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Removal of Mg^{2+} inhibition of cardiac ryanodine receptor by palmitoyl coenzyme A

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Abstract $^{45}Ca^{2+}$ fluxes and planar bilayer recordings indicated that the fatty acid metabolite palmitoyl coenzyme A, but not free coenzyme A or palmitic acid, stimulated the cardiac ryanodine receptor channel of pig heart sarcoplasmic reticulum. Palmitoyl CoA reactivated channels inhibited by concentrations of cytoplasmic free Mg^{2+} in the physiological range. Reactivation by palmitoyl CoA in the presence of Mg^{2+} was stimulated by myoplasmic free Ca^{2+} in the micromolar range. Acyl coenzyme A derivatives may be utilized by cardiac muscle cells to compensate for the severe Mg^{2+} inhibition of ryanodine receptors which would otherwise leave Ca^{2+} stores unresponsive to Ca^{2+} and to other cytosolic ligands involved in signal transduction.

Key words: Fatty acid metabolite; Ryanodine receptor; Sarcoplasmic reticulum; Ca^{2+} release channel

1. Introduction

Cardiac myocytes release massive amounts of Ca^{2+} from the sarcoplasmic reticulum (SR) in response to cell depolarization. Ca^{2+}-induced Ca^{2+} release appears to be the main mechanism by which the Ca^{2+} permeability of the SR is increased during this process [1]. Evidence favoring the participation of ryanodine receptors in Ca^{2+}-induced Ca^{2+} release has been provided by studies demonstrating that ligands that potentiate or inhibit Ca^{2+}-induced Ca^{2+} release, such as caffeine and adenine nucleotides or Mg^{2+} and procaine, respectively open or close ryanodine receptor channels (reviewed in [2]). Furthermore, ryanodine receptors can be rapidly activated by submicromolar Ca^{2+} concentrations in minimal solutions typically used in single channel recordings [3].

Although ryanodine receptors are intrinsically activated by Ca^{2+}, it is likely that other cell constituents or biochemical reactions within the cell play a role in the control of the Ca^{2+} sensitivity of this channel. This must be the case because Mg^{2+} ions are strong inhibitors of ryanodine receptors [2] and the cytosolic free Mg^{2+} concentration of striated muscle cells is unlikely to be lower than 0.5 to 1 mM although it could be as high as 6 mM [4]. At free Mg^{2+} concentrations in the millimolar range, skeletal and cardiac ryanodine receptor channels incorporated into planar bilayers respond poorly to Ca^{2+} [5,6]. Other approaches, such as $^{45}Ca^{2+}$ flux and [^{3}H]ryanodine binding kinetics, have also concluded that ryanodine receptors should be unavailable for opening in physiological solutions of Mg^{2+} [2].

The cellular mechanism by which ryanodine receptors remain sensitive to Ca^{2+} in the presence of cytosolic free Mg^{2+} is unknown. Previous studies suggested that fatty acid metabolites such as palmitoyl carnitine or palmitoyl CoA may serve as an endogenous modulator of the skeletal ryanodine receptor [7,8]. Here we show that long-chain acyl coenzyme A (CoA) derivatives specifically activated the cardiac ryanodine receptor. Furthermore, the acyl CoA activated channel was sensitive to micromolar myoplasmic Ca^{2+} in the presence of millimolar concentrations of Mg^{2+}. Acyl CoA metabolites normally found in the cytoplasm of cardiac cells may thus participate in the control the Ca^{2+} sensitivity of the cardiac ryanodine receptor.

2. Materials and methods

2.1. Preparation of cardiac SR

Total SR was prepared as described [9]. Adult swine left ventricular free wall was homogenized in 0.1 M NaCl, 5 mM Tris-Maleate pH 6.8 and centrifuged at 2600 × *g* for 30 min. The supernatant was centrifuged at 100,000 × *g* for 60 min and the resulting pellet was resuspended in 0.3 M sucrose, 0.4 M KCl, 20 μM $CaCl_2$, 5 mM Tris-MES pH 6.8, snap frozen and stored at −80°C.

2.2. Bilayer recordings

Planar bilayer formation and recording was described previously [10]. Bilayers were composed of equal concentrations of brain PE and PS dissolved in decane at 20 mg/ml. Total SR [9] was added to the *cis* (cytosolic) solution composed of 500 mM Cs-methanesulfonate, 10 mM CsCl and 10 mM HEPES-Tris pH 7.2. The *trans* (lumenal) solution was 50 mM CsMethanesulfonate, 10 mM CsCl, and 10 mM HEPES-Tris pH 7.2. The contaminant free Ca^{2+} of the *cis* chamber was in the range of 1 to 3.6 μM (indicated in the text as ≈3 μM) and was measured by Ca^{2+} electrode. The free Ca^{2+} of the *cis* solution in the range of pCa 9 to pCa 7 was adjusted with 1 mM Na_2EGTA plus $CaCl_2$ and, when appropriate $MgCl_2$, according to a computer calculation described elsewhere [10]. The free Ca^{2+} in the range of pCa 6 to pCa 4 was adjusted without EGTA and was verified by a Ca^{2+} electrode. Fatty acids were added to the *cis* solution from stock solutions prepared in methanol. The final concentration of methanol of the *cis* solution was ≥5% v/v and had no effect on channel activity.

