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Calcium-induced Calcium Release from Purified Cardiac Sarcoplasmic Reticulum Vesicles

GENERAL CHARACTERISTICS*

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Isolated canine cardiac sarcoplasmic reticulum exhibits Ca^{2+}-induced Ca^{2+} release from both actively and passively loaded vesicles. The rate and extent of Ca^{2+} release depend on the extravesicular ionized Ca^{2+} concentration ($[Ca^{2+}]_o$) at the onset of release. Maximal release following ATP-dependent, phosphate-facilitated Ca^{2+} loading (up to 360 nmol of Ca^{2+}/mg of protein/min at 37 °C) occurs at 1.5–2 μM $[Ca^{2+}]_o$, with reduced release at both lower and higher Ca^{2+} concentrations (half-maximal Ca^{2+} release at approximately 0.8 and 5.5 μM $[Ca^{2+}]_o$). Only a portion of the accumulated Ca^{2+} is released and the release is followed by reuptake of Ca^{2+}. A similar Ca^{2+} dependence is obtained in the absence of ATP and P_i by measuring unidirectional Ca^{2+} efflux from passively loaded vesicles (maximal Ca^{2+} efflux at 1 μM $[Ca^{2+}]_o$; half-maximal Ca^{2+}-dependent efflux at approximately 0.15 and 13 μM $[Ca^{2+}]_o$). Although the Ca^{2+} release rates observed in this study are several orders of magnitude lower than the rate of Ca^{2+} release which occurs in muscle cells *in vivo*, this Ca^{2+} release phenomenon may be related to the Ca^{2+}-induced Ca^{2+} release which has been described for skinned cardiac cells (Fabiato, A. (1983) *Am. J. Physiol.* 245, C1–C14).

Ca^{2+} release occurs in the presence of an ATP-regenerating system and is not accompanied by a reduction in ATP hydrolysis. Also, since unidirectional Ca^{2+} efflux (as high as 860 nmol of Ca^{2+}/mg of protein/min at 37 °C) exceeds net Ca^{2+} release under similar conditions, Ca^{2+} influx proceeds during the period of net Ca^{2+} release. Therefore, Ca^{2+} release does not involve reversal or cessation of inward Ca^{2+} pumping. Other data indicate that Ca^{2+} release is not mediated through the Ca^{2+} pump protein, but occurs through a separate Ca^{2+}-dependent efflux pathway, possibly a channel.

The SR[1] is a specialized membrane system in muscle which regulates the myoplasmic Ca^{2+} level thereby controlling muscle contraction and relaxation (2). Release of Ca^{2+} from the SR triggers contraction and active Ca^{2+} reuptake by the SR produces relaxation. Ca^{2+} uptake, mediated by a membrane-bound Ca^{2+} pump protein, has been extensively studied for both cardiac and skeletal muscle SR (2–5). On the other hand, the mechanism by which depolarization of the surface membrane causes Ca^{2+} release from the SR is not well understood (6, 7).

Additional complexity is present in the heart where systems in the sarcolemma such as a "slow" inward Ca^{2+} channel (8, 9), a Ca^{2+} pump (10–12), and a Na^+-Ca^{2+} exchanger (13) also participate in the regulation of intracellular Ca^{2+} levels. Studies on skinned cardiac cells indicate that in the mammalian heart, the trans-sarcolemmal Ca^{2+} influx is insufficient to activate the myofilaments and that Ca^{2+} release from the SR is essential for contraction (14, 15). Fabiato postulates that the trans-sarcolemmal Ca^{2+} influx acts as a trigger to induce Ca^{2+} release from the SR and has amassed convincing evidence for this hypothesis of Ca^{2+}-induced Ca^{2+} release using skinned heart cells (14, 15). In this report, we describe a Ca^{2+}-induced Ca^{2+} release from isolated cardiac SR vesicles with a Ca^{2+} concentration response similar to that described for skinned heart fibers.

EXPERIMENTAL PROCEDURES

Antipyrylazo III, Na_2ATP, Na_2-phosphocreatine, creatine phosphokinase type I, NADH, phosphoenolpyruvate, pyruvate kinase, and lactic dehydrogenase were from Sigma. $^{45}CaCl_2$ was obtained from New England Nuclear and from ICN Pharmaceuticals, Inc.

Cardiac SR was isolated from canine ventricles as previously described (16) and stored in liquid N_2. Net Ca^{2+} uptake and release were observed by dual wavelength spectrophotometry, using the metallochromic indicator antipyrylazo III (17) and monitoring ΔA ($A_{710} - A_{790}$) with a Hewlett-Packard model 8450A spectrophotometer. Antipyrylazo III was less inhibitory than arsenazo III in the Ca^{2+} loading assay; *i.e.* at indicator concentrations producing adequate signal-to-noise characteristics, loading rates measured in the presence of antipyrylazo III were higher than those determined in the presence of arsenazo III. At 275 μM antipyrylazo III, Ca^{2+} loading rates were reduced only 5–10% and the patterns of Ca^{2+} uptake and release were essentially unaltered, when compared to results at threshold concentrations of antipyrylazo III (100–200 μM) where the signal-to-noise characteristics were poor. The wavelength pair ($A_{710} - A_{790}$) was optimal for following Ca^{2+} uptake and release under our assay conditions and light scattering changes in normal differential absorbance measurements (at 180° with respect to the incident light and without dye) were insignificant compared to the dye-related signals at the low sample concentrations employed. The standard 1-ml uptake/release assay contained 35–40 μg of SR protein, 1 mM Na_2ATP, 1 mM $MgSO_4$, 3 mM Na_2-phosphocreatine, 20 μg (2–3 units) of creatine phosphokinase, and 275 μM antipyrylazo III in 120 mM K^+, 75 mM P_i, pH 7.0 at 37 °C. Ca^{2+} uptake was initiated by the addition of Ca^{2+} as indicated in the figures.

