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Kinetics of Rapid Ca^{2+} Release by Sarcoplasmic Reticulum. Effects of Ca^{2+}, Mg^{2+}, and Adenine Nucleotides†

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ABSTRACT: A radioisotope flux–rapid-quench–Millipore filtration method is described for determining the effects of Ca^{2+}, adenine nucleotides, and Mg^{2+} on the Ca^{2+} release behavior of "heavy" sarcoplasmic reticulum (SR) vesicles. Rapid $^{45}Ca^{2+}$ efflux from passively loaded vesicles was blocked by the addition of Mg^{2+} and ruthenium red. At pH 7 and 10^{-9} M Ca^{2+}, vesicles released $^{45}Ca^{2+}$ with a low rate ($k = 0.1$ s^{-1}). An increase in external Ca^{2+} concentration to 4 μM or the addition of 5 mM ATP or the ATP analogue adenosine 5′-(β,γ-methylenetriphosphate) (AMP-PCP) resulted in intermediate $^{45}Ca^{2+}$ release rates. The maximal release rate was observed in media containing 4 μM Ca^{2+} and 5 mM AMP-PCP and had a first-order rate constant of 30–100 s^{-1}. Mg^{2+} partially inhibited Ca^{2+}- and nucleotide-induced $^{45}Ca^{2+}$ efflux. In the absence of AMP-PCP, $^{45}Ca^{2+}$ release was fully inhibited at 5 mM Mg^{2+} or 5 mM Ca^{2+}. The composition of the release media was systematically varied, and the flux data were expressed in the form of Hill equations. The apparent n values of activation of Ca^{2+} release by ATP and AMP-PCP were 1.6–1.9. The Hill coefficient of Ca^{2+} activation ($n = 0.8$–2.1) was dependent on nucleotide and Mg^{2+} concentrations, whereas the one of Mg^{2+} inhibition ($n = 1.1$–1.6) varied with external Ca^{2+} concentration. These results suggest that heavy SR vesicles contain a "Ca^{2+} release channel" which is capable of conducting Ca^{2+} at rates comparable with those found in intact muscle. Ca^{2+}, AMP-PCP (ATP), and Mg^{2+} appear to act at noninteracting or interacting sites of the channel.

Sarcoplasmic reticulum (SR)[1] forms a distinct intracellular membrane compartment that regulates the contraction–relaxation cycle of muscle by releasing and reabsorbing Ca^{2+} [for reviews, see Ebashi (1976), Endo (1977), Winegrad (1982), Martonosi & Beeler (1983), and Inesi (1985)]. Release of Ca^{2+} from SR is triggered by an action potential at the neuromuscular junction that is communicated to SR via the T system. The mechanism by which Ca^{2+} is released from SR, however, has remained unclear. Mechanisms proposed to explain physiological release of Ca^{2+} include induction by Ca^{2+}, "depolarization" of the SR membrane, a change in membrane surface charge, and/or a pH gradient (Ebashi, 1976; Endo, 1977; Winegrad, 1982).

Of particular relevance to this study is the Ca^{2+}-induced Ca^{2+} release hypothesis which states that a small amount of Ca^{2+} moving into the sarcoplasm during an action potential induces the release of sufficient Ca^{2+} from SR to activate muscle contraction. In support of this hypothesis, skinned muscle fibers (Stephenson, 1981; Fabiato, 1983) and "heavy" SR vesicles obtained by centrifugation between 2000g and 10000g (Onishi, 1981; Yamamoto & Kasai, 1982; Miyamoto & Racker, 1982; Kirino et al., 1983; Morii & Tonomura, 1983; Nagasaki & Kasai, 1983; Kim et al., 1983; Meissner, 1984) possess a Ca^{2+} permeability mechanism which is activated by micromolar concentrations of Ca^{2+}. Caffeine and adenine nucleotides (ATP, ADP, AMP, adenosine, adenine) potentiated Ca^{2+}-induced Ca^{2+} release, whereas Mg^{2+} was inhibitory.

In this report, we describe a radioisotope flux–rapid-quench–Millipore filtration technique to determine the effects of Ca^{2+}, adenine nucleotides, and Mg^{2+} on the Ca^{2+} release

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[1] Abbreviations: SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; AMP-PCP, adenosine 5′-(β,γ-methylenetriphosphate); Pipes, 1,4-piperazinediethanesulfonic acid.

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behavior of heavy SR vesicles. Our studies suggest that heavy SR vesicles possess a "Ca^{2+} release" channel which contains interacting regulatory binding sites for Ca^{2+}, adenine nucleotides, and Mg^{2+}. In the presence of millimolar concentrations of AMP-PCP, a nonhydrolyzable ATP analogue, and micromolar concentrations of Ca^{2+}, the vesicles released Ca^{2+} with rates close to those expected to occur in muscle. A preliminary account of part of this work has been presented (Meissner & Darling, 1985).

Materials and Methods

Reagents. $^{45}Ca^{2+}$ was purchased from ICN Pharmaceuticals, Irvine, CA. The ATP analogue AMP-PCP was obtained from Sigma, St. Louis, MO. All other reagents were of reagent grade.

Isolation of SR Vesicles. Rabbit skeletal muscle sarcoplasmic reticulum was fractionated into "heavy Ca^{2+} release" and "light control" vesicle fractions by differential and sucrose gradient centrifugation as described previously (Meissner, 1984). Homogenization and sucrose gradient solutions contained the protease inhibitor phenylmethanesulfonyl fluoride at concentrations of 2 and 0.2 mM, respectively. Ca^{2+} release fractions were recovered from the 36–45% region of sucrose gradients that contained membranes sedimenting at 2600–35000*g*. Control fractions were recovered in the 30–34% region of sucrose gradients containing membranes obtained by differential pelleting at 35000–130000*g*.

Isotope Flux Measurements. $^{45}Ca^{2+}$ efflux rates from vesicles passively loaded with $^{45}Ca^{2+}$ were determined by Millipore filtration, as previously described (Meissner, 1984). Briefly, $^{45}Ca^{2+}$ was equilibrated across the vesicle membranes by incubation for 2 h at 22 °C in a medium containing 20 mM K-Pipes, pH 7, 0.1 M KCl, 0.1 mM EGTA, and either 0.6 or 5.1 mM $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ efflux was initiated by diluting vesicles into isoosmolal unlabeled release media containing varying concentrations of free Ca^{2+}, adenine nucleotide, and Mg^{2+}. $^{45}Ca^{2+}$ efflux was terminated by placing the vesicles on 0.45-μm HAWP Millipore filters followed by rapid rinsing to remove extravesicular $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ radioactivity retained by the vesicles on the filters was determined by liquid scintillation counting.

An Update System 1000 chemical quench apparatus (Madison, WI) was used in experiments which required resolving times in the millisecond range. The Update System consists of an electronically driven ram which was programmed to push three or four syringes simultaneously with a speed of 1 cm/s. $^{45}Ca^{2+}$ release in the absence of nucleotide was measured by using three syringes and two Update four-grid acrylic mixing chambers with 1.6-μL dead volume. Unless otherwise indicated, four syringes and three mixing chambers were used to measure rapid nucleotide-stimulated $^{45}Ca^{2+}$ efflux from SR vesicles passively loaded with 0.5 or 5 mM $^{45}Ca^{2+}$. One 0.5-mL syringe was filled with the vesicle suspension and three 2-mL syringes with three mixing solutions to achieve the following sequence of conditions: (1) dilution of vesicles into a medium containing EGTA to yield a final free Ca^{2+} concentration of 5 μM or less; (2) stimulation of $^{45}Ca^{2+}$ efflux by the addition of ATP or the nonhydrolyzable ATP analogue AMP-PCP; and (3) inhibition of $^{45}Ca^{2+}$ efflux by the addition of Mg^{2+} and ruthenium red. Reaction times were determined by varying the lengths of the aging hoses between the mixing chambers. Millipore filtration separated untrapped and released $^{45}Ca^{2+}$ from $^{45}Ca^{2+}$ retained by the vesicles.

$^{45}Ca^{2+}$ efflux measurements were carried out at least in duplicate with three or more time points. For a given preparation, the standard errors were ±10% or less.

