\) 10\(^{-6}\) M Mg\(^{2+}\) and 2 \(\times\) 10\(^{-4}\) M Ca\(^{2+}\).

An argument against the Ca\(^{2+}\)-triggering hypothesis has been that Ca\(^{2+}\)-induced Ca\(^{2+}\) release from skinned muscle fibers and SR vesicles is inhibited by physiological concentrations of Mg\(^{2+}\) (1, 2, 6). Additional parameters have therefore been considered to control rapid release of Ca\(^{2+}\) from SR such a change in transmembrane potential (23, 45), surface charge (46), or pH (47). This study shows that adenine nucleotides dramatically stimulate Ca\(^{2+}\) release from passively and actively loaded SR vesicles. Involvement of nucleotides in the Ca\(^{2+}\) release process has been previously reported. At a very low level of free Mg\(^{2+}\), ATP stimulated the release of Ca\(^{2+}\) in skinned muscle fibers (48). Ogawa and Ebashi (37) found that AMP-PCP induced the release of Ca\(^{2+}\) from actively loaded vesicles (57 nmol/mg of protein/30 s). Chiesi and Wen (39) observed that the rapid phase of ATP-induced Ca\(^{2+}\) release from vesicles passively loaded with 10 mm Ca\(^{2+}\) was composed of two components, one involving the phosphorylated intermediate of the Ca\(^{2+}\)-ATPase and the other also being induced by AMP-PCP (10 nmol of Ca\(^{2+}\)/mg of protein/5 s), and therefore apparently requiring only binding of the nucleotide to the catalytic sites of the enzyme. Whether rapid release of Ca\(^{2+}\) was mediated by binding of the nucleotide to the Ca\(^{2+}\)-ATPase or to another component of SR is not clear from the data presented in the previous studies. We observed that Ca\(^{2+}\)-induced Ca\(^{2+}\) release action of AMP-PCP paralleled that of Ca\(^{2+}\)-induced Ca\(^{2+}\) release in that similar amounts of Ca\(^{2+}\) were rapidly released from passively and actively loaded vesicles in media containing micromolar concentrations of free Ca\(^{2+}\) and no or 1.5 mM Mg\(^{2+}\) plus 1 mM AMP-PCP. The ability of AMP-PCP to stimulate \(^{45}\)Ca\(^{2+}\) efflux from vesicles capable of Ca\(^{2+}\)-induced Ca\(^{2+}\) release, but not from vesicles that lack this mechanism but nevertheless contain the Ca\(^{2+}\)-ATPase, suggests that AMP-PCP exerted its effect through a structure different from that of the Ca\(^{2+}\)-ATPase. Since both types of vesicles contained the Ca\(^{2+}\)-ATPase, it could not be excluded, however, that the Ca\(^{2+}\)-ATPase in conjunction with another component formed the Ca\(^{2+}\) channel of the Ca\(^{2+}\)-release vesicles. In addition, it may be noted that we could not rule out the possibility that rapid Ca\(^{2+}\) release observed in the presence or absence of adenine nucleotides is mediated by two separate rather than a single pathway.

During the review of the manuscript we became aware that Morii and Tomonura (49) had also found that various adenine nucleotides (ATP, AMP-PCP, ADP, AMP) accelerated the release of Ca\(^{2+}\) from passively loaded vesicles. Other nucleotides (CTP, GTP, ITP, UTP) and caffeine had no effect on the release of Ca\(^{2+}\). Rather one of these (CTP) inhibited the releasing effect of AMP-PCP. In addition and in disagreement with the present study, CAMP and adenosine were found to be ineffective. The degrees of effectiveness of some of the nucleotides may be due to differences in the assay conditions used by us and Morii and Tomonura (49). Their measurements of Ca\(^{2+}\) release at 0 °C in media containing 5 mM Mg\(^{2+}\) resulted in relatively low Ca\(^{2+}\) release rates of 1–5 nmol/mg of protein/s. Kinetic studies of the Ca\(^{2+}\)-releasing action of the adenine nucleotides indicated that Ca\(^{2+}\) efflux was activated by AMP with a Hill coefficient of 1 and an apparent dissociation constant of 2 mM (49). In the presence of 5 mM Mg\(^{2+}\), the addition of 1 mM ATP or 1 mM AMP increased the amount of Ca\(^{2+}\) released from 0 to 60%, as the free Ca\(^{2+}\) concentration was increased from 0.06 to 0.24 μM. Morii and Tomonura proposed that the Ca\(^{2+}\)-release channel of SR was activated by the binding of one molecule of adenine nucleotide. Different external Ca\(^{2+}\) concentrations were thought to be required to open a heterogeneous population of activated channels in an all or none fashion. We observed that both in the absence and presence of 1.5 mM Mg\(^{2+}\) plus 1 mM AMP-PCP, the rate but not the total amount of Ca\(^{2+}\) release depended on the external Ca\(^{2+}\) concentration. This observation would seem to favor a model in which all channels are capable of opening, however, where frequency or duration of channel opening or the rate of ion movement through the
channel are regulated by external Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, and adenine nucleotide. The role of Mg\textsuperscript{2+} in the Ca\textsuperscript{2+} release process was not considered by Morii and Tonomura (49).