For each experiment, a calibration was performed to correlate

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[1] The abbreviations used are: SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; $[Ca^{2+}]_o$, extravesicular ionized Ca^{2+} concentration; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

absorbance changes with extravesicular ionized Ca^{2+} concentration, $[Ca^{2+}]_0$, by adding varying amounts of $CaCl_2$ to the standard assay mixture and noting the absorbance change. For each *total* $CaCl_2$ concentration, the *ionized* Ca^{2+} concentration was determined using the association constants and calculations previously described (18). In addition, binding of Ca^{2+} and Mg^{2+} to antipyrylazo III was included in the calculations. An apparent stability constant of 8330 M^{-1} for the Ca^{2+}-antipyrylazo III complex used (17). A median value of 3000 M^{-1} was used for the apparent stability constant of the Mg^{2+}-antipyrylazo III complex (19, 20).

ATPase values were determined spectrophotometrically, by monitoring the oxidation of NADH in a coupled enzyme system (21). This assay required a variant of the standard uptake/release medium containing an alternate ATP-regenerating system. A 1-ml assay contained 35–40 μg of SR protein, 1 mM Na_2ATP, 1 mM $MgSO_4$, 0.2 mM NADH, 2 mM phosphoenolpyruvate, 8.75 units of pyruvate kinase, and 12.5 units of lactic dehydrogenase in 120 mM K^+, 75 mM P_i, pH 7.0 at 37 °C. For the paired experiments of Fig. 3, Ca^{2+} uptake and release were determined in this medium containing 275 μM antipyrylazo III. Reactions were initiated by the addition of $CaCl_2$. The "basal" ATPase measured in the presence of 4 mM EGTA was subtracted from the total ATPase to give Ca^{2+}-dependent ATPase.

In the estimation of unidirectional Ca^{2+} efflux from actively loaded SR vesicles, uptake of $^{45}CaCl_2$ in 2 ml of the standard uptake/release assay medium was monitored spectrophotomterically. At the onset of Ca^{2+} release, a $^{40}CaCl_2$/EGTA buffer containing an excess of $^{40}CaCl_2$ was added to fix $[Ca^{2+}]_0$ at 2×10^{-8} M, 2×10^{-6} M, or 2×10^{-4} M at a constant pH of 7.0. Concentrated buffer (40 μl) was added to give final concentrations of 0.5 mM $CaCl_2$, 10.5 mM EGTA; 3 mM $CaCl_2$, 3.58 mM EGTA; or 3 mM $CaCl_2$, 2.02 mM EGTA, respectively. At intervals thereafter, 100-μl aliquots were removed from the cuvette and rapidly filtered through 0.2-μm pore size nitrocellulose filters in a microfilter kit (Schleicher and Schuell) using air pressure. ^{45}Ca in the filtrate was monitored to estimate Ca^{2+} efflux.[2] The specific activity (approximately 12,000 cpm/nmol) was determined from the ^{45}Ca *inside* the SR (measured isotopically) and from the total Ca^{2+} accumulated by the SR (determined spectrophotometrically) at the onset of release prior to adding to $^{40}CaCl_2$/EGTA buffer. When $[Ca^{2+}]_0$ was fixed at 2×10^{-8} M, where Ca^{2+} uptake is negligible, ^{45}Ca was diluted 6- to 7-fold with ^{40}Ca. When $[Ca^{2+}]_0$ was fixed at 2×10^{-6} M and 2×10^{-4} M, where considerable Ca^{2+} uptake can occur, ^{45}Ca was diluted 31- to 35-fold with ^{40}Ca. Thus, reuptake of ^{45}Ca would be reduced by the same factor and the increase in extravesicular ^{45}Ca is a reasonable estimate of unidirectional Ca^{2+} efflux. Although EGTA is not an efficient buffer of ionized Ca^{2+} at 2×10^{-4} M, the total extravesicular $CaCl_2$ changed by less than 1% during the efflux measurements at 2×10^{-4} M Ca^{2+}, so the ionized Ca^{2+} concentration remained within 5% of 2×10^{-4} M.

To determine Ca^{2+} efflux from passively loaded SR in the absence of ATP and P_i, SR vesicles (4.7–6.7 mg/ml) in 0.29 M sucrose, 0.2 M KCl, 10 mM imidazole-HCl (pH 6.7 at 25 °C) were passively loaded with Ca^{2+} by incubating for 16–18 h at 0 °C in 2 mM $^{45}CaCl_2$ (9000 cpm/nmol). Efflux was initiated by adding 0.11 ml of this mixture to 9.9 ml of 0.3 M sucrose, 0.1 M KCl, 5 mM K_2-EGTA, 20 mM Hepes, pH 7.0, at 25 °C containing various concentrations of $MgCl_2$ and $^{40}CaCl_2$ to produce a constant ionized Mg^{2+} concentration of 0.1 mM at ionized Ca^{2+} concentrations ranging from 10^{-9} M to 10^{-4} M. After mixing, 0.95-ml aliquots were withdrawn and rapidly filtered through 0.22-μm pore size Millipore filters. The filters were immediately washed with 2 ml of ice-cold 10 mM $LaCl_3$, 20 mM $MgCl_2$ (22). After drying, the filters were dissolved and counted in 10 ml of Bray's solution (Research Products International Corp., Mount Prospect, IL). The first order rate constant of the *fast initial component* (first 35 s) of the Ca^{2+} efflux was determined by linear regression analysis of a semilog plot of ^{45}Ca remaining in the SR *versus* time (*cf.* Fig. 5A).

