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Lack of effects of calcium · calmodulin-dependent phosphorylation on Ca^{2+} release from cardiac sarcoplasmic reticulum

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Canine cardiac sarcoplasmic reticulum is phosphorylated by an endogenous calcium · calmodulin-dependent protein kinase and phosphorylation occurs mainly on a 27 kDa proteolipid, called phospholamban. To determine whether this phosphorylation has any effect on Ca^{2+} release, sarcoplasmic reticulum vesicles were phosphorylated by the calcium · calmodulin-dependent protein kinase, while non-phosphorylated vesicles were preincubated under identical conditions but in the absence of ATP to avoid phosphorylation. Both non-phosphorylated and phosphorylated vesicles were centrifuged to remove calmodulin, and subsequently used for Ca^{2+} release studies. Calcium loading was carried out either by the active calcium pump or by incubation with high (5 mM) calcium for longer periods. Phosphorylation of sarcoplasmic reticulum by calcium · calmodulin-dependent protein kinase had no appreciable effect on the initial rates of Ca^{2+} released from cardiac sarcoplasmic reticulum vesicles loaded under passive conditions and on the apparent $^{45}Ca^{2+}$–$^{40}Ca^{2+}$ exchange from cardiac sarcoplasmic reticulum vesicles loaded under active conditions. Thus, it appears that calcium · calmodulin-dependent protein kinase mediated phosphorylation of cardiac sarcoplasmic reticulum is not involved in the regulation of Ca^{2+} release and $^{45}Ca^{2+}$–$^{40}Ca^{2+}$ exchange.

Introduction

The calcium pump in cardiac sarcoplasmic reticulum (SR) appears to be regulated through phosphorylation of phospholamban, a 27 kDa polymeric proteolipid. Phospholamban is phosphorylated by cAMP-dependent protein kinase and this phosphorylation is associated with stimulation of the initial rates of Ca^{2+} transport and Ca^{2+}-ATPase activity by SR [1,2]. Phospholamban is also phosphorylated by an endogenous calcium · calmodulin-dependent protein kinase on a site, which appears distinct form that phosphorylated by cAMP-dependent protein kinase [3,4]. Phosphorylation by the calcium · calmodulin-dependent protein kinase is half-maximally stimulated by 3.8 ± 0.3 μM calcium and it is absolutely dependent on exogenous calmodulin for

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mes, 4-morpholineethanesulfonic acid.

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activation (EC_{50} = 49 nM) [5]. Calcium · calmodulin-dependent phosphorylation is also associated with an increased rate of Ca^{2+} transport [5,6]. The stimulatory effect of the protein kinases on Ca^{2+} transport may be reversed by a SR associated protein phosphatase, which can dephosphorylate both the cAMP-dependent as well as the calcium · calmodulin-dependent site on phospholamban [7,8]. Thus, phospholamban appears to be a regulator for the calcium pump in cardiac SR.

Although a considerable amount of evidence has been accumulated for the effects of phosphorylation on Ca^{2+}-transport, very little is known about its effects on Ca^{2+} release from cardiac SR. It has previously been shown that cAMP-dependent phosphorylation of cardiac SR was associated with stimulation of Ca^{2+} efflux [9]. In the present study, we report that calcium · calmodulin-dependent phosphorylation of SR has no appreciable effect on Ca^{2+} release from cardiac SR, loaded under passive conditions and on the apparent $^{45}Ca^{2+}$–$^{40}Ca^{2+}$ exchange from cardiac SR loaded under active conditions.

Methods

Preparation of sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum was prepared from dog cardiac muscle and the purity of the preparations was evaluated by various enzyme marker assays, as we have described previously [10,11]. Mitochondrial contamination was 3–5%, sarcolemmal contamination was less than 10%, and cytosolic contamination was 0.2%.