Biochemical Assays. Protein was determined by the Lowry method using bovine serum albumin as a standard. Free Ca^{2+} and Mg^{2+} concentrations were calculated according to a computer program using binding constants published by Fabiato (1981). Measurements with an Orion divalent cation selective electrode in a buffer containing 0.1 M NaCl and 20 mM NaPipes, pH 7, indicated a similar apparent association constant for the Mg–ATP complex [$K = (1.0 \pm 0.6) \times 10^4$ M^{-1}] and Mg–AMP-PCP complex [$K = (0.9 \pm 0.6) \times 10^4$ M^{-1}]. Free Mg^{2+} concentrations in the presence of AMP-PCP were therefore calculated by using the ATP binding constants published by Fabiato.

Results

Effect of Ca^{2+} and AMP-PCP on $^{45}Ca^{2+}$ Release. Figure 1 compares the $^{45}Ca^{2+}$ efflux rates of a heavy SR vesicle fraction passively loaded with 5 mM $^{45}Ca^{2+}$ and diluted into different media. Between 80 and 100 nmol of $^{45}Ca^{2+}$/mg of protein was retained by the vesicles when samples were diluted into a medium containing 6 mM Mg^{2+} and 0.1 μM free Ca^{2+} (Figure 1A). About 90% of the trapped $^{45}Ca^{2+}$ could be released within 30 s by diluting vesicles into Mg^{2+}-free media containing ~5 μM free Ca^{2+} and 0 or 2.5 mM AMP-PCP. The nonhydrolyzable ATP analogue AMP-PCP was used rather than ATP in order to avoid reuptake of the released $^{45}Ca^{2+}$ by the SR Ca^{2+} pump. Data of Figure 1A are in accord with previous findings that isolated heavy SR vesicles contain a Ca^{2+} release channel which is activated in the absence of Mg^{2+} by micromolar concentrations of external Ca^{2+}. The small amount of $^{45}Ca^{2+}$ remaining with the vesicles 1–2 min after dilution indicated that a small fraction of the vesicles lacked the channel (Meissner, 1984).

Two effective inhibitors of Ca^{2+}-induced Ca^{2+} release from SR are Mg^{2+} and ruthenium red (Stephenson, 1981; Onishi, 1981; Yamamoto & Kasai, 1982; Miyamoto & Racker, 1982; Meissner, 1984). We have used these two compounds to stop Ca^{2+} release in a rapid mixing apparatus at time intervals ranging from 25 to 1000 ms. $^{45}Ca^{2+}$ efflux measurements in nucleotide-containing media involved either two or three mixing steps. Either vesicles were diluted directly into the nucleotide-containing medium or the external free Ca^{2+} concentration was initially lowered to 5 μM or less before addition of the nucleotide medium in a second mixing step. In Figure 1A, vesicles present in 5 mM $^{45}Ca^{2+}$ were diluted directly into a medium containing 2.5 mM AMP-PCP. EGTA and Ca^{2+} concentrations and the pH of the dilution medium were adjusted so that after mixing the vesicles were present in 4 μM free Ca^{2+} at pH 7. In a second mixing chamber, $^{45}Ca^{2+}$ efflux was stopped after 25 ms by the addition of 6 mM Mg^{2+} and 10 μM ruthenium red. The untrapped and released $^{45}Ca^{2+}$ was removed by the relatively slow Millipore filtration technique. Figure 1 A shows that the addition of Mg^{2+} and ruthenium red at 25 ms effectively blocked $^{45}Ca^{2+}$ release from vesicles initially present in a medium containing 4 μM free Ca^{2+} and 2.5 mM AMP-PCP. Ca^{2+} release from the Ca^{2+}-permeable vesicle fraction in the absence of the two quenching agents followed first-order kinetics, being nearly completed within 0.1 s (Figure 1B). In media containing 5 μM Ca^{2+}, the vesicles released $^{45}Ca^{2+}$ with an initial rate of about 0.1 μmol of Ca^{2+} (mg of protein)$^{-1}$ s^{-1} ($k = 1.15$ s^{-1}). Addition of 2.5 mM AMP-PCP to the release medium increased the initial $^{45}Ca^{2+}$ release rate to 3 μmol of Ca^{2+} (mg of protein)$^{-1}$ s^{-1} ($k = 35$ s^{-1}).

Similar rate constants of $^{45}Ca^{2+}$ release were obtained when the external free Ca^{2+} concentration was lowered to 4 μM before the nucleotide was added, or when vesicles were diluted

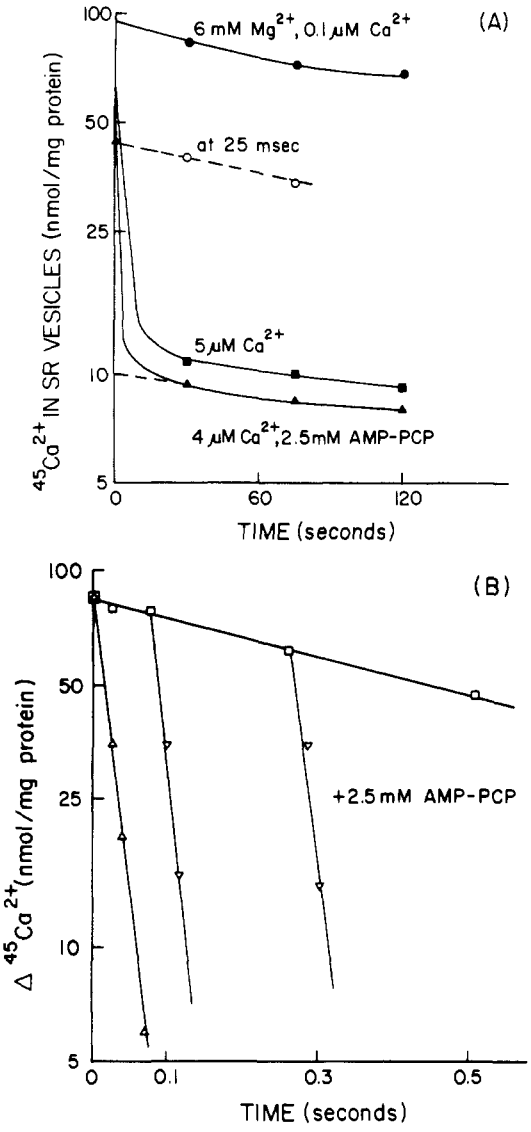

FIGURE 1: Measurement of $^{45}Ca^{2+}$ efflux rates. A heavy SR Ca^{2+} release fraction, preincubated for 2 h at 22 °C in the presence of 5 mM $^{45}Ca^{2+}$, was diluted into 20 mM K-Pipes, pH 7.0, and 0.1 M KCl medium containing amounts of Mg^{2+}, EGTA, and AMP-PCP so that release media after the addition of the vesicles contained 6 mM Mg^{2+} plus 0.1 μM free Ca^{2+} [(●) in panel A], 5 μM free Ca^{2+} [5 mM EGTA plus 4.65 mM Ca^{2+}; (■) in panel A, (□) in panel B], or 4 μM free Ca^{2+} plus 2.5 mM AMP-PCP [(▲) in panel A, (△) in panel B]. An Update System 1000 chemical quench apparatus was used in experiments which required a resolving time of less than 1 s. Vesicles were diluted directly into the release medium [(○) in panel A, (□, △) in panel B] or were initially mixed with 4 volumes of a 6.25 mM EGTA–4.45 mM Ca^{2+} medium to lower the free external Ca^{2+} concentration from 5 mM to 4 μM before adding the nucleotide medium in a second mixing step after 72 or 260 ms [(▽) in panel B]. Rapid $^{45}Ca^{2+}$ efflux was inhibited by the addition of 6 mM Mg^{2+} and 10 μM ruthenium red. Vesicles were subsequently placed on 0.45-μm Millipore filters and rinsed with a medium containing 6 mM Mg^{2+} and 10 μM ruthenium red. The amount of $^{45}Ca^{2+}$ initially trapped by all of the vesicles (95 nmol/mg of protein) as well as the amount not readily released by a subpopulation of vesicles (10 nmol/mg of protein) was obtained by back-extrapolation to the time of vesicle dilution. In panel B, the time course of $^{45}Ca^{2+}$ efflux from the vesicle population capable of rapid Ca^{2+} release (85 nmol/mg of protein) was obtained by subtracting the amount not readily released (10 nmol/mg of protein).