RESULTS

Ca^{2+} Release from Actively Loaded SR—Ca^{2+}-induced Ca^{2+} release from cardiac SR vesicles is shown in Fig. 1. In this experiment, the SR was preloaded with three additions of

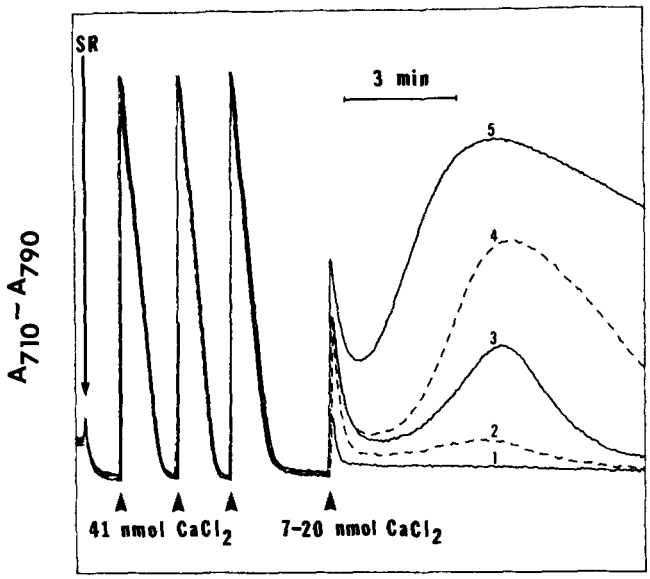

FIG. 1. **Ca^{2+}-induced Ca^{2+} release from cardiac SR vesicles.** In figures where Ca^{2+} uptake and release were determined spectrophotometrically, the absorbance is proportional to $[Ca^{2+}]_0$. Thus, net Ca^{2+} uptake by the SR is accompanied by a *decrease* in absorbance and net Ca^{2+} release is reflected by an *increase* in absorbance. The data are from five separate reactions. At the *arrow*, SR (35 μg of protein) was added to standard uptake/release medium (final volume after addition of $CaCl_2$: 1 ml) at 37 °C as described under "Experimental Procedures" and allowed to accumulate contaminating Ca^{2+} from the medium (approximately 4 nmol). Following three additions of 41 nmol of $CaCl_2$ (*arrowheads*), the amount of $CaCl_2$ in a fourth addition was successively increased in the separate reactions from 7 (*trace 1*) to 20 nmol (*trace 5*). For all but the smallest of these additions, net Ca^{2+} uptake ceased before all the Ca^{2+} was accumulated by the SR and a period of net Ca^{2+} release ensued. After 2–3 min of Ca^{2+} release, Ca^{2+} reuptake by the SR occurred.

$CaCl_2$. The $CaCl_2$ in a fourth addition was varied so that net Ca^{2+} uptake ceased and net Ca^{2+} release began at varying $[Ca^{2+}]_0$. The rate and extent of release depended on $[Ca^{2+}]_0$ at the onset of release for SR vesicles loaded to the same capacity (Table I). After 2–3 min of Ca^{2+} release, Ca^{2+} reuptake by the SR occurred (Fig. 1).

In this assay, Ca^{2+} release occurs only after preloading to a specific capacity (*e.g.* see Table I) which varied among several SR preparations (2.6–4.7 μmol of Ca^{2+}/mg of protein). However, the rate and extent of release do not depend upon the value of this "trigger" capacity (data not shown). Rather, Ca^{2+} release is dependent upon $[Ca^{2+}]_0$ after each preparation accumulates $CaCl_2$ to its trigger capacity. This capacity is not the maximum Ca^{2+} loading capacity, since under defined conditions the SR can accumulate Ca^{2+} to at least twice this level (see "Discussion").

The dependence of the rate of Ca^{2+} release on $[Ca^{2+}]_0$ at the onset of release is presented in Fig. 2. A similar curve is obtained for the extent of release (data not shown). Maximum net release (up to 360 nmol of Ca^{2+}/mg of protein/min) occurs when $[Ca^{2+}]_0$ is 1.5–2 μM. Ca^{2+} release declines at both lower and higher $[Ca^{2+}]_0$. Under optimal conditions, as much as 20% of the accumulated Ca^{2+} is released prior to reuptake.

Ca^{2+} release is not dependent upon depletion of ATP since it occurs in the presence of an ATP-regenerating system (see "Experimental Procedures") and reuptake of released Ca^{2+} is observed (Fig. 1). Ca^{2+} release does not appear to be mediated by reversal of Ca^{2+} pumping since this process (in skeletal muscle SR) requires $[Ca^{2+}]_0$ less than 0.1 μM and ADP in excess over ATP (23). The Ca^{2+} release described here is

[2] In control experiments (in the absence of EGTA), simultaneous monitoring of Ca^{2+} loading by the spectrophotomteric procedure and by filtration gave identical values, indicating that the filtration procedure completely removed SR vesicles and the intravesicular Ca^{2+}.

TABLE I

Ca^{2+}-induced Ca^{2+} release from cardiac SR vesicles

The data are derived from Fig. 1. For each trace in the figure, the extravesicular ionized Ca^{2+} concentration, $[Ca^{2+}]_o$, at the onset of release was determined as described under "Experimental Procedures" using the absorbance value at the point when net Ca^{2+} uptake ceases and net Ca^{2+} release begins. Also, for each trace in Fig. 1, the *maximum rate of change* of absorbance (dA/dt during the 1–2 min when it is constant) and the *maximum extent* of the absorbance change (ΔA) following the onset of net release were determined and converted to units of Ca^{2+} as described under "Experimental Procedures" to yield, respectively, the maximum release rate and the maximum release extent. The values are for *net* Ca^{2+} release and reflect the summation of unidirectional influx and efflux. The rate and extent of net Ca^{2+} release are dependent upon $[Ca^{2+}]_o$ for SR loaded to approximately the same total capacity.

Trace in Fig. 1	Capacity at onset of release	$[Ca^{2+}]_o$ at onset of release	Maximum release	
			Rate	Extent
	μmol Ca^{2+}/mg protein	*μM*	*nmol Ca^{2+}/mg/min*	*nmol Ca^{2+}/mg*
1	3.82	0.26[a]	0	0
2	3.97	0.55	18	43
3	3.95	0.79	128	261
4	3.94	0.92	262	526
5	3.87	1.99	291	594

[a] $[Ca^{2+}]_o$ at the absorbance baseline was calculated to be 0.26 μM by a parallel loading in the presence of $^{45}CaCl_2$ and measuring extravesicular ^{45}Ca following filtration.