Phosphorylation of cardiac sarcoplasmic reticulum. Calcium · calmodulin-dependent phosphorylation of SR (0.5 mg/ml) was carried out, as previously described [4,12], at 30 °C in 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM $MgCl_2$, 0.5 mM EGTA, 0.478 mM $CaCl_2$ (10 μM free Ca^{2+}), 1.0 μM calmodulin (isolated from bovine testis [13]), and 0.5 mM [γ-^{32}P]ATP. Reactions were terminated by the addition of 7% cold perchloric acid containing 7% polyphosphoric acid. After addition of SR carrier protein (2.5 mg/ml reaction solution) the samples were washed, and the amounts of [^{32}P]P_i incorporated were determined as previously described [2]. Phosphate incorporation due to calcium · calmodulin-dependent protein kinase has been characterized as a phosphoester bond based on its stability to hydroxylamine and NaOH; thus, this bond can easily be distinguished from the acylphosphate intermediate of the Ca^{2+}-ATPase [4,12].

For investigation of the effects of phosphorylation on Ca^{2+} release, SR vesicles were phosphorylated under the same conditions as above using unlabeled ATP. The phosphorylation reaction was initiated by the addition of ATP. Non-phosphorylated vesicles were also incubated under identical conditions, but in the absence of ATP. After 2 min of incubation at 30 °C, the reaction mixture was diluted 2-fold with ice-cold 20 mM Tris-maleate, 0.3 M sucrose (pH 7.0) (Buffer A), and centrifuged at 100000 × g for 30 min. The pellet was resuspended in Buffer A, and the protein was determined by the method of Lowry et al. [14]. The conditions used for preincubation (phosphate buffer, $MgCl_2$, $CaCl_2$, EGTA, temperature, time of incubation) and subsequent washing of SR vesicles were found not to influence the rate of transport [5] or the levels of total Ca^{2+} accumulated and released. Vesicles, which were kept on ice (0 °C), expressed the same amount of Ca^{2+} released as those exposed to the preincubation conditions described above. Thus, non-phosphorylated vesicles for this study were preincubated simultaneously with phosphorylated vesicles under identical conditions (including calmodulin) but in the absence of ATP to avoid phosphorylation.

Calcium release form passively loaded sarcoplasmic reticulum. Sarcoplasmic reticulum vesicles (5–10 mg/ml) in 0.3 M sucrose, 0.1 M KCl, 20 mM Tris-maleate (pH 7.0) were passively loaded with 5 mM $CaCl_2$ by preincubation for 5–6 h at 4 °C. The Ca^{2+}-loaded SR (0.06 mg/ml) was then incubated in 0.15 M KCl, 20 mM Mes (pH 6.8), 5 mM $CaCl_2$, and 20 μM chlorotetracycline at 20 °C for 20 min (Syringe A). Calcium release was induced by mixing (1 : 1) the contents of syringe A with the contents of syringe B, which contained 0.15 M KCl, 20 mM Mes (pH 9.3), 20 μM chlorotetracycline, and various concentrations of EGTA to measure changes in fluorescence intensity as indicator of Ca^{2+} release (excitation, 395 nm; emission, 510 nm) [15–18]. The final pH after

mixing was 6.8. The initial rate of Ca^{2+} release was calculated from the initial slope of the Ca^{2+} release curves.

To correlate changes in fluorescence intensity with the amount of Ca^{2+} release, the time-course of Ca^{2+} release was monitored using the rapid filtration apparatus (Bio-Logic Co., Zisrst, 38240 Meylan, France) [19]. Sarcoplasmic reticulum vesicles, which were loaded with 5 mM $^{45}CaCl_2$ as described above, were diluted with 5 mM $CaCl_2$ and about 0.2 mg of the SR vesicles were placed on a Millipore filter (pore size, 0.65 μm). Calcium release was started by flow of a solution containing 20 mM Mes (pH 6.8), 0.15 M KCl, 5 mM $CaCl_2$ and 5.56 mM EGTA and at various times the release was terminated by stopping flow of the solution. The filters were dried and the radioactivity on the filters was counted by liquid scintillation counting.