directly into a 4 μM Ca^{2+} medium containing 2.5 mM AMP-PCP (Figure 1B). Other control experiments indicated that the time course of $^{45}Ca^{2+}$ release was not significantly affected when mixing rates were varied by changing the ram speed of the rapid-quench apparatus from 1 cm/s to 0.8 or 1.6 cm/s (cf. Materials and Methods). On the other hand, the direct dilution method yielded a higher initial release rate when the final free Ca^{2+} concentration was less than 0.1 μM (not shown). This suggested that at low Ca^{2+} concentrations, chelation of Ca^{2+} by EGTA was not sufficiently rapid to prevent Ca^{2+}-induced, nucleotide-stimulated Ca^{2+} release (see below). Therefore, in the experiments described below, we studied the kinetics of AMP-PCP activation of Ca^{2+} release by lowering the free Ca^{2+} concentration from 0.5 or 5 mM to 5 μM or less in an initial mixing step. Minimal nucleotide-stimulated $^{45}Ca^{2+}$ efflux rates were obtained when the nucleotide was added 50–100 ms after the Ca^{2+} concentration was lowered to below 10^{-8} M by the addition of EGTA.

Table I: Ca^{2+} Release Properties of a Heavy SR Vesicle Fraction[a]

additions to release medium			$^{45}Ca^{2+}$ efflux, k
free Ca^{2+} (M)	Mg^{2+} (M)	AMP-PCP (M)	(s^{-1})
10^{-8}	5×10^{-3}		0.004
10^{-8}			0.1
10^{-8}		5×10^{-3}	24
10^{-8}	8×10^{-3}	5×10^{-3}	0.04
4×10^{-6}	5×10^{-3}		0.004
4×10^{-6}			1.5
4×10^{-6}		5×10^{-3}	90
4×10^{-6}	8×10^{-3}	5×10^{-3}	1.1
5×10^{-3}			0.01

[a] Heavy SR vesicles were passively loaded with 5 mM $^{45}Ca^{2+}$, and the time course of $^{45}Ca^{2+}$ efflux from the Ca^{2+}-permeable vesicle population was determined as indicated in Figure 1. $^{45}Ca^{2+}$ release in the presence of the nucleotides was determined by initially lowering the free Ca^{2+} concentration to 4 μM or less before adding the nucleotide medium in a second mixing step. Of the trapped $^{45}Ca^{2+}$, 90% (105 nmol/mg of protein) was rapidly released in a Mg^{2+}-free medium containing 4 μM free Ca^{2+}.

It is conceivable that release of positively charged Ca^{2+} resulted in formation of a large membrane potential, which in turn might have slowed Ca^{2+} efflux. However, addition of the K^+ ionophore valinomycin (15 μM) to a release medium containing 4 μM free Ca^{2+} and 2.5 mM AMP-PCP did not appreciably affect $^{45}Ca^{2+}$ efflux (data not shown). The intrinsic permeability of the vesicles to K^+, H^+, and Cl^- (Meissner, 1983) was probably sufficient to prevent formation of a retarding membrane potential during Ca^{2+} release.

In agreement with a previous study (Meissner, 1984), a majority of light SR vesicles were found to lack a permeation system that mediates rapid Ca^{2+} release under the experimental conditions of Figure 1 (not shown). In another control experiment using [^{14}C]sucrose, we found that Ca^{2+} and AMP-PCP did not exert their activating effects by nonspecifically breaking down the permeability barrier of the vesicles (not shown). A slight increase in the [^{14}C]sucrose permeability of the Ca^{2+} release vesicles was, however, observed in media containing AMP-PCP, raising the possibility that sucrose slowly permeates through the "open" channel.

Table I summarizes the results of rapid-quench experiments carried out with a heavy SR vesicle fraction passively loaded with 5 mM $^{45}Ca^{2+}$. The concentrations of three components in the release medium—free Ca^{2+}, Mg^{2+}, and nucleotide—dramatically affected the rate constant of $^{45}Ca^{2+}$ release. A maximal rate constant of 90 s^{-1} with an initial release rate of 8 μmol of Ca^{2+} (mg of protein)$^{-1}$ s^{-1} was observed for vesicles passively loaded with 5 mM $^{45}Ca^{2+}$ and diluted into a release medium containing 4 μM free Ca^{2+} and 5 mM AMP-PCP. Thus, in vitro SR vesicles demonstrate rates of Ca^{2+} release close to those measured in muscle (Baylor et al., 1983; Melzer et al., 1984). Lowering the free Ca^{2+} concentration from 4 μM to 10^{-8} M and omission of the nucleotide decreased the