2.3. Multiple channel activity index

Because the total number of channels in a given bilayer could not be determined in all cases, we used $\Sigma\, np_L$ as an index of channel activity where, n, is the conductance level corresponding to the number of channels simultaneously open (n = 0, 1, 2, etc.) and, p_L, is the experimentally observed fraction of the total recording time (typically 90 s) spent in a given conductance level. For each experimentally observed conductance level we computed the product $n \times p_L$ and this product was

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Abbreviations: EGTA, (ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetra acetic acid); HEPES, (*N*-2-Hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid); MES, (2-[*N*-Morpholino] ethanesulfonic acid); Tris, (Tris[hydroxymethyl] aminomethane).

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summed over all observed conductance levels. Since the index scored the sum of the probabilities of each conductance level, if two channels were open all the time, its numerical value would be 2.

2.4. Plot of cumulative activity vs. time

Recordings at 0 mV containing one channel during the control and test periods were used. Control recording was 60 to 80 s and test recording was for the same time or less depending on bilayer stability. Open probability (P_o) was measured continuously in time and averaged within intervals of 680 ms. The P_o during an interval of 680 ms was summed to the P_o of the next interval and the sum was plotted as a function of time.

2.5. $^{45}Ca^{2+}$ fluxes

Cardiac SR was actively loaded with $^{45}Ca^{2+}$ as described previously [11,12] by incubation of ≈90 μg of protein in 100 μl of 150 mM K^+-Gluconate, 5 mM Mg^{2+}-acetate, 100 μM $^{45}Ca^{2+}$-acetate, 1 mM MgATP, 20 mM MES-Tris pH 6.8, for 3 min at 37°C. Release was initiated by dilution of 90 μl of the $^{45}Ca^{2+}$-loaded sample into 1 ml of 150 mM K^+-Gluconate, 5 mM Mg^{2+}-acetate, 20 mM MES-Tris pH 6.8 without or with 50 μM fatty acid. Diluted samples were filtered after 5 s on 0.8 μm nitrocellulose filters (Millipore, Danvers, MA) and washed twice with 3 ml of 150 mM K^+-gluconate, 5 mM Mg^{2+}-acetate, 20 μM Ruthenium red, 20 mM MES-Tris pH 6.8.

2.6. Chemicals

Free and esterified fatty acids were from Sigma (St. Louis, MO). Stock solutions were prepared in distilled water, 95% methanol or DMSO (dimehylsulfoxide) when appropriate and kept at 4°C. Bovine brain PE (phosphotidylethanolamine) and PS (phosphatidylserine) were from Avanti Polar Lipids (Alabaster, AL).

3. Results and discussion

To determine which fatty acid metabolites affected the Ca^{2+} permeability of cardiac SR, we screened the Ca^{2+} releasing ability of palmitoyl derivatives with different esterifying head groups. Screening was done using a transport assay in which $^{45}Ca^{2+}$ was actively loaded into cardiac microsomes for 3 min at 37°C and the $^{45}Ca^{2+}$ content was measured after dilution of the sample for 5 s in 150 mM K^+-gluconate, 5 mM Mg^{2+}-acetate, 20 mM MES-Tris pH 6.8 with or without (control) 50 μM fatty acid. The results shown in Fig. 1 (left panel) indicated that only palmitoyl CoA and to a lesser extent cholesteryl palmitate, were capable of releasing SR-stored $^{45}Ca^{2+}$. Derivatives containing head groups with an amino alcohol (sphingosine, sphingomyelin), a neutral sugar (cerebroside), or carnitine, were ineffective. In addition, dipalmitoyl phosplolipids and triglycerides were also ineffective (data not shown). In order to compare different SR preparations, the $^{45}Ca^{2+}$ content in the presence of a fatty acid was normalized to the $^{45}Ca^{2+}$ loading capacity of a control sample from the same preparation under the same conditions. As shown in the right panel of Fig. 1, a decrease in $^{45}Ca^{2+}$ content (relative to control) was found when SR was exposed to long-chain (myristoyl C_{14}, palmitoyl C_{16}) acyl CoA derivatives but not to the short-chain (octanoyl C_8) derivatives, free CoA or palmitic acid. In three preparations, 50 μM palmitoyl CoA decreased the $^{45}Ca^{2+}$ content to 67.5 ± 13% (mean ± S.D.) of control and myristoyl CoA decreased the $^{45}Ca^{2+}$ content to 60.7 ± 5.3% of control. In both cases the decrease was statistically significant to $P < 0.05$ using a Student's *t*-test for paired observations. From these results we concluded that Ca^{2+} release from cardiac SR was stimulated by long-chain acyl CoA derivatives and that the fatty acid required esterification to the head group to be effective.