Calcium release from actively loaded sarcoplasmic reticulum. Calcium efflux was measured by a modification of the Millipore filtration technique of Martonosi and Feretos [20]. The reaction mixture consisted of 20 mM Mes (pH 6.8), 0.15 M KCl, 2 mM $MgCl_2$, 10 mM NaN_3, 0.12 mM $^{45}CaCl_2$ (10^4 cpm/nmol), and 0.26 mM EGTA (0.37 μM free Ca^{2+}). Phosphorylated or non-phosphorylated SR vesicles (0.05 mg/ml) were preincubated at 37 °C for 1 min in the above buffer. The reaction was initiated by addition of ATP (2 mM final) and incubation continued for 9.5 additional min. Calcium efflux was measured by two different procedures. In the first procedure, $CaCl_2$ or $^{45}CaCl_2$ was added to give the desired free Ca^{2+} concentration as calculated by a computer program [21]. Addition of $CaCl_2$ did not result in any significant pH changes under the present experimental conditions. At certain intervals thereafter, 0.5 ml aliquots of the reaction mixture were rapidly filtered through 0.45 μm pore size Millipore filters. The filters were immediately washed with 20 ml of 20 mM Mes (pH 6.8) containing 0.15 M KCl, 30 mM EGTA, and 15 μM ruthenium red (washing buffer). In the second procedure, calcium efflux was assayed under conditions which prevented ATP-dependent calcium uptake (i.e., in the absence of Mg^{2+} and ATP). An aliquot (0.2 ml) of SR vesicles, loaded with calcium as described above, was filtered through a 0.45 μm Millipore filter followed by either two 10-s rinses (10 ml) with washing buffer, which inhibited $^{45}Ca^{2+}$ efflux, or one 10-s rinse (5 ml) with 20 mM Mes (pH 6.8), 0.15 M KCl, 0.26 mM EGTA, and 0.213 mM $^{40}CaCl_2$ or $^{45}CaCl_2$ (2 μM free Ca^{2+}), which promoted $^{45}Ca^{2+}$ efflux, followed by two 10-s rinses (10 ml) with washing buffer. After drying, the radioactivity retained on the filters was counted in 10 ml of Budget-Solve liquid scintillation cocktail (Research Products International Corp.) using a Beckman model LS 8100 liquid scintillation counter.

Miscellaneous. Calcium content of all solutions was determined by atomic absorption spectroscopy using a Perkin-Elmer 4000 atomic absorption spectrophotometer. Calcium-EGTA buffers contained various concentrations of EGTA and $CaCl_2$. Free Ca^{2+} concentrations at pH 6.8 were based upon the EGTA association constants reported by Martell and Smith [22] and they were calculated by the use of a computer program [21]. When the final Ca^{2+} concentration used to induce Ca^{2+} release was calculated, the amount of Ca^{2+} depletion in the medium due to Ca^{2+} transport was taken into consideration.

Results

Stability of the phosphoprotein

To study the effects of calcium · calmodulin-dependent phosphorylation on Ca^{2+} release, cardiac SR vesicles were phosphorylated under optimal conditions, in the presence of 10 μM Ca^{2+} ($C_{1/2}$: 3.8 ± 0.3 μM) and 1 μM calmodulin ($C_{1/2}$: 49 nM). Phosphorylation occurred mainly (over 90%) on phospholamban, a 27 kDa polymeric proteolipid, which upon boiling in sodium dodecyl sulfate migrated as a 9000–11000 M_r phosphoprotein in sodium dodecyl sulfate gels [8]. Other minor phosphoproteins formed by the calcium · calmodulin-dependent protein kinase had molecular masses of 45 and 55 kDa. In order to investigate the effects of calcium · calmodulin-dependent phosphorylation of SR on Ca^{2+} release, it is essential to determine whether or not the level of phosphoprotein is maintained throughout the course of Ca^{2+} release experiments. It was found that the level of phosphoprotein (1556 ± 127 pmol/mg SR: 100 ± 3%;

$n = 4$) formed by the calcium · calmodulin-dependent protein kinase was stable ($88 \pm 5\%$) during the washing of the phosphorylated SR. The phosphoprotein levels also remained stable during active Ca^{2+} loading with Mg-ATP at 37 °C and subsequent Ca^{2+} efflux assays ($87 \pm 3\%$; $n = 3$) as well as during passive Ca^{2+} loading, in 5 mM Ca^{2+} for 5 h at 4 °C ($85 \pm 1\%$; $n = 3$). Thus, it appears that the phosphoprotein levels were well maintained throughout the course of Ca^{2+} release experiments.