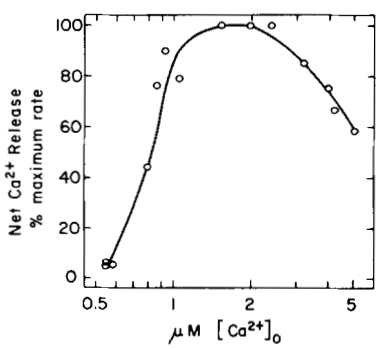

FIG. 2. **Net Ca^{2+} release at various $[Ca^{2+}]_o$.** The data are from three experiments similar to that described in Fig. 1 and Table I, using two different cardiac SR preparations. The *rate* of net Ca^{2+} release, expressed as a *percentage* of the maximal value at optimum $[Ca^{2+}]_o$, is ploted *versus* the $[Ca^{2+}]_o$ at the *onset* of release. $[Ca^{2+}]_o$ and the rates of net Ca^{2+} release were calculated as described in the legend to Table I. For these experiments, the 100% values at 1.5–2 μM $[Ca^{2+}]_o$ varied between 240 and 290 nmol of Ca^{2+}/mg of protein/min. Half-maximal release occurred at approximately 0.8 and 5.5 μM $[Ca^{2+}]_o$.

optimal at 1.5–2 μM $[Ca^{2+}]_o$ and occurs in 1 mM ATP in the presence of a regenerating system to prevent ADP accumulation. Furthermore, as shown in Fig. 3, the Ca^{2+}-dependent ATPase rate is not diminished during Ca^{2+} release, but increases somewhat over the rate observed during Ca^{2+} preloading.

Unidirectional Ca^{2+} Efflux from Actively Loaded SR—The data thus far have been for *net Ca^{2+} release*. We also measured *unidirectional ^{45}Ca efflux* as a function of fixed $[Ca^{2+}]_o$ (Fig. 4). Two preloading conditions were chosen to vary $[Ca^{2+}]_o$ at the onset of release prior to adding $CaCl_2$/EGTA buffers to fix $[Ca^{2+}]_o$. In Fig. 4*A*, $[Ca^{2+}]_o$ at the onset of release was 1.9 μM, near the optimal for net Ca^{2+} release (see Fig. 2). In Fig. 4*B*, $[Ca^{2+}]_o$ at the onset of release was 0.6 μM, near the minimum threshold for inducing Ca^{2+} release. For both conditions, $[Ca^{2+}]_o$ was fixed immediately after the onset of

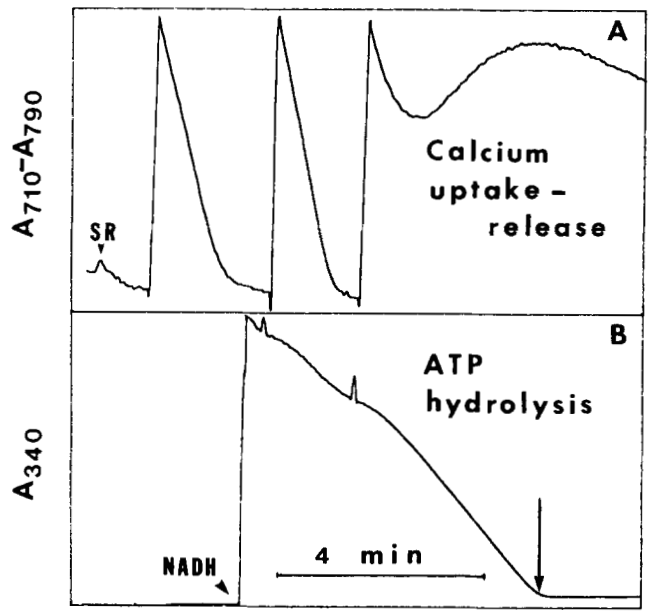

FIG. 3. **ATP hydrolysis during Ca^{2+} uptake and release.** In paired experiments, net Ca^{2+} uptake and release (*A*) and ATP hydrolysis, *i.e.* oxidation of NADH (*B*), were monitored as described under "Experimental Procedures." SR (38 μg of protein) was allowed to accumulate contaminating Ca^{2+} in the medium prior to three successive additions of 40 nmol of $CaCl_2$. Ca^{2+} release occurred after the third addition of $CaCl_2$. NADH (0.2 mM) was not added until after the first addition of $CaCl_2$ had been accumulated by the SR in order to ensure that there would be sufficient NADH to monitor ATP hydrolysis during the Ca^{2+} release phase. At the *arrow*, A_{340} levels off because the NADH has been depleted, not because ATP hydrolysis has ceased (as confirmed in a parallel experiment in which NADH was not added until after the second addition of $CaCl_2$ had been accumulated by the SR). The rates of ATP hydrolysis were 1.10 and 1.32 μmol/mg/min during Ca^{2+} preloading and during Ca^{2+} release, respectively.

release at 2×10^{-8} M, 2×10^{-6} M, and 2×10^{-4} M.

As observed for net release, unidirectional Ca^{2+} efflux is dependent upon $[Ca^{2+}]_o$. For both preloading conditions, maximum efflux occurred when $[Ca^{2+}]_o$ was fixed at 2×10^{-6} M, with the initial efflux rates at 2×10^{-8} M and 2×10^{-4} M, approximately 35% and 15%, respectively, of the maximum rate. Moreover, the initial rate of unidirectional Ca^{2+} efflux (856 nmol/mg/min) at 2 μM $[Ca^{2+}]_o$ exceeded that of net release (325 nmol/mg/min) at 1.9 μM $[Ca^{2+}]_o$ (Fig. 4*A*) indicating that Ca^{2+} influx occurs during release. The difference between net Ca^{2+} release and unidirectional Ca^{2+} efflux indicates that Ca^{2+} pumping proceeds at about 60% of the rate measured during preloading.