Based on these results we investigated if palmitoyl CoA could directly open cardiac ryanodine receptor channels incorporated into planar bilayers. Fig. 2 shows recordings from the same bilayer during a control period in a cytosolic (*cis*) solution containing 0.1 μM free Ca^{2+} and following *cis* addition of two concentrations of palmitoyl CoA. Palmitoyl CoA produced a large increase in the single channel mean open time which resulted in one channel open most of the time at a concentration of 20 μM, and two channels open at a concentration of 50 μM. As shown in top left panel of Fig. 2, the increase in channel activity became significant at concentrations above 5 μM and resulted in a ≈90-fold change at 20 μM, and ≈500-fold change

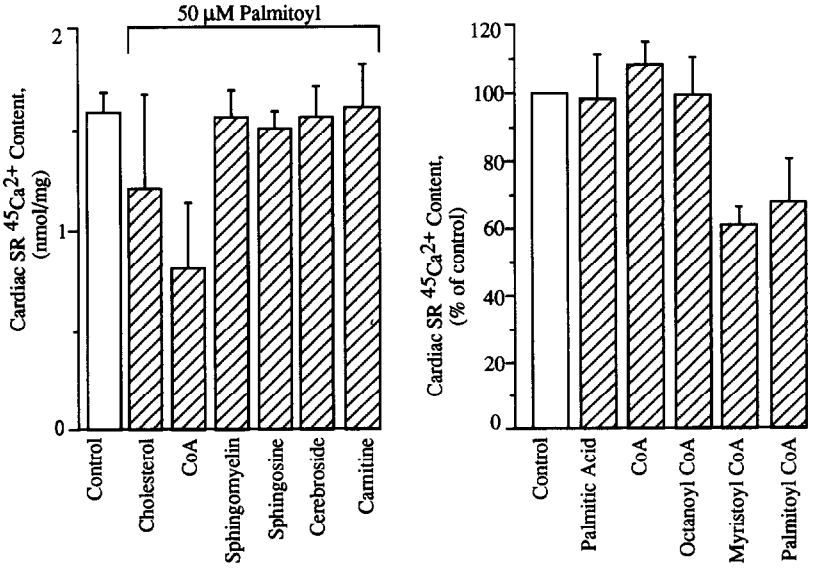

Fig. 1. $^{45}Ca^{2+}$ release from cardiac SR induced by acyl CoA derivatives. Control corresponds to $^{45}Ca^{2+}$ content after 3 min of ATP-dependent uptake at 37°C. Compounds were tested at a concentration of 50 μM during 5 s of exposure at 37°C. Left panel correspond to averages ($n = 3$) in duplicate from the same SR preparation. Right pannel shows normalized content (control = 100%) averaged ($n = 3$ to 8) in three separate SR preparations. Bars corresponds to the S.D.