Effect of phosphorylation on Ca^{2+} release after passive loading

Passive Ca^{2+} loading was completed within 5 h of incubation with $^{45}Ca^{2+}$ at 4 °C. The calcium · calmodulin-dependent phosphorylation did not have any appreciable effect on the level of Ca^{2+} loading (data not shown). To induce Ca^{2+} release cardiac SR vesicles, loaded with 5 mM $^{45}Ca^{2+}$, were mixed rapidly with a solution containing various concentrations of EGTA or EGTA-$CaCl_2$ mixture to create various $[Ca^{2+}]_{final}$. The time-course of Ca^{2+} release was then monitored by two different methods: stopped-flow fluorometry using chlorotetracycline (CTC), as an indicator of the intravesicular Ca^{2+} [15–18], and a rapid filtration method using $^{45}Ca^{2+}$ [19]. As seen in Fig. 1, the time-courses of Ca^{2+} release obtained by stopped-flow fluorometry and rapid Ca^{2+} filtration were similar in nature. Fig. 2 illustrates plots of p*Ca* (Ca^{2+}_{final}) vs. the initial rates of Ca^{2+} release determined by the fluorometric method. A 'bell-shaped' $[Ca^{2+}]$-dependence curve was observed and it was essentially the same as that of Ca^{2+} release in skeletal muscle SR [23]. An increase of $[Ca^{2+}]_{final}$ from 0.55 to 2.2 μM increased the Ca^{2+} release rates in the non-phosphorylated SR, while further increase of $[Ca^{2+}]_{final}$ decreased the rate (Fig. 2). However, even the maximum Ca^{2+} release rate obtained was much lower than that which occurs in vivo [24]. The basis for the reduced rates of Ca^{2+} release, which are observed in vitro [9,25], is not presently known but it may be due to assay conditions or possible loss of essential components from the system as previously suggested [25]. Calcium · calmodulin-dependent phosphorylation had no significant effect on the

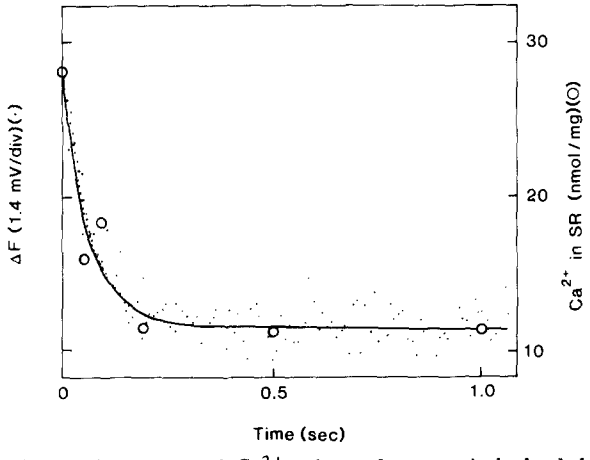

Fig. 1. Time-course of Ca^{2+} release, from passively loaded cardiac sarcoplasmic reticulum (SR) vesicles, monitored by stopped-flow fluorometry using chlorotetracycline (CTC) (· · · · · ·) and rapid filtration method using ^{45}Ca (O). Cardiac SR vesicles (5–10 mg/ml) were passively loaded with 5 mM $CaCl_2$ for 5–6 h at 4 °C. For stopped-flow fluorometry Ca^{2+} loaded SR (0.06 mg/ml) was incubated in 0.15 M KCl, 20 mM Mes (pH 6.8), 5 mM $CaCl_2$, and 20 mM CTC at 20 °C for 20 min. Calcium release was induced by addition of EGTA to make 2 μM free Ca^{2+}. Calcium release was also induced by the rapid filtration method [19] as described under Methods and each point represents the average of two separate experiments.