The unidirectional Ca^{2+} effluxes are similar for preloading conditions which were chosen, respectively, to maximize (Fig. 4*A*) and minimize (Fig. 4*B*) net Ca^{2+} release. The control traces verify that this goal was achieved. However, when $[Ca^{2+}]_o$ is fixed soon after the onset of release, Ca^{2+} efflux depends upon the new fixed $[Ca^{2+}]_o$ and not upon the $[Ca^{2+}]_o$ at the onset of release. Thus, under these experimental conditions, the Ca^{2+} release process does not have a "memory" as has been suggested (24).

Ca^{2+} Efflux from Passively Loaded SR—We next studied Ca^{2+} efflux from SR vesicles passively loaded with Ca^{2+} in the absence of ATP and P_i. This Ca^{2+} efflux was also dependent upon $[Ca^{2+}]_o$ (Fig. 5). Since the Ca^{2+} efflux rate in this assay is dependent upon the Ca^{2+} gradient across the SR membrane, the results (Fig. 5*B*) are expressed as the first order rate constant. The maximum rate constant for Ca^{2+} efflux (k =

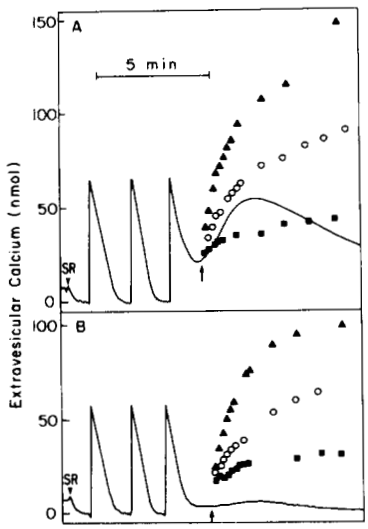

FIG. 4. **Unidirectional Ca^{2+} efflux from actively loaded SR at different $[Ca^{2+}]_0$.** SR vesicles (71 μg of protein) were loaded with $^{45}CaCl_2$ in 2 ml of the standard uptake/release assay medium. The *lines* are spectrophotometric traces of $A_{710} - A_{790}$ indicating net Ca^{2+} uptake and release by control samples. The rates of Ca^{2+} preloading are 942 and 915 nmol of Ca^{2+}/mg of protein/min for *traces A* and *B*, respectively. The *symbols* are isotopically determined values of extravesicular $CaCl_2$ following addition of $CaCl_2$/EGTA buffers as described under "Experimental Procedures." *A*, after three successive additions of 65 nmol of $CaCl_2$, net Ca^{2+} release (325 nmol of Ca^{2+}/mg of protein/min) began at 1.91 μM $[Ca^{2+}]_0$. *B*, after three successive additions of 57.5 nmol of $CaCl_2$, net Ca^{2+} release (21 nmol of Ca^{2+}/mg of protein/min) began at 0.59 μM $[Ca^{2+}]_0$. For both *A* and *B*, $^{40}CaCl_2$/EGTA buffers were added immediately after the onset of release (*arrows*) to fix $[Ca^{2+}]_0$ at 2×10^{-8} M (○), 2×10^{-6} M (▲), or 2×10^{-4} M (■). When $[Ca^{2+}]_0$ was fixed at 2×10^{-6} M, approximately 60% of the accumulated ^{45}Ca was released within 3.5 min.

1.1–1.3 min^{-1}) is observed at 1 μM $[Ca^{2+}]_0$. Ca^{2+} efflux is slower at both higher and lower $[Ca^{2+}]_0$, with a portion of this efflux (that observed at 1 nM $[Ca^{2+}]_0$) apparently independent of $[Ca^{2+}]_0$.

Inhibition of Ca^{2+}-induced Ca^{2+} Release by Mg^{2+}—The data in Fig. 6 demonstrate that net Ca^{2+} release from actively loaded SR is inhibited by increasing total magnesium from 1 to 4 mM, resulting in an increase in ionized Mg^{2+} from 0.12 to 0.74 mM. Reduced Ca^{2+} release could result from increased inward Ca^{2+} pumping. However, no increase in Ca^{2+} pumping is observed. Rather, a small decrease in the Ca^{2+} loading rate is seen at the higher Mg^{2+} concentration (Fig. 6). A similar Mg^{2+} inhibition was also demonstrated for unidirectional Ca^{2+} efflux from passively loaded vesicles in the absence of Ca^{2+} pumping (Table II). Elevation of ionized Mg^{2+} from 0.10 to 0.72 mM at a constant 1 μM $[Ca^{2+}]_0$ reduces Ca^{2+} efflux to the level previously seen at 1 nM $[Ca^{2+}]_0$ which is presumed to be Ca^{2+}-independent efflux.

Dependence of Ca^{2+}-induced Release upon P_i—Net Ca^{2+} release is pronounced after energized Ca^{2+} accumulation in the presence of 75 mM P_i as a precipitating anion, but it is significantly reduced in 50 mM P_i and does not occur in 25 mM P_i (Fig. 7). This effect could be attributed to either a direct effect of the P_i concentration or upon a higher intravesicular ionized Ca^{2+} concentration at lower P_i concentrations.

It is likely that the requirement for high P_i is a peculiarity of the assay for Ca^{2+} release from actively loaded SR and not a requirement for Ca^{2+} release, *per se*. Ca^{2+} release from passively loaded cardiac SR (in the absence of P_i) shares many similarities with Ca^{2+} release from actively loaded SR including Ca^{2+} dependence (compare Figs. 2 and 5*B*), inhibition by Mg^{2+} (compare Fig. 6 and Table II), and inhibition by a wide variety of chemical compounds (see accompanying report, Ref. 25). These similarities suggest that the two assays reflect Ca^{2+} efflux through identical pathways or "channel(s)," and that the Ca^{2+}-dependent opening of the channel(s) can occur in the absence of P_i. Thus, although it is doubtful that the release from actively loaded SR which occurs only with high P_i concentrations is itself a "physiological" process, the physiological Ca^{2+} release may involve the same Ca^{2+} efflux pathway.