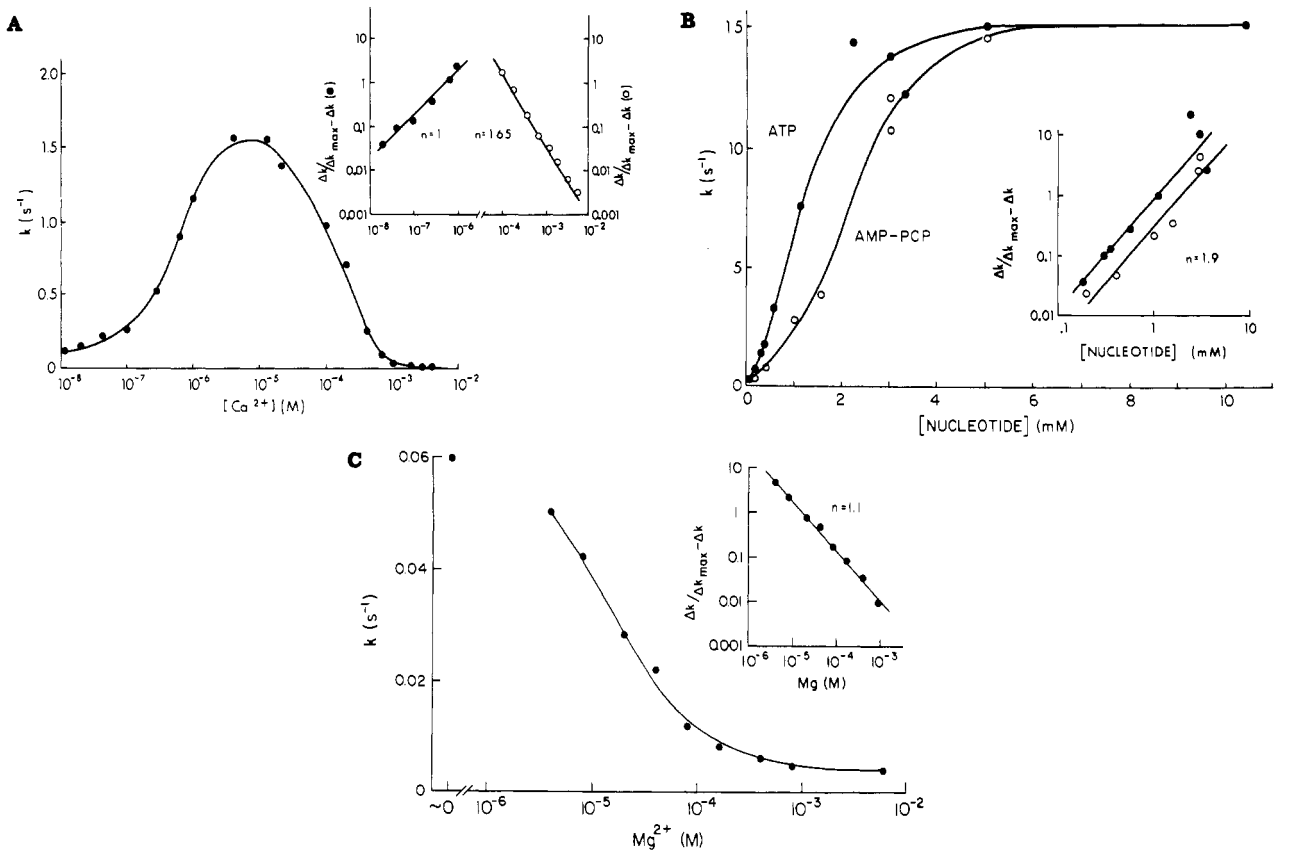

FIGURE 2: Dependence of $^{45}Ca^{2+}$ efflux rate constants on external Ca^{2+}, adenine nucleotide, and Mg^{2+} concentrations. (A) A heavy SR Ca^{2+} release fraction, perincubated for 2 h at 22 °C with 5 mM $^{45}Ca^{2+}$, was diluted into 20 mM K-Pipes, pH 7.0, and 0.1 M KCl release media containing amounts of EGTA and Ca^{2+} to yield the indicated final concentrations of free Ca^{2+}. The time course of rapid $^{45}Ca^{2+}$ efflux from the Ca^{2+}-permeable vesicle fraction was determined by using the Update rapid-quench apparatus with two mixing chambers. The apparent n value of Ca^{2+} activation of Ca^{2+} release was calculated according to the equation $\Delta k/(\Delta k_{max} - \Delta k) = K[Ca]^{n_{Ca}}$ where Δk indicates the difference in rate constants at a given free Ca^{2+} concentration and a minimally activating (10^{-8} M) concentration of free Ca^{2+}. Δk_{max} is the difference in rate constants at maximally and minimally activating concentrations of free Ca^{2+}. n_{Ca} indicates the apparent number of activating Ca^{2+} binding sites per channel, and K is an apparent binding constant. The apparent n value of Ca^{2+} inactivation of Ca^{2+} release was calculated by subtracting from the measured rate constants the rate constant calculated in the presence of 10 μM ruthenium red plus 6 mM Mg^{2+} (k = 0.005 s^{-1}). (B) The time course of $^{45}Ca^{2+}$ efflux was determined with the Update rapid-quench apparatus using three mixing chambers. Vesicles passively loaded with 0.5 mM $^{45}Ca^{2+}$ were rapidly mixed with 4 volumes of a medium containing 25 mM EGTA. After 200 ms, 4 volumes of release medium was added containing 20 mM EGTA and 2.25 times the indicated concentrations of ATP (●) or AMP-PCP (○). Hill coefficients were determined by using a Δk_{max} value of 15 s^{-1}. (C) Vesicles passively loaded with 5 mM $^{45}Ca^{2+}$ were diluted 250-fold into media containing 8 mM EGTA and 2 mM EDTA ("0" Mg^{2+}) or the indicated concentrations of Mg^{2+}. The rate constants of $^{45}Ca^{2+}$ release were obtained by measuring amounts of $^{45}Ca^{2+}$ remaining with the vesicles at times ranging from 5 to 150 s. Time points of 5, 10, and 15 s were determined by inhibiting $^{45}Ca^{2+}$ efflux by the addition of 6 mM Mg^{2+} and 10 μM ruthenium red. The apparent n value of Mg^{2+} inhibition was determined by using a Δk_{max} value of 0.056 s^{-1}.

$^{45}Ca^{2+}$ efflux rate constant by a factor of 900. An increase in the free Ca^{2+} concentration back to 4 μM or addition of AMP-PCP to the low-Ca^{2+} medium resulted in intermediate release rates. Mg^{2+} and high concentrations of Ca^{2+} had strong inhibitory effects. Addition of 8 mM Mg^{2+} to the 5 mM AMP-PCP media containing 10^{-8} M or 4 μM Ca^{2+} reduced the release rates to values seen in the absence of nucleotide. In the absence of nucleotide, both Mg^{2+} and Ca^{2+} fully inhibited $^{45}Ca^{2+}$ release at a concentration of 5 mM. Some variations in the $^{45}Ca^{2+}$ efflux rates were noted. In the presence of 4 μM free Ca^{2+}, vesicles typically released $^{45}Ca^{2+}$ with a first-order rate constant of 1–2.5 s^{-1}. Addition of 5 mM AMP-PCP to the release medium increased the first-order rate constant to 30–100 s^{-1}. The difference in the release activity of the vesicle fractions can be most easily explained by assuming that the more active vesicle fractions contained a larger number of Ca^{2+} release channels per vesicle.

Regulation of Ca^{2+} Release by Ca^{2+}, Adenine Nucleotides, and Mg^{2+}. We have formulated a simple model (see Figure 5) to analyze the effects of Ca^{2+}, adenine nucleotide, and Mg^{2+} on Ca^{2+} release as shown in Table I. The model assumes that the SR Ca^{2+} release channel (E) is an allosteric enzyme which contains interacting regulatory binding sites for Ca^{2+}, adenine nucleotide, and Mg^{2+}. The channel is present in its ligand-free form, designated E_0, in the absence of external Ca^{2+} ($<10^{-8}$ M), ATP (AMP-PCP), and Mg^{2+}. Distinct channel intermediates, e.g., E_{Ca}, E_A, and E_{Mg}, are assumed to be formed by diluting the vesicles into media containing optimally activating concentrations of Ca^{2+} or nucleotide or inhibitory concentrations of Mg^{2+}. The kinetics of channel activation and inhibition have been investigated by systematically varying the Ca^{2+}, nucleotide, and Mg^{2+} concentrations in the release media and expressing the flux data in the form of simplifield equations for allosteric enzymes, i.e., Hill equations. Hill plot analysis has been found useful in determining the permeation behavior of another ligand-gated ion channel, the acetylcholine receptor ion channel. Ion flux and direct binding studies have indicated that the channel is opened by the binding of two molecules of agonist to two interacting subunits of the channel (Walker et al., 1982; Neubig et al., 1982).

Activation and Inhibition of the Ligand-Free E_0 Channel Form. Data of Table I indicate that the ligand-free E_0 form

Table II: Regulation of $^{45}Ca^{2+}$ Release by Ca^{2+}, AMP-PCP, and Mg^{2+}

reaction	ligands: free Ca^{2+} (M)	AMP-PCP (M)	Mg^{2+} (M)	n_{app}[a]	L_{50}[b] (M)
$E_0 \rightleftharpoons E_{Ca}$	10^{-9}–10^{-5}			1.0	5×10^{-7}
$E_A \rightleftharpoons E_{A,Ca}$	10^{-9}–10^{-5}	5×10^{-3}		0.8	9×10^{-7}
$E_{Mg} \rightleftharpoons E_{Mg,Ca}$	10^{-9}–10^{-5}		10^{-3}	1.35	1.3×10^{-6}
$E_{A,Mg} \rightleftharpoons E_{A,Mg,Ca}$	10^{-9}–10^{-5}	5×10^{-3}	5×10^{-3}	2.1	2×10^{-6}[c]
$E_0 \rightleftharpoons E_A$	10^{-9}	$(0–5) \times 10^{-3}$		1.9	2×10^{-3}
$E_{Ca} \rightleftharpoons E_{Ca,A}$	4×10^{-6}	$(0–5) \times 10^{-3}$		1.6	1.6×10^{-3}
$E_0 \rightleftharpoons E_{Mg}$	10^{-9}		$(0–5) \times 10^{-3}$	1.1	2×10^{-5}
$E_{Ca} \rightleftharpoons E_{Ca,Mg}$	5×10^{-6}		$(0–5) \times 10^{-3}$	1.55	1.0×10^{-4}
$E_{Ca} \rightleftharpoons E^{i}_{Ca}$	(5×10^{-6})–(5×10^{-3})			1.65	1.5×10^{-4}

[a] n_{app} was determined as indicated in Figures 2 and 4. [b] L_{50} indicates the concentration of the variable ligand that resulted in a half-maximal increase or reduction of the rate constants of $^{45}Ca^{2+}$ release. [c] Vesicles were passively loaded with 5 mM $^{45}Ca^{2+}$.

represents a low Ca^{2+} release state of the channel. Activation of E_0 by Ca^{2+} in the absence of nucleotide and Mg^{2+} is shown in Figure 2A. $^{45}Ca^{2+}$ efflux was maximal with a rate constant of about 1.5 s^{-1} in a medium containing about 5 μM free Ca^{2+} and was half-maximally activated at an external Ca^{2+} concentration of 5×10^{-7} M. A Hill plot of the data yielded an apparent n value of 1 for Ca^{2+} activation of Ca^{2+} release (inset of Figure 2A, Table II). This suggests that in the absence of nucleotide and Mg^{2+} the channel does not contain interacting activating binding sites for Ca^{2+}. Figure 2A further shows that $^{45}Ca^{2+}$ efflux was greatly inhibited at millimolar concentrations of Ca^{2+}. As discussed below, the inhibition of Ca^{2+} release appears to be due to the presence of low-affinity inhibitory Ca^{2+} and Mg^{2+} binding sites.