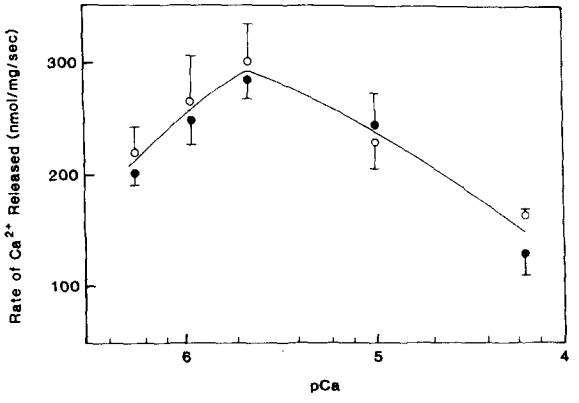

Fig. 2. The initial rates of Ca^{2+} release from passively loaded cardiac sarcoplasmic reticulum (SR) vesicles at various free Ca^{2+} concentrations. Cardiac SR vesicles were passively loaded with calcium and treated as described in Fig. 1. Calcium release, monitored by stopped-flow fluorometry, occurred at final Ca^{2+} concentration of: 0.55 μM ($n = 3$), 1.1 μM ($n = 5$), 2.2 μM ($n = 6$), 8.0 μM ($n = 3$) and 63 μM ($n = 2$). Each value represents the mean of n paired experiments ± S.E. for phosphorylated (O) and non-phosphorylated (●) SR.

initial rates of Ca^{2+} release and the extent of the $[Ca^{2+}]$-activation of Ca^{2+} release appeared to be the same in the phosphorylated and non-phosphorylated SR. Thus, these findings on the passively loaded vesicles suggest that phosphorylation of cardiac SR had no apparent effect on Ca^{2+} release.

Effect of phosphorylation on Ca^{2+} efflux after active loading

Active Ca^{2+} transport by SR vesicles at $[Ca^{2+}]_0$ of 0.37 μM was completed within a few minutes, and the steady-state level of transport was sustained for more than 10 min (Fig. 3). Calcium · calmodulin-dependent phosphorylation of the SR membranes resulted in an increase of the initial rates of Ca^{2+} transport and an increase in the Ca^{2+} levels accumulated. However, the stimulatory effect observed in this study was obtained in the absence of a Ca^{2+} precipitating anion, and it was small compared to previous reports in which oxalate was used [5,8]. An abrupt change of the calcium concentration in the media, from 0.37 μM to 2 μM by addition of $^{40}CaCl_2$, induced Ca^{2+} efflux, which was completed within 30 s in both phosphorylated and non-phosphorylated SR vesicles (Fig. 3). The level of Ca^{2+} efflux from phosphorylated SR vesicles (6.8 ± 0.5 nmol/mg; $n = 6$) was significantly larger than that from non-phosphorylated SR vesicles (5.3 ± 0.6 nmol/mg; $n = 6$) but the fraction of $^{45}Ca^{2+}$ efflux (level of Ca^{2+} efflux/level of Ca^{2+} loaded) from phosphorylated and non-phosphorylated SR vesicles was similar (Fig. 3, inset). Furthermore, when the relative $^{45}Ca^{2+}$ efflux was obtained as a function of the final calcium concentration after the $^{40}Ca^{2+}$ jump, there was no appreciable difference between the phosphorylated and non-phosphorylated SR