DISCUSSION

Purified cardiac SR vesicles exhibit a pronounced Ca^{2+}-induced Ca^{2+} release. This phenomenon has been demonstrated for SR actively loaded with Ca^{2+} in the presence of ATP and P_i and for passively loaded SR. Ca^{2+} efflux from actively and passively loaded vesicles appears to proceed via the same channels since both phenomena are characterized by similar $[Ca^{2+}]_0$ dependence, inhibition by Mg^{2+}, and (as

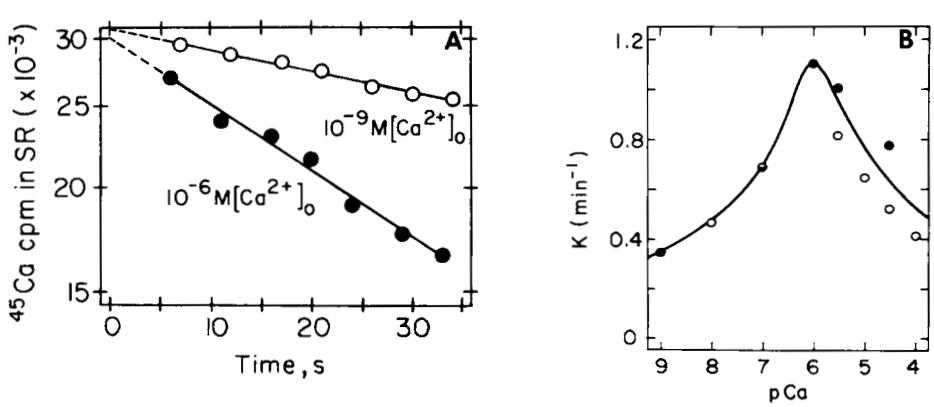

FIG. 5. **Unidirectional Ca^{2+} efflux from passively loaded SR vesicles at different $[Ca^{2+}]_0$.** SR vesicles were passively loaded with $^{45}CaCl_2$ and efflux was initiated by dilution into various ionized $^{40}Ca^{2+}$ concentrations as described under "Experimental Procedures." *A*, semilog plot of ^{45}Ca counts/min remaining in SR (*i.e.* trapped on filters) *versus* time after initiation of efflux by dilution. Data are shown only for dilution into media containing 10^{-9} M $[Ca^{2+}]_0$ and 10^{-6} M $[Ca^{2+}]_0$. *B*, the first order rate constants of the fast initial phase of Ca^{2+} efflux were determined from linear regression analyses of data as shown in *A* and plotted as a function of the pCa of the dilution medium. *Open* and *closed symbols* represent data from two separate experiments. *Ca^{2+}-dependent* efflux was half-maximal at approximately 0.15 and 13 μM $[Ca^{2+}]_0$.

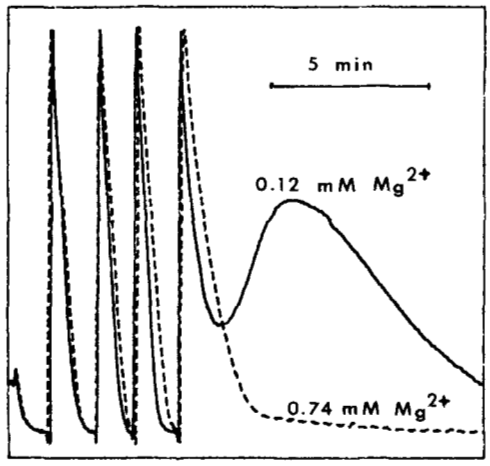

FIG. 6. **Inhibition of Ca^{2+}-induced Ca^{2+} release by Mg^{2+}.** SR (40 μg of protein) was added either to standard uptake/release assay medium (1 mM total magnesium, *solid line*) or to medium containing 4 mM total magnesium (*dashed line*); *i.e.* the ionized Mg^{2+} concentrations were 0.12 and 0.74 mM, respectively. Net Ca^{2+} uptake/release was monitored after four successive additions of 40 nmol of $CaCl_2$ (final volume, 1 ml).

TABLE II

Inhibition of Ca^{2+} efflux from passively loaded SR by Mg^{2+}

SR vesicles were passively loaded with $^{45}CaCl_2$ and unidirectional efflux was initiated by dilution as described under "Experimental Procedures." The ionized Mg^{2+} concentration in the dilution buffer (essentially equivalent to the total magnesium under these conditions) was varied at a constant 1 μM $[Ca^{2+}]_0$. The first order rate constant of the fast initial phase of the Ca^{2+} efflux varied as a function of ionized Mg^{2+}. At 0.72 mM Mg^{2+}, Ca^{2+} efflux was reduced to the level previously measured at 1 nM $[Ca^{2+}]_0$ and 0.10 mM Mg^{2+} (Fig. 5).

$[Ca^{2+}]_0$	Ionized Mg^{2+}	k
μM	mM	min^{-1}
1	0.10	1.30
1	0.72	0.37

reported in the companion paper, Ref. 25) inhibition by a wide variety of chemical compounds. The Ca^{2+} concentrations which promote Ca^{2+} release are in the physiological range, with half-maximal Ca^{2+} release at submicromolar $[Ca^{2+}]_0$ (Figs. 2 and 5). Elevation of $[Ca^{2+}]_0$ to 5–10 μM significantly inhibits Ca^{2+} release. The characteristics of this release are thus similar to those previously observed for Ca^{2+}-induced Ca^{2+} release in skinned cardiac fibers (14, 15). The rates of Ca^{2+} release observed in this study are several orders of magnitude lower than Ca^{2+} release *in vivo* (26), but the physiological Ca^{2+} release may involve the same Ca^{2+} efflux pathway.