Activation of the ligand-free E_0 form of the channel by AMP-PCP and ATP was investigated at a free extravesicular Ca^{2+} concentration (10^{-9} M) which minimally activated the SR Ca^{2+} release channel. A free Ca^{2+} concentration of 10^{-9} M was achieved in the rapid-mixing experiments by lowering the Ca^{2+} concentration in the vesicle incubation medium to 0.5 mM $^{45}Ca^{2+}$ and by diluting vesicles into 20 mM EGTA medium. Ca^{2+} release was half-maximally activated at an AMP-PCP concentration of 2 mM with an apparent n value of 1.9 (Figure 2B, Table II). ATP was somewhat more effective in activating Ca^{2+} release than AMP-PCP, Ca^{2+} efflux being half-maximally activated at 1 mM ATP with an apparent n value of 1.9. At 10^{-9} M free Ca^{2+}, ATP did not appear to exert its effect by activating the Ca^{2+} pump of sarcoplasmic reticulum. Uptake media contained 0 or 5 mM Mg^{2+}, 5 mM ATP, and 10 μM ruthenium red.

Figure 2C shows the effects of Mg^{2+} concentration on the Ca^{2+} release behavior of vesicles at 10^{-9} M Ca^{2+} in the absence of adenine nucleotide. In the absence of Mg^{2+}, a rate constant of 0.06 s^{-1} with an initial release rate of 3 nmol of Ca^{2+} (mg of protein)$^{-1}$ s^{-1} was observed. The initial release rate was half-maximally reduced at an external Mg^{2+} concentration of 2×10^{-5} M with an apparent n value of 1.1 and was lowered to a limiting value of about 0.2 nmol (mg of protein)$^{-1}$ s^{-1} (k = 0.004 s^{-1}) by raising the Mg^{2+} concentration to 5 mM. A similar low rate was measured for control vesicles which lack the channel, suggesting that the small amounts of $^{45}Ca^{2+}$ released at millimolar concentrations of Mg^{2+} were not mediated by the channel.

Activation of E_{Ca} by AMP-PCP. As shown in Table I, both Ca^{2+} and AMP-PCP are required to optimally activate $^{45}Ca^{2+}$ release from SR vesicles. The dependence of the Ca^{2+} release rate on AMP-PCP concentration in the presence of Ca^{2+} was determined at an optimally activating Ca^{2+} concentration of 4 μM. As was observed at 10^{-9} M Ca^{2+} (Figure 2B), a fairly high concentration of nucleotide in the 5–10 mM concentration range was required to optimally activate Ca^{2+}-induced Ca^{2+} release (not shown). Ca^{2+} release was half-maximally activated at 1.6 mM AMP-PCP with an apparent n value of 1.6 (Table II).

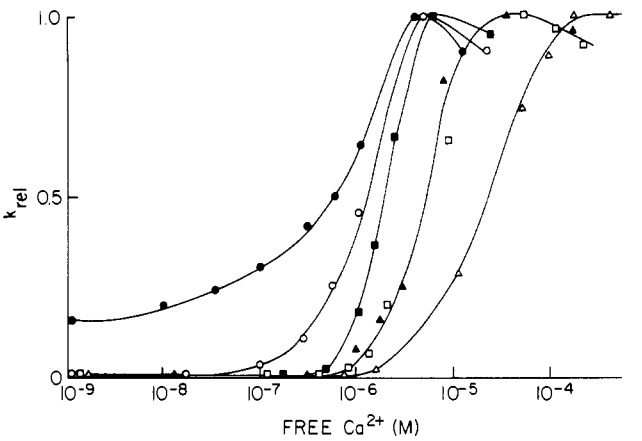

FIGURE 3: Activation of $^{45}Ca^{2+}$ efflux rate by Ca^{2+} in the presence of AMP-PCP and Mg^{2+}. Heavy SR release vesicles were preincubated for 2 h at 22 °C with 0.5 (●, ▲, △) or 5 mM (○, ■, □) $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ efflux rates from the Ca^{2+}-permeable vesicle fraction were determined as indicted in Figure 1. Vesicles were diluted into media containing the indicated concentrations of free Ca^{2+} and 5 mM AMP-PCP (●), 1 mM Mg^{2+} (○), 5 mM AMP-PCP plus 5 mM Mg^{2+} (▲, ■), or 5 mM AMP-PCP plus 10 mM Mg^{2+} (△, □).

Activation of E_A, E_{Mg}, and $E_{A,Mg}$ by Ca^{2+}. The effects of activating concentrations of Ca^{2+} on the Ca^{2+} release behavior of the vesicles in the presence of AMP-PCP and Mg^{2+} are illustrated in Figure 3. The flux data were normalized to account for the large differences in $^{45}Ca^{2+}$ efflux rates seen in media containing nucleotide or Mg^{2+} (Table I). For vesicles diluted into an optimally activating concentration of 5 mM AMP-PCP, the $^{45}Ca^{2+}$ efflux rate constant increased about 6-fold as the external free Ca^{2+} concentration was increased from 10^{-9} to 5×10^{-6} M. $^{45}Ca^{2+}$ efflux was half-maximally activated at 9×10^{-7} M Ca^{2+}. The apparent n value of Ca^{2+} activation was 0.8 as compared to 1 in the absence of nucleotide (Table II). Substitution of 5 mM AMP-PCP by 1 mM Mg^{2+} greatly reduced the $^{45}Ca^{2+}$ efflux rate constant at micromolar concentrations of external Ca^{2+} (cf. Table I). The apparent n value of Ca^{2+} activation increased to 1.35, and about a 1.5-fold higher Ca^{2+} concentration (1.3×10^{-6} M) was required to half-maximally activate $^{45}Ca^{2+}$ efflux (Table II).

Figure 3 further shows four Ca^{2+} activation curves which were obtained with vesicles diluted into media containing both AMP-PCP and Mg^{2+}. Vesicles filled with 0.5 or 5 mM $^{45}Ca^{2+}$ were diluted into media containing 5 mM AMP-PCP and either 5 or 10 mM total Mg^{2+} (0.7 and 5 mM free Mg^{2+}, respectively). A series of similarly shaped Ca^{2+} activation

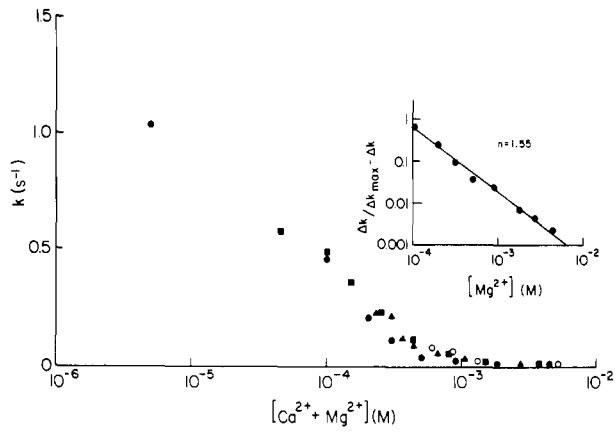

FIGURE 4: Inhibition of $^{45}Ca^{2+}$ efflux by Mg^{2+} and Ca^{2+}. $^{45}Ca^{2+}$ efflux rates were determined as indicated in Figure 2A. Vesicles were passively loaded with 5 mM $^{45}Ca^{2+}$; EGTA, Ca^{2+}, and Mg^{2+} concentrations and the pH of the dilution media were adjusted so that after mixing the vesicles were present in media containing the indicated concentrations of Mg^{2+} plus free Ca^{2+} at pH 7. Free Ca^{2+} concentrations were 5 (●), 45 (■), 250 (▲), and 600 (○) μM.

curves was obtained that were shifted to higher Ca^{2+} activating concentrations as the Mg^{2+} concentration in the release medium was increased. A shift to the right was also observed, as the Ca^{2+} concentration in the vesicle medium was decreased. In the presence of AMP-PCP and Mg^{2+}, a relatively high Ca^{2+} concentration of (5×10^{-7})–10^{-6} M was required before a noticeable increase in the flux rates could be observed. Above 10^{-6} M Ca^{2+}, $^{45}Ca^{2+}$ efflux became very sensitive to external Ca^{2+} concentration. The Hill plots of the four Ca^{2+} activation curves yielded apparent n values of 1.7–2.1. These results indicated that in the presence of AMP-PCP and Mg^{2+}, there occurred a significant change in the cooperativity of Ca^{2+} activation of Ca^{2+} release, with Ca^{2+} likely acting at two or more interacting activating sites.