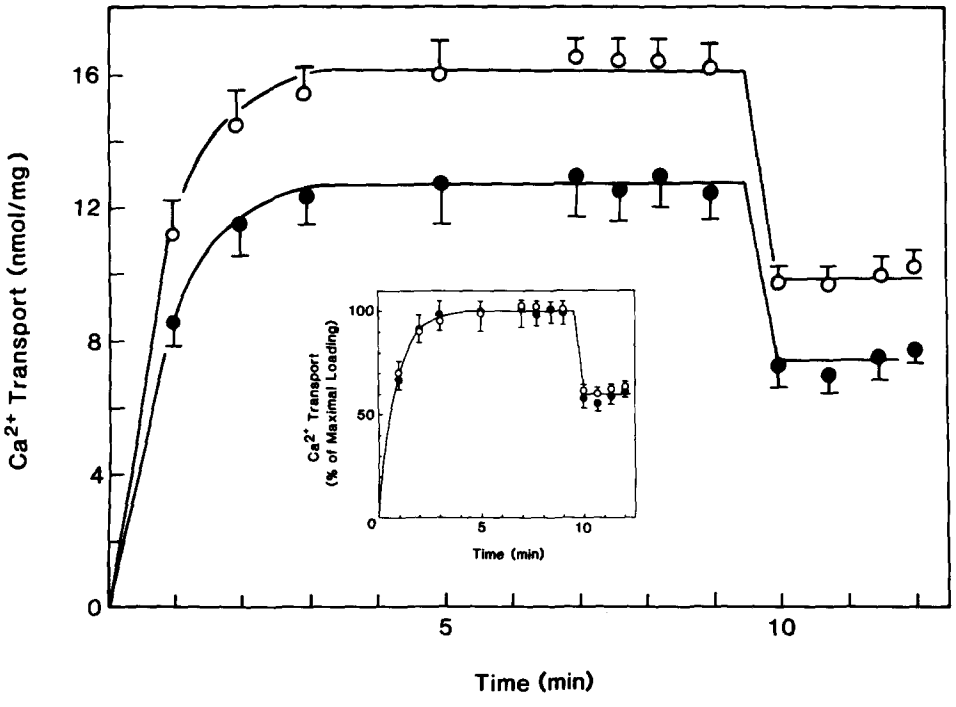

Fig. 3. Time-course of Ca^{2+} transport and release by phosphorylated and non-phosphorylated cardiac sarcoplasmic reticulum (SR) vesicles. Cardiac SR vesicles were prephosphorylated by calcium·calmodulin-dependent protein kinase (○). Non-phosphorylated vesicles (●) were treated under identical conditions but in the absence of ATP. SR vesicles (0.05 mg/ml) were assayed for calcium transport (0.26 mM EGTA/0.12 mM $^{45}CaCl_2$; 0.37 μM free Ca^{2+}) as described under Methods. At 9.5 min 92.2 μM $CaCl_2$ was added (2 μM final Ca^{2+}). Each value represents the mean of six paired experiments ± S.E. Inset. Ca^{2+} transport values are expressed as percent of maximal loading for phosphorylated (○) and non-phosphorylated (●) cardiac SR vesicles, respectively.

preparations, in both the relative amount and the $[Ca^{2+}]$-dependence of $^{45}Ca^{2+}$ efflux (Fig. 4).

The level of active Ca^{2+} loading is inevitably higher in the phosphorylated SR than the non-phosphorylated SR if the reaction is carried out under the same conditions as described above. In order to achieve the same level of Ca^{2+} loading, therefore, we carried out the active Ca^{2+} transport reaction at 0.37 μM Ca^{2+} for the phosphorylated SR and at 0.63 μM Ca^{2+} for the non-phosphorylated SR (amount of Ca^{2+} loaded: 15.3 ± 0.6 vs. 15.6 ± 0.6 nmol Ca^{2+}/mg for non-phosphorylated vs. phosphorylated SR; $n = 3$). The calcium concentration was then increased to 2 μM in both preparations to induce $^{45}Ca^{2+}$ efflux. The level of $^{45}Ca^{2+}$ efflux was about the same for non-phosphorylated (5.0 ± 0.6 nmol Ca^{2+}/mg) and phosphorylated SR (5.7 ± 1.2 nmol Ca^{2+}/mg).