Following ATP-dependent Ca^{2+} loading in the presence of 75 mM P_i, Ca^{2+} release occurs after SR vesicles have accumulated Ca^{2+} to a trigger capacity. This capacity varies from SR preparation to preparation and is not the maximum Ca^{2+} loading capacity since the SR can accumulate Ca^{2+} to at least twice this level in the presence of 10 mM total magnesium or when P_i is reduced to 25 mM (data not shown). The rate and extent of net Ca^{2+} release are dependent upon $[Ca^{2+}]_0$ when the trigger capacity is attained (Table I). Maximum Ca^{2+} release occurs when $[Ca^{2+}]_0$ is 15.–2 μM (Fig. 2). Ca^{2+} release occurs in the presence of 1 mM ATP and an ATP-regenerating system, *i.e.* under conditions that prevent ATP depletion and Ca^{2+} pump reversal. Ca^{2+}-dependent ATP hydrolysis continues unabated during Ca^{2+} release (Fig 3). Paired experiments measuring net Ca^{2+} release and unidirectional Ca^{2+} efflux

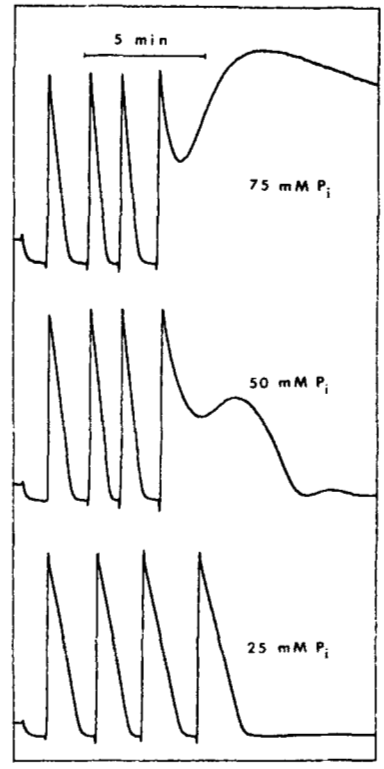

FIG. 7. **Dependence of Ca^{2+}-induced Ca^{2+} release on P_i concentration.** SR (40 μg of protein) was added either to standard uptake/release assay medium (75 mM P_i) or to media containing 50 or 25 mM P_i, in which the total potassium concentration was maintained at 120 mM by addition of KCl. Net Ca^{2+} uptake/release was monitored after four successive additions of 40 nmol of $CaCl_2$ (final volume, 1 ml).

confirm the Ca^{2+} dependence of Ca^{2+} efflux (Fig. 4) and indicate that inward Ca^{2+} pumping continues during Ca^{2+} release at about 60% of the initial rate prior to release. After 2–3 min of net Ca^{2+} release, Ca^{2+} reuptake by the SR occurs (Fig. 1). The reuptake of released Ca^{2+} indicates that release is not mediated by irreversible damage to the membrane permeability barrier. Net Ca^{2+} release is inhibited by increasing ionized Mg^{2+} from 0.12 to 0.74 mM (Fig. 6) or by decreasing P_i from 75 to 25 mM (Fig. 7). Unidirectional Ca^{2+} efflux from passively loaded vesicles exhibits similar dependence on $[Ca^{2+}]_0$ (Fig. 5) and inhibition by Mg^{2+} (Table II). It occurs in the absence of P_i, indicating that high levels of P_i are not required for Ca^{2+}-induced Ca^{2+} release, *per se*.

There have been numerous investigations of Ca^{2+}-induced Ca^{2+} release using SR vesicles isolated from either skeletal (27–35) or cardiac (24, 36–39) muscle. There is some doubt about the physiological role of Ca^{2+}-induced Ca^{2+} release in skeletal muscle (6), but experiments on skinned heart cells provide convincing evidence for a physiologically significant role for Ca^{2+}-induced Ca^{2+} release from mammalian cardiac SR (14, 15). In skinned heart cells, increasing $[Ca^{2+}]_0$ results in increased Ca^{2+} release up to an optimum $[Ca^{2+}]_0$ beyond which further increases progressively inhibit Ca^{2+} release (15, 40). These observations are consistent with the decreased contraction of intact heart fibers at high $[Ca^{2+}]_0$ (41). For skinned fibers from adult dog ventricle, the optimum $[Ca^{2+}]_0$ for Ca^{2+} release is 0.6–0.7 μM (14).

In previous reports on Ca^{2+}-induced Ca^{2+} release from cardiac SR vesicles, the optimum $[Ca^{2+}]_0$ varied from 5–30 μM and there was little or no inhibition of Ca^{2+} release by higher $[Ca^{2+}]_0$ (24, 37–39). The Ca^{2+} release from SR vesicles de-

scribed in this report more closely resembles that observed with skinned heart cells. Maximum net Ca^{2+} release is observed at 1.5–2 μM $[Ca^{2+}]_0$ and declines at higher $[Ca^{2+}]_0$ such that at 5 μM $[Ca^{2+}]_0$ it is only 60% of the maximum (Fig. 2). Similarly, the *Ca^{2+}-dependent component* of Ca^{2+} efflux from passively loaded SR vesicles decreases about 50% as $[Ca^{2+}]_0$ is increased from 1 to 10 μM (Fig. 5). It should be noted again, however, that the maximum Ca^{2+} efflux rates observed in this study, like many others previously observed *in vitro* using SR vesicles, is several orders of magnitude lower than the rate of Ca^{2+} release which occurs in muscle cells *in vivo* (26). The basis for reduced efflux *in vitro* may be the different conditions of the isolated system, including suboptimal assay conditions or the possible loss of a component(s), and is yet to be resolved.