Inhibition of E_{Ca} by Mg^{2+} and Ca^{2+}. Ca^{2+}-induced Ca^{2+} release is inhibited by millimolar concentrations of Ca^{2+} (Figure 2A). Figure 4 demonstrates that the E_{Ca} form of the channel is inhibited in a similar manner by Mg^{2+}. $^{45}Ca^{2+}$ efflux was half-maximally inhibited at 1.5×10^{-4} M Ca^{2+} or 10^{-4} M Mg^{2+}. Apparent n values of 1.65 for Ca^{2+} and 1.55 for Mg^{2+} suggested that the two divalent cations acted on the channel by binding to two or more interacting inhibitory sites. One possibility was that Ca^{2+} and Mg^{2+} bound to the same sites. This was tested by measuring $^{45}Ca^{2+}$ efflux in the presence of varying concentrations of external Ca^{2+} and Mg^{2+}. Vesicles were diluted into media containing increasing concentrations of Mg^{2+} and either 5, 45, 250, or 600 μM free Ca^{2+}. As shown in Figure 4, nearly identical inactivation curves were obtained when the $^{45}Ca^{2+}$ efflux rate constants were plotted against the sum of the concentrations of the two divalent cations. That the inactivation curves were superimposable suggested that Ca^{2+} and Mg^{2+} likely inhibited $^{45}Ca^{2+}$ release by acting at the same sites.

Inhibition of $E_{Ca,A}$ by Mg^{2+}. The free Mg^{2+} concentration in mammalian skeletal muscle is not known but has been estimated to range from 0.2 to 4 mM in frog skeletal muscle (Gupta & Moore, 1980; Baylor et al., 1982). Table III shows that the rate constant of Ca^{2+}- and nucleotide-induced Ca^{2+} release was greatly reduced when the free Mg^{2+} concentration in the release medium was raised from 0 to 4 mM. Varying concentrations of Mg^{2+} were added to media containing 4 μM free Ca^{2+} and 5 mM AMP-PCP so that the free Mg^{2+} concentration ranged from about 0.1 to 4 mM (experiment 1 of Table III). In the presence of 0.14 mM free Mg^{2+}, the rate

Table III: Effect of Mg^{2+} Concentration on Ca^{2+}-Induced and Adenine Nucleotide Induced $^{45}Ca^{2+}$ Release[a]

additions to release medium			$^{45}Ca^{2+}$ efflux, k (s^{-1})
free AMP-PCP (M)	free Mg^{2+} (M)	Mg–AMP-PCP (M)	
	Experiment 1		
5.0×10^{-3}	0	0	56
2.1×10^{-3}	1.4×10^{-4}	2.9×10^{-3}	30
1.3×10^{-3}	2.9×10^{-4}	3.7×10^{-3}	19
6.5×10^{-4}	6.6×10^{-4}	4.3×10^{-3}	14
2.2×10^{-4}	2.2×10^{-3}	4.8×10^{-3}	1.8
1.2×10^{-4}	4.1×10^{-3}	4.9×10^{-3}	1.3
	Experiment 2		
0	0	0	1.7
0	2×10^{-3}	0	0.01
10^{-4}	0	0	3.75
10^{-4}	2×10^{-3}	1.9×10^{-3}	0.8

[a] Heavy SR vesicles were passively loaded with 5 mM $^{45}Ca^{2+}$. Release media contained after the addition of the vesicles 4×10^{-6} M free Ca^{2+} and the indicated concentrations of nucleotide and Mg^{2+}. The first-order rate constants of Ca^{2+} release from the Ca^{2+}-permeable vesicle population were determined as indicated in Figure 1.

constant of $^{45}Ca^{2+}$ efflux was reduced from 56 to 30 s^{-1}. A more dramatic decrease of k to 1.3 s^{-1} was observed when the free Mg^{2+} concentration was increased to 4 mM by the addition of 9 mM Mg^{2+}.

One complicating factor of studying the effects of Mg^{2+} in the presence of nucleotide is the formation of a Mg–AMP-PCP complex which may interact differently with the channel. The possibility that the Mg–AMP-PCP complex activates the channel was tested by diluting vesicles into media that contained, in addition to Mg–AMP-PCP, defined concentrations of free Mg^{2+} and nucleotide (experiment 2 of Table III). The rate constant of Ca^{2+} release was reduced 170-fold by the addition of 2 mM Mg^{2+} to the 5 μM release medium. By contrast, only a 5-fold reduction was observed when vesicles were diluted into media containing 0.1 mM free nucleotide and 0 or 2 mM free Mg^{2+} plus 1.9 mM Mg AMP-PCP. The concentration of free nucleotide in the presence and absence of 2 mM Mg^{2+} was kept constant by increasing total nucleotide and Mg^{2+} concentrations to 2 and 3.9 mM, respectively. One explanation of the data of Table III is that the Mg–AMP-PCP complex activates the channel, thereby minimizing inhibition by Mg^{2+}. Another, less likely possibility would be that low concentrations of free nucleotide modify the channel so that its sensitivity to Mg^{2+} is decreased. Regardless of the exact mechanism of nucleotide activation in the presence of Mg^{2+}, one important conclusion to be drawn from the data of Table III is that nucleotides render the channel less sensitive to inhibition by Mg^{2+}.

The effects of intravesicular Mg^{2+} on Ca^{2+} release were studied by passively loading the vesicles with 2 mM $^{45}Ca^{2+}$ in the presence or absence of 5 mM Mg^{2+}. Vesicles were subsequently diluted into release media containing 4 μM free Ca^{2+}, 1 mM AMP-PCP, and 1 mM Mg^{2+}. Vesicles incubated in the absence of Mg^{2+} released $^{45}Ca^{2+}$ with a rate constant of 3.5 s^{-1}. Addition of 5 mM Mg^{2+} to the vesicle medium reduced the amount of $^{45}Ca^{2+}$ trapped by the vesicles from 60 to 40 nmol/mg of protein; however, there occurred no change in the rate constant of $^{45}Ca^{2+}$ release. Intravesicular Mg^{2+} appears, therefore, at least under our experimental conditions, not to inhibit $^{45}Ca^{2+}$ efflux. This observation is in accordance with a recent report indicating that Mg^{2+} can rapidly permeate through the Ca^{2+}-activated channel of SR (Nagasaki & Kasai, 1984).

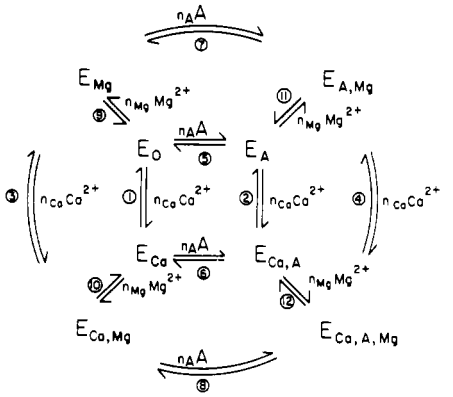

FIGURE 5: Model of regulation of the SR Ca^{2+} release channel by Ca^{2+}, Mg^{2+}, and adenine nucleotide. Abbreviations: E, SR Ca^{2+} release channel; *n*, apparent number of binding sites per channel; A, adenine nucleotide.