The apparent $^{45}Ca^{2+}$ efflux induced by a $^{40}Ca^{2+}$ jump was not observed when $^{45}CaCl_2$ was used to induce the efflux of calcium from SR vesicles. The lack of any observable $^{45}Ca^{2+}$ efflux could be either due to increased ATP-dependent Ca^{2+} uptake or due to $^{45}Ca^{2+}$–$^{45}Ca^{2+}$ exchange. To distinguish between these possibilities, vanadate (sodium vanadate, ortho, from Fischer) was added to the Ca^{2+} transport assay, after loading of the SR vesicles (at 9 min), at a concentration (100 μM) which inhibits the Ca^{2+}-ATPase activity by over 70%. Addition of vanadate had no effect on the levels of $^{45}Ca^{2+}$ associated with the SR vesicles following the $^{45}Ca^{2+}$ jump, suggesting that the lack of observable $^{45}Ca^{2+}$ efflux was not due to increased ATP-dependent Ca^{2+} uptake. This was further supported by filtration experiments in which calcium efflux was induced by $^{45}Ca^{2+}$ in the absence of Mg^{2+} and ATP to prevent ATP-dependent Ca^{2+} uptake (second procedure above). The levels of $^{45}Ca^{2+}$ remaining in these SR vesicles were similar when the $^{45}Ca^{2+}$ jump occurred in the absence or presence of Mg^{2+} and ATP, which would support ATP-dependent Ca^{2+} uptake.

From these findings, it is tentatively suggested that the apparent $^{45}Ca^{2+}$ efflux, observed upon a $^{40}Ca^{2+}$ jump under the active-loading conditions, is due to $^{45}Ca^{2+}$–$^{40}Ca^{2+}$ exchange (see Discussion). Thus, it appears that the apparent exchange reaction as well as Ca^{2+} release are not affected by calcium · calmodulin-dependent phosphorylation of cardiac SR.

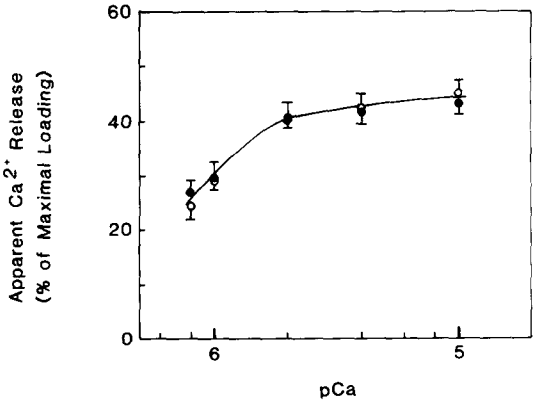

Fig. 4. Calcium release from actively loaded cardiac sarcoplasmic reticulum (SR) vesicles, at various free Ca^{2+} concentrations. The conditions for phosphorylation and transport are the same as in Fig. 3. At 9.5 min, $CaCl_2$ was added, and final concentrations were: 0.8 μM, 1 μM, 2 μM, 4 μM and 10 μM. Each value represents the mean of three paired experiments ± S.E. for phosphorylated (○) and non-phosphorylated (●) SR vesicles.

Discussion

Previously, Kirchberger and Wong [9] reported that cAMP-dependent phosphorylation increased the rates of Ca^{2+} released from cardiac SR. In that study, Ca^{2+} release was induced from Ca^{2+}-loaded SR vesicles in the presence of oxalate or phosphate. In the present study, the effects of calcium · calmodulin-dependent phosphorylation were studied using SR vesicles preloaded with Ca^{2+} under active or passive conditions in the absence of any Ca^{2+} precipitating anions. Phosphorylation did not increase the rates of Ca^{2+} release from cardiac SR vesicles loaded with Ca^{2+} under passive conditions and there was no appreciable difference in the $[Ca^{2+}]$-dependence between phosphorylated and non-phosphorylated SR vesicles. The reason for the discrepancy between previous reports and the present results is not clear, but it might be due to differences in the sites phosphorylated by the cAMP-dependent versus the calcium · calmodulin-dependent protein kinases and in reaction conditions, e.g., presence or absence of Ca^{2+} precipitating anions.