The Ca\textsuperscript{2+} releasing action of adenine is of interest, since caffeine, a related compound, is known to cause Ca\textsuperscript{2+} release from skinned frog skeletal muscle and SR vesicles (1, 2, 6).

In addition to external activating and inhibiting divalent cations sites, it has been suggested that the Ca\textsuperscript{2+}-release channel of SR possesses an activating receptor site for caffeine more physiological assay conditions such as an intracellular concentration of 20 mM can be as high as 10\textsuperscript{-7}--10\textsuperscript{-6} M, Ca\textsuperscript{2+} concentration has been raised to about 10\textsuperscript{-6}--10\textsuperscript{-5} M in 5 ms is required to initiate muscle activity (51). This corresponds to a release rate of 4-8 nmol/mg of protein/s, using an SR content of 5 mg of protein/g of muscle (52) and assuming that Ca\textsuperscript{2+} release occurs over the whole reticular structure. Following the suggestion that Ca\textsuperscript{2+} release is limited to the terminal cisterna region of SR (29, 30) and that it accounts for one-third of the reticular structure (53), a release rate of 12-24 pmol of Ca\textsuperscript{2+}/mg of protein/s is estimated. Thus, maximal release rates observed in this study for vesicles passively loaded with 5 mM Ca\textsuperscript{2+} would correspond to less than 1% of the in vitro rate. A release rate of 40 nmol of Ca\textsuperscript{2+}/mg of protein/s corresponds to an ion flux rate of about 10\textsuperscript{6} Ca\textsuperscript{2+}/channel/ s, assuming that there is on an average only one channel/vesicle, an average vesicle diameter of 0.1 \mu m (26) and an intravesicular volume of 2 \mu l/mg of protein (54). Since the turnover of an ion channel with a half-saturation concentration of 20 mM can be as high as 10\textsuperscript{7} s\textsuperscript{-1} (55), it may be that more physiological assay conditions such as an increase of nucleotide concentration from 1 to 5 mM, temperature from 22 to 37 °C, and pH from 6.8 to 7 or 7.2 significantly increases in vitro Ca\textsuperscript{2+}-release rates. However, to measure such rapid rates, it will be necessary to carry out the release experiments on a shorter time scale than has been possible in the present study.

In media containing ATP and a relatively low concentration of free Mg\textsuperscript{2+} and Ca\textsuperscript{2+} at a concentration of 10\textsuperscript{-6}--10\textsuperscript{-8} M, Ca\textsuperscript{2+}-release vesicles rapidly released their Ca\textsuperscript{2+}, resulting in a low steady state level of Ca\textsuperscript{2+} uptake during active transport (Refs. 10 and 23; Table IV). This observation raises an important question: how is Ca\textsuperscript{2+} release interrupted once the myoplasmic Ca\textsuperscript{2+} concentration has been raised to about 10\textsuperscript{-6}--10\textsuperscript{-8} M during the event of excitation-contraction coupling? One possibility would be that under intracellular conditions, the Ca\textsuperscript{2+}-transport system of SR can sufficiently remove Ca\textsuperscript{2+} near release sites to cause a rapid inactivation of the Ca\textsuperscript{2+}-release channels. Alternatively, it is conceivable that a protein responsible for terminating Ca\textsuperscript{2+} release from SR or another regulatory factor has been removed during vesicle isolation. In this regard it is of interest that exposure of the membrane fractions to salt results in dissociation of SR and T-system membranes as well as loss of "foot" proteins (19, 40, 56) suggesting that perhaps a component of the triad missing from the Ca\textsuperscript{2+}-release vesicles mediates the termination of Ca\textsuperscript{2+} release.

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Ca²⁺ Release in Sarcoplasmic Reticulum