DISCUSSION

We have described a radioisotope flux–rapid-quench–Millipore filtration method for the measurement of rapid release of Ca^{2+} from isolated sarcoplasmic reticulum (SR) vesicle fractions. Using this method, we were able to demonstrate that heavy rabbit skeletal muscle SR vesicles, thought to be derived from the terminal cisternae of sarcoplasmic reticulum (Meissner, 1984), are capable of releasing Ca^{2+} with rates comparable to those expected to occur in muscle. Release rates with a first-order rate constant of 30–100 s^{-1} were obtained for vesicles diluted into media containing micromolar concentrations of Ca^{2+} and a 5 mM concentration of the nonhydrolyzable ATP analogue AMP-PCP. In control experiments, using light SR vesicles and [^{14}C]sucrose, it was established that Ca^{2+} and AMP-PCP did not exert their activating effects by nonspecifically breaking down the permeability barrier of the vesicles.

A major aim of this study was to determine the effects of Ca^{2+}, adenine nucleotide, and Mg^{2+} on the Ca^{2+} release channel of heavy SR vesicles. Dose–response curves were obtained (Table II) which described the formation of six of the seven hypothetical ligand-occupied states of the channel shown in Figure 5. An additional reaction not indicated in Figure 5 was inhibition of E_{Ca} by elevated concentrations of Ca^{2+} (Figure 2A and Table II). The kinetics of Mg^{2+} inhibition of E_A and $E_{Ca,A}$ (reactions 11 and 12) and of nucleotide activation of E_{Mg} and $E_{Ca,Mg}$ (reactions 7 and 8) channel states were not determined. Interpretation of these reactions is complicated due to the formation of a Mg–AMP-PCP complex which may interact differently with the channel.

In the kinetic studies, we were in part guided by single channel measurements with a nucleotide-activated Ca^{2+} conducting channel from rabbit skeletal muscle. Using the fusion technique of Miller & Racker (1976) for inserting SR proteins into planar lipid bilayers, we have identified an unusually large (170 pS in 50 mM Ba^{2+}) divalent conducting channel in heavy SR vesicle fractions (Smith et al., 1985). ATP or AMP-PCP activated the channel in the presence of micromolar concentrations of Ca^{2+} by increasing the open time of the channel without affecting single channel conductance. Taken together, our vesicle flux and lipid bilayer measurements suggest that Ca^{2+} and ATP affect the gating mechanism of the channel in a saturable and cooperative or noncooperative manner, depending on the experimental conditions. The SR Ca^{2+} release channel rarely opens in the absence of Ca^{2+} and adenine nucleotide. Ca^{2+} or ATP by themselves partially activates the channel by opening it for intermittent times. The presence of both ATP and Ca^{2+} fully activates Ca^{2+} release, and the channel is open essentially all the time. Millimolar concentrations of Ca^{2+} and Mg^{2+} inhibit the channel in the presence and absence of nucleotide by a mechanism that, as discussed below, is not well understood at present.

Stimulation of Ca^{2+} release by Ca^{2+}, adenine nucleotide, and caffeine and inhibition of release by Mg^{2+} and high concentrations of Ca^{2+} have been reported by several laboratories (Stephenson, 1981; Onishi, 1981; Winegrad, 1982; Yamamoto & Kasai, 1982; Miyamoto & Racker, 1982; Kirino et al., 1983; Morii & Tonomura, 1983; Nagasaki & Kasai, 1983; Kim et al., 1983; Meissner, 1984). In most of the previous vesicle studies, Ca^{2+} release was slow, i.e., less than 1% of the estimated in vivo rate. Ikemoto et al. (1984) recently reported that T-tubule depolarization triggers rapid Ca^{2+} release (10–20 nmol/mg of protein, $k_1 \sim 100$ s^{-1}) from an actively loaded vesicle fraction containing "triads" (T-tubule/SR junctions). In a preliminary presentation, it was reported that ATP also stimulated rapid release of Ca^{2+} from this triad fraction (Ikemoto & Antoniu, 1985). Nagasaki & Kasai (1983) used the fluorescent indicator dye chlorotetracycline to follow rapid Ca^{2+} release from passively loaded vesicles and estimated a maximal release rate constant of 80 s^{-1} in the presence of 12.5 mM caffeine and 10 mM ATP. In the absence of caffeine, the ATP-stimulated Ca^{2+} release rate constant was 20 s^{-1}; this latter constant is somewhat lower than the rate constant of 30–100 s^{-1} observed in the presence of 5 mM AMP-PCP in this study.

Regulation of the SR Ca^{2+} release channel by Ca^{2+}, adenine nucleotide, and Mg^{2+} has been previously investigated. Using a light-scattering method, Yamanouchi et al. (1984) found that at free Ca^{2+} concentrations ranging from 2×10^{-7} to 10^{-5} M, ATP activation of choline(1+) influx into SR vesicles followed a single-site titration curve with a dissociation constant of about 1 mM. In contrast, our data suggest cooperative binding of ATP to the SR Ca^{2+} release channel, as indicated by a Hill coefficient of 1.6–1.9 for ATP and AMP-PCP activation of Ca^{2+} release at 10^{-9} and 4×10^{-6} M free Ca^{2+}. Our observation that ATP activates the channel at very low concentrations of Ca^{2+} is of interest since it indicates that the channel can be partially activated by either Ca^{2+} or adenine nucleotide.

In agreement with a previous report (Yamamoto & Kasai, 1982), Ca^{2+} efflux by the ligand-free E_0 form of the channel was activated by Ca^{2+} with a Hill coefficient of 1 (Figure 2A). Kirino et al. (1983) reported a Hill coefficient of 1.2. In the absence of Mg^{2+} and nucleotide, the channel therefore does not appear to be regulated by interacting activating Ca^{2+} binding sites. The addition of a second ligand such as caffeine, adenine nucleotide, or Mg^{2+} enhanced or decreased the Ca^{2+} release rate, and in the case of caffeine and Mg^{2+} changed the affinity of the Ca^{2+} activation sites for Ca^{2+}. However, none of the ligands when added alone was reported to have an appreciable effect on the Hill coefficient of Ca^{2+}-activated Ca^{2+} release (Nagasaki & Kasai, 1983; Kirino et al., 1983). In contrast, there occurs, as shown in this study, a marked increase in the cooperativity of Ca^{2+} activation of Ca^{2+} release when the vesicles are diluted into a Mg^{2+}- and nucleotide-containing medium. That nucleotides and Mg^{2+} render the channel sensitive to external Ca^{2+} in a quite narrow concentration range is probably biologically important and provides a clue for a role of ATP and Mg^{2+} in the process of Ca^{2+}-induced Ca^{2+} release by SR. Exactly how ATP and Mg^{2+} increase the cooperativity of channel activation by Ca^{2+} is not

clear at present. One possible explanation is that the Mg–AMP-PCP complex, which forms in the presence of millimolar concentrations of AMP-PCP and Mg^{2+}, is the physiological effector of the channel. This possibility is supported by the fact that in muscle most of the ATP is present in the form of a Mg–ATP complex (Gupta & Moore, 1980).

We are aware of only one other group (Morii & Tonomura, 1983) that has carried out a detailed study of the kinetics of Ca^{2+} activation of Ca^{2+} release in the presence of adenine nucleotide and Mg^{2+}. Measurement of the Ca^{2+}-releasing action of AMP in the presence of 15 μM Ca^{2+} and 5 mM Mg^{2+} at 0 °C indicated an apparent dissociation constant of 2 mM for AMP and a Hill coefficient of 1. The amount of Ca^{2+} released decreased from 60% to 0 as the free Ca^{2+} concentration in the release medium was decreased from 2.4×10^{-7} to 6×10^{-8} M. These results led Morii and Tonomura to suggest the presence of a channel which is inactive in the absence of nucleotide. Binding of one molecule of nucleotide shifted the equilibrium toward an active but closed form of the channel. Different external Ca^{2+} concentrations were required to open a heterogeneous population of activated channels in an all or none fashion. Our results do not support their model. We observed that adenine nucleotide activated the channel at Ca^{2+} concentrations of less than 10^{-8} M. Further, the rate but not the total amount of Ca^{2+} release depended on external Ca^{2+} concentration. These observations would seem to favor a model in which all channels are capable of opening at low Ca^{2+} concentrations, where, however, frequency and/or duration of channel opening are regulated by external Ca^{2+} concentration.