As demonstrated here, the actively loaded

$^{45}Ca^{2+}$ was released by a Ca^{2+} jump using $^{40}Ca^{2+}$, whereas no appreciable $^{45}Ca^{2+}$ release was observed if the same type of Ca^{2+} jump was performed using $^{45}Ca^{2+}$. The possibility that this might represent rapid reuptake of the released $^{45}Ca^{2+}$, due to activation of the Ca^{2+} pump by the increase of $[Ca^{2+}]$, was excluded by the findings that conditions (addition of the transport inhibitor vanadate or removal of Mg ATP by filtration), which would prevent reuptake of calcium, had no effect. Thus, it appears that the $^{40}Ca^{2+}$ jump-induced $^{45}Ca^{2+}$ release, shown in the experiments of Fig. 3, actually represents a $^{45}Ca^{2+}$–$^{40}Ca^{2+}$ exchange. However, because of the rather low kinetic resolution of the assay method for release of actively loaded $^{45}Ca^{2+}$, it is possible that a rapid $^{45}Ca^{2+}$ release preceded a slow Ca^{2+}–Ca^{2+} exchange but it was not detectable in this study. Furthermore, since the amount of apparent Ca^{2+}–Ca^{2+} exchange (6–7 nmol Ca^{2+}/mg) is of the same order of magnitude as Ca^{2+} binding to the Ca^{2+}-ATPase [26,27] it is also possible that, in the $^{45}Ca^{2+}$ jump experiments, $^{45}Ca^{2+}$ release may have been hindered by binding of an additional amount of $^{45}Ca^{2+}$ to the Ca^{2+}-ATPase. Thus, although there was no observable release of the actively loaded $^{45}Ca^{2+}$ in the $^{45}Ca^{2+}$ jump experiments, it is likely that $^{45}Ca^{2+}$ release occurred but it was hindered by other processes such as exchange of the intravesicular with the extravesicular Ca^{2+} under the active loading-release conditions. In fact, previous reports in skinned fibers indicated that a Ca^{2+} jump could induce Ca^{2+} release from cardiac SR [28,29].

Recently, calcium · calmodulin-dependent phosphorylation of a 60 kDa protein of the skeletal muscle SR was shown to decrease the Ca^{2+} release rates [18]. This effect of calcium · calmodulin-dependent phosphorylation is different from the one reported here on cardiac SR, in which phosphorylation occurs mainly on phospholamban. Phosphorylation of cardiac SR by the calcium · calmodulin-dependent protein kinase was associated with increased levels of Ca^{2+} efflux from the cardiac SR. This increase was due to the increased amount of Ca^{2+} transported by phosphorylation of cardiac SR and it may mislead one to the conclusion that phosphorylation might activate Ca^{2+} release [30].

Previously, it was shown that inclusion of calmodulin in the Ca^{2+} release assays resulted in reduction of the rates of Ca^{2+} release and it was suggested that the effect of calmodulin was mediated by its direct interaction with SR and did not involve protein phosphorylation [31]. In the present study, calmodulin was present in the preincubation reaction for both non-phosphorylated and phosphorylated SR. However, the amounts of calmodulin remaining with the washed SR vesicles during Ca^{2+} release assays were less than 700 ng/mg of SR, or less than 2 nM calmodulin, as determined by radioimmunoassay [5]. This concentration of calmodulin is almost 1000-fold lower than that previously reported to directly affect Ca^{2+} release by cardiac SR.

Therefore, in contrast to the stimulatory effect of calcium · calmodulin-dependent phosphorylation of cardiac SR on the Ca^{2+} pump, phosphorylation had no appreciable effect on the kinetics of calcium release from vesicles passively loaded with $^{45}Ca^{2+}$. It had no effect on release of actively loaded $^{45}Ca^{2+}$, induced by a $^{40}Ca^{2+}$ jump, either. However, when $^{45}Ca^{2+}$ was used to produce the calcium jump, there was no appreciable release indicating that rather complicated processes, such as Ca^{2+}–Ca^{2+} exchange are involved under active loading conditions. The present results suggest that calcium · calmodulin-dependent phosphorylation of SR is not involved in the regulation of the putative Ca^{2+} channels [25,31].

Acknowledgments

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