Inhibition of Ca^{2+}-induced Ca^{2+} release, *in vitro*, by Mg^{2+} has previously been described for both skeletal muscle (6, 22, 27–29, 42) and cardiac (37, 38, 43) SR. Nevertheless, the inhibition of Ca^{2+} release by 0.74 mM ionized Mg^{2+} might cast doubt upon the physiological relevance of the phenomenon. In skinned heart cells, the threshold $[Ca^{2+}]_0$ for release is elevated as ionized Mg^{2+} is increased, but Ca^{2+} release still occurs at a presumed physiological level of 3.2 mM ionized Mg^{2+} (14, 15, 44). Similarly, in one previous study with cardiac SR vesicles, decreased sensitivity of EGTA-induced Ca^{2+} efflux to $[Ca^{2+}]_0$ was observed as ionized Mg^{2+} was increased from 0.1 to 1.0 mM (38). Ca^{2+} efflux was reduced 5-fold at the original optimum $[Ca^{2+}]_0$ of 3 μM, but at the new optimum $[Ca^{2+}]_0$ of 13 μM, *Ca^{2+} efflux in the absence of Ca^{2+} pumping* occurred at 80% of the original rate at 0.1 mM Mg^{2+}, 3μM $[Ca^{2+}]_0$.

Our experiments indicate that Ca^{2+}-induced Ca^{2+} *efflux* does indeed occur when the trigger capacity is attained at 10 μM $[Ca^{2+}]_0$ following active Ca^{2+} loading in the presence of 0.74 mM ionized Mg^{2+} (data not shown). In this regard, our data parallel the earlier results using cardiac SR vesicles (38). However, the magnitude of the efflux is insufficient to produce *net Ca^{2+} release* and our data (and possibly the earlier results also since net uptake/release data are not presented in Ref. 38) differ from the results obtained with skinned heart cells where net Ca^{2+} release can occur at high ionized Mg^{2+} concentrations (14, 15, 44). The significance of the inhibition of Ca^{2+} release by 0.74 mM ionized Mg^{2+} is difficult to evaluate since the physiological level of ionized Mg^{2+} has not been unambiguously determined. Although the presumed physiological level is about 3 mM ionized Mg^{2+} (14, 15, 44), in one recent report (45), the ionized Mg^{2+} level in heart muscle was determined to be only 0.2–0.6 mM.

In some respects, the Ca^{2+} release described in this report resembles that from skeletal muscle SR described by Katz and co-workers (27, 30, 31). Because of some similarities in the requirements for Ca^{2+} pumping and Ca^{2+} release, especially those for Ca^{2+} and Mg^{2+}, they concluded that this Ca^{2+} release is mediated by the Ca^{2+} pump protein (27, 30). The same conclusion based on similar considerations was also reached regarding Ca^{2+}-induced Ca^{2+} release from cardiac SR (24, 38).

Our studies do not support this conclusion. The Ca^{2+} and Mg^{2+} requirements for Ca^{2+} pumping and Ca^{2+} release are distinctly different. The K_m for Ca^{2+} pumping by our cardiac SR preparation is 2.6 μM $[Ca^{2+}]_0$ (data not shown) while net Ca^{2+} release is half-maximal at 0.8 μM $[Ca^{2+}]_0$ (Fig. 2) and Ca^{2+}-dependent Ca^{2+} efflux after passive loading is half-maximal at 0.1–0.15 μM $[Ca^{2+}]_0$ (Fig. 5*B*). Moreover, at 5–10 μM $[Ca^{2+}]_0$ where Ca^{2+} pumping is maximal, both net Ca^{2+} release and Ca^{2+}-dependent efflux after passive loading are reduced approximately 50% from maximum values. Also, elevation of ionized Mg^{2+} from 0.12 to 0.74 mM inhibits Ca^{2+} release (Fig. 6, Table II) but has little effect on Ca^{2+} pumping (Fig. 6). Furthermore, a variety of chemicals completely block Ca^{2+}-induced Ca^{2+} release at concentrations which have little effect on the turnover of the Ca^{2+}-dependent ATPase (25). We conclude from these considerations that Ca^{2+}-induced Ca^{2+} efflux occurs through specific channel(s) distinct from the Ca^{2+} pump protein.[3]

Palade *et al.* (46) described a spontaneous Ca^{2+} release from skeletal muscle SR which differs from the Ca^{2+}-induced Ca^{2+} release described by Katz *et al.* (27, 30, 31) but which also shares some characteristics with the Ca^{2+}-induced Ca^{2+} release from heart SR described here. Both types of release occur in the presence of 1 mM ATP and a regenerating system and are followed by reuptake of the released Ca^{2+}. Both types of release are reduced either by increasing Mg^{2+} or by decreasing P_i (47). Also, during both types of release, Ca^{2+}-dependent ATPase continues unabated and inward Ca^{2+} pumping continues at a reduced rate. Since similar results were previously observed for Ca^{2+}-induced Ca^{2+} release from both cardiac and skeltal muscle (24, 27, 30, 31, 36, 38), these features may be common to a variety of modes of Ca^{2+} release *in vitro*. However, the spontaneous Ca^{2+} release from skeletal muscle SR differs fundamentally from the Ca^{2+}-induced Ca^{2+} release from cardiac SR in at least two important respects, the dependence upon $[Ca^{2+}]_0$ (this report and Ref. 46) and the inhibition of Ca^{2+} release by various chemical compounds (25, 48). These results indicate diversity, either in the Ca^{2+} channels themselves or in the modulation of the channels.

This report provides the most detailed study to date of Ca^{2+}-induced Ca^{2+} release from isolated cardiac SR vesicles. In contrast to previous results (24, 37–39, 43), Ca^{2+} efflux was elicited by relatively low, *i.e.* physiological Ca^{2+} concentrations and was significantly inhibited by higher $[Ca^{2+}]_0$. Our results are similar to those achieved with skinned cardiac fibers (14, 15). Also, for the first time, Ca^{2+}-induced Ca^{2+} efflux was demonstrated for both actively and passively loaded cardiac SR vesicles. Finally, in contrast to a previous conclusion (24, 38), the characteristics of the phenomenon indicate that Ca^{2+} efflux occurs via channels distinct from the Ca^{2+} pump protein.

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[3] In further support of this conclusion, preliminary studies indicate that it is possible to separate cardiac SR vesicles containing the Ca^{2+}-gated Ca^{2+} channels from vesicles which do not have the channels but which do contain the Ca^{2+} pump protein (B. K. Chamberlain, unpublished data).

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