Ca^{2+} release from SR vesicles is inhibited by Mg^{2+} in at least two ways. One effect of Mg^{2+} is to increase Ca^{2+} concentrations required to maximally activate Ca^{2+} release in the presence or absence of caffeine (Nagasaki & Kasai, 1983; Kirino et al., 1983) or adenine nucleotide (Figure 3). Mg^{2+} also drastically reduces the maximal Ca^{2+} release rates seen in the presence or absence of micromolar concentrations of external Ca^{2+} or millimolar concentrations of nucleotide. Mg^{2+} has been suggested to inhibit Ca^{2+} release by binding competitively to the Ca^{2+} activating sites (Nagasaki & Kasai, 1983), by decreasing their apparent affinity (Kirino et al., 1983), or by acting as a blocker of the channel as it binds to the transport sites of the channel (Yamamoto & Kasai, 1982). Another possibility that cannot be ruled out at present is that Mg^{2+} binds to external low-affinity regulatory sites distinct from the Ca^{2+} activation and transport sites of the channel.

Whether Mg^{2+} and elevated concentrations of Ca^{2+} inhibit Ca^{2+} release by acting at interacting or noninteracting sites has remained controversial (Yamamoto & Kasai, 1982; Nagasaki & Kasai, 1983; Kirino et al., 1983). Our study shows that the cooperativity of Mg^{2+} inhibition is dependent on external Ca^{2+} concentration (Table II). At 10^{-9} M Ca^{2+}, Ca^{2+} efflux was inhibited half-maximally at 2×10^{-5} M Mg^{2+} with a Hill coefficient of 1.1. In the presence of 5×10^{-6} M Ca^{2+}, a 5-fold higher Mg^{2+} concentration was required to half-maximally inhibit Ca^{2+} release with a Hill coefficient of 1.55. Our explanation for the differences in the effectiveness and cooperativity of Mg^{2+} inhibition is that at low external Ca^{2+} concentration, Mg^{2+} inhibits the channel primarily by binding to noninteracting activating Ca^{2+} binding sites. By comparing the half-maximally activating Ca^{2+} and inactivating Mg^{2+} concentrations, it is estimated that Ca^{2+} activating sites favor Ca^{2+} binding over Mg^{2+} by a factor of about 40. Occupation of the activating sites by Ca^{2+} changes the pattern of Mg^{2+} inhibition, in part because, at elevated Mg^{2+} concentrations, binding to additional low-affinity sites appears to become important. That Mg^{2+} binds to additional sites of the channel is supported by the observation that the inhibitory effects of Mg^{2+} are closely matched by elevated concentrations of Ca^{2+} (Figure 4). Exactly how Mg^{2+} inhibits Ca^{2+}-induced Ca^{2+} release is not clear at present and may require combining vesicle studies with single channel recordings using the lipid bilayer technique. The bilayer studies can reveal more direct information about the process of channel inhibition, such as whether Mg^{2+} acts primarily by binding to the transport sites or to regulatory sites on the channel.

In the absence of Mg^{2+}, heavy SR vesicles released $^{45}Ca^{2+}$ with a maximal first-order rate constant of 30–100 s^{-1}. By comparison, in frog skeletal muscle following an action potential, Ca^{2+} has been estimated to be released with a rate constant of about 50–100 s^{-1} (Baylor et al., 1983; Melzer et al., 1984). However, Ca^{2+} release in muscle likely occurs in the presence of significant albeit unknown concentrations of free Mg^{2+}. When conditions considered to approximate those in frog muscle [0.2–4 mM free Mg^{2+} (Gupta & Moore, 1980; Baylor et al., 1982), 5 μM free Ca^{2+}, and 5 mM nucleotide (Kushmerick, 1983)] were used, $^{45}Ca^{2+}$ was released from rabbit skeletal muscle SR vesicles at 22 °C with a rate constant ranging from about 25 to 1 s^{-1} (Table III). At 37 °C, the rate constants are probably 2–3 times greater than at 22 °C. Heavy SR vesicles therefore demonstrate Ca^{2+}- and nucleotide-induced Ca^{2+} release rates which, at least in the presence of low free Mg^{2+} concentrations, appear to approach those in muscle.

Registry No. AMP-PCP, 3469-78-1; ATP, 56-65-5; Ca, 7440-70-2; Mg, 7439-95-4.

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Evidence of a Role for Calmodulin in the Regulation of Calcium Release from Skeletal Muscle Sarcoplasmic Reticulum†

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ABSTRACT: The effect of calmodulin and calmodulin inhibitors on the "Ca^{2+} release channel" of "heavy" skeletal muscle sarcoplasmic reticulum (SR) vesicles was investigated. SR vesicles were passively loaded with $^{45}Ca^{2+}$ in the presence of calmodulin and its inhibitors, followed by measurement of $^{45}Ca^{2+}$ release rates by means of a rapid-quench–Millipore filtration method. Calmodulin at a concentration of 2–10 μM reduced $^{45}Ca^{2+}$ efflux rates from passively loaded vesicles by a factor of 2–3 in media containing 10^{-6}–10^{-3} M Ca^{2+}. At 10^{-9} M Ca^{2+}, calmodulin was without effect. $^{45}Ca^{2+}$ release rates were varied 1000-fold ($k_1 \simeq 0.1$–100 s^{-1}) by using 10^{-5} M Ca^{2+} with either Mg^{2+} or the ATP analogue adenosine 5′-(β,γ-methylenetriphosphate) in the release medium. In all instances, a similar 2–3-fold reduction in release rates was observed. At 10^{-5} M Ca^{2+}, $^{45}Ca^{2+}$ release was half-maximally inhibited by about 2×10^{-7} M calmodulin, and this inhibition was reversible. Heavy SR vesicle fractions contained 0.1–0.2 μg of endogenous calmodulin/mg of vesicle protein. However, the calmodulin inhibitors trifluoperazine, calmidazolium, and compound 48/80 were without significant effect on $^{45}Ca^{2+}$ release at concentrations which inhibit calmodulin-mediated reactions in other systems. Studies with actively loaded vesicles also suggested that heavy SR vesicles contain a Ca^{2+} permeation system that is inhibited by calmodulin.

Muscle contracts when the free Ca^{2+} concentration of the myofibrillar space reaches 10^{-6}–10^{-5} M by the release of Ca^{2+} stores from sarcoplasmic reticulum (SR)[1] via a putative Ca^{2+} channel (Ebashi, 1976; Endo, 1977; Winegrad, 1982). Muscle relaxes again when Ca^{2+} concentration is decreased below 10^{-7} M through the action of the membrane-bound Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase or Ca^{2+} pump of SR (Tada et al., 1978; Ikemoto, 1982; Martonosi & Beeler, 1983; Inesi, 1985). Release of Ca^{2+} by SR is triggered by an action potential at the neuromuscular junction that is communicated to SR via an extension of the surface membrane, the T system. Although the molecular basis of T-system depolarization-induced Ca^{2+} release by SR has remained obscure, recent studies have indicated the presence of a ligand-gated SR Ca^{2+} release channel which is activated by Ca^{2+} and adenine nucleotides and inhibited by Mg^{2+} (Stephenson, 1981; Onishi, 1981; Yamamoto & Kasai, 1982; Miyamoto & Racker, 1982; Fabiato, 1983; Kirino et al., 1983; Morii & Tonomura, 1983; Nagasaki & Kasai, 1983; Kim, et al., 1983; Meissner, 1984; Meissner et al., 1986).

The influence of calmodulin on Ca^{2+} uptake and release in SR has been studied primarily by using isolated vesicle fractions. In cardiac muscle, Ca^{2+}-stimulated ATPase and Ca^{2+} uptake activities are stimulated by calmodulin- and cAMP-dependent protein kinases. Both kinases exert their activating effects by phosphorylation of a 22 000-dalton protein called phospholamban (Tada et al., 1982). Skeletal muscle SR vesicles also have been found to contain a calmodulin-dependent protein kinase as well as calmodulin; however, activation of the Ca^{2+} transport system by calmodulin has not yet been shown (Campbell & MacLennan, 1981; Chiesi & Carafoli, 1982, 1983; Seiler et al., 1984; Eibschutz et al., 1984).

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[1] Abbreviations: SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; AMP-PCP, adenosine 5′-(β,γ-methylenetriphosphate); Pipes, 1,4-piperazinediethanesulfonic acid; cAMP, adenosine cyclic 3′,5′-phosphate.

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