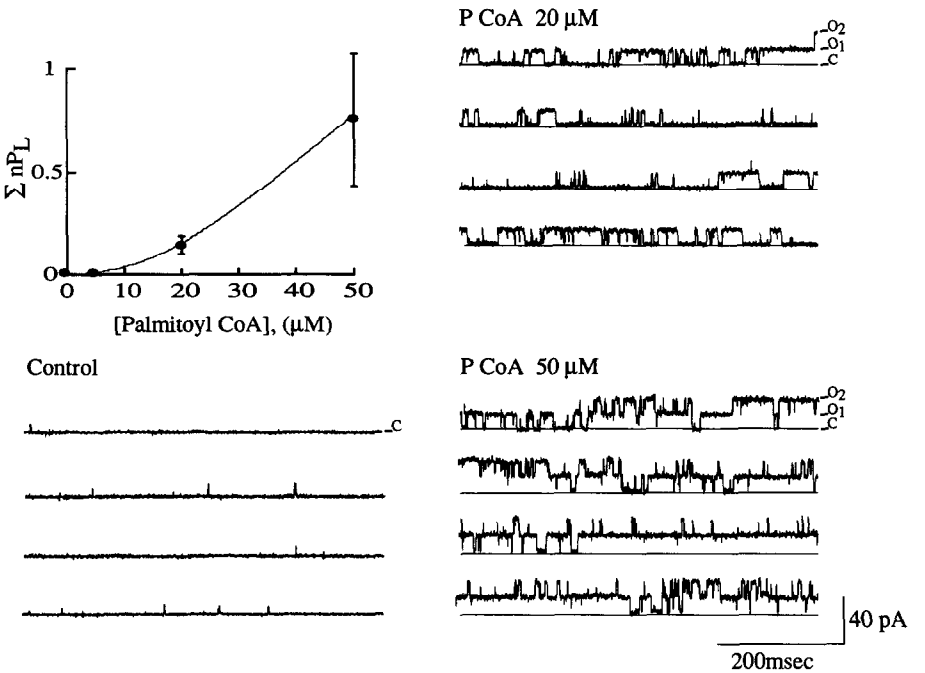

Fig. 2. Dose-dependent activation of cardiac ryanodine receptor by palmitoyl CoA. Recordings are from the same bilayer at 0 mV and *cis* pCa 7 plus indicated *cis* concentrations of palmitoyl CoA. Dose–response curve is the average of two separate bilayer recordings each with multiple channels.

at 50 μM palmitoyl CoA. In three separate recordings, 50 μM palmitoyl CoA increased $\Sigma\ np_L$ from a control value of 0.0015 ± 0.00034 (mean ± S.D.) to 0.75 ± 0.25 within 2 min of compound addition. This increase was statistically significant to $P < 0.05$ using a Student's *t*-test for paired observations.

In Fig. 3 we further examined if channel activation was specifically produced by the long chain acyl CoA derivatives as predicted by the $^{45}Ca^{2+}$ flux assay. Plots of cumulative P_o as a function of time, in which an increase or decrease in slope reflected and increase or decrease in P_o, are shown for the control period (*C*) and following addition of 50 μM metabolite (see section 2). To collect a significant number of openings during the control period, recordings were made in *cis* solutions containing a free Ca^{2+} concentration of ≈3 μM present as a contaminant. Although control and test periods followed each other in time, for the purpose of comparison, they were plotted with the same origin. Palmitic acid (PA), free CoA (CoA), and octanoyl CoA (OCoA) produced relatively minor changes in activity relative to control. In one case (PA), there was a ≈2-fold increase in activity at early times ($t < 20$ s) whereas in another (CoA), there was a ≈2-fold increase at late times ($t > 40$ s). These changes were not consistently observed. On the other hand, palmitoyl and myristoyl CoA produced a large and sustained increase in P_o that resulted in a significant increase in the slope of the cumulative plot. The increase in P_o occurred without a lag and resulted in an almost permanent opening of the channel to $P_o > 0.85$ in each case. The average change in P_o during the test period in two separate recordings of each metabolite (open bars) and three additional recordings in the presence of palmitoyl CoA in 0.1 μM *cis* free Ca^{2+} (hatched bars) are shown in the bottom right panel of Fig. 3. The large activation produced by palmitoyl CoA at pCa 7 reflected the low P_o during the control period when the free Ca^{2+} was in the submicromolar range. These results were in agreement with those obtained in the SR $^{45}Ca^{2+}$ release assays and suggested that the macroscopic increase in SR Ca^{2+} permeability induced by long-chain acyl CoA derivatives was due to an increase in cardiac ryanodine receptor channel activity.

We next examined if palmitoyl CoA stimulated channels in the presence of inhibitory concentrations of *cis* Mg^{2+}. Fig. 4 shows a control recording containing two cardiac channels activated by *cis* ≈3 μM freeCa^{2+} and the subsequent inhibition to virtually zero activity produced by *cis* 3 mM Mg^{2+}. Addition of 20 μM palmitoyl CoA to the Mg^{2+}-inhibited channels resulted in a recovery of approximately 50% of the activity seen during the control period. As shown by the dose response curve of Fig. 4 top left, whereas 3 mM Mg^{2+} inhibited cardiac channels in the control solution, up to 9 mM Mg^{2+} was required to inhibit channels activated by 20 μM palmitoyl CoA. Thus, palmitoyl CoA made the cardiac ryanodine receptor channel less sensitive to inhibition by Mg^{2+}.

Activation of ryanodine receptors by cytosolic free Ca^{2+} in the micromolar range is virtually eliminated by millimolar free Mg^{2+} [2,5]. Therefore it was of interest to investigate if palmitoyl CoA, in the presence of Mg^{2+}, was capable of restoring the Ca^{2+} dependence of the channel. To test the efficacy of palmitoyl CoA, channels were severely inhibited by 5 mM free Mg^{2+} which is a relatively high physiological concentration [4]. In addition, according to Fig. 2, we chose a moderate concentration of 20 μM palmitoyl CoA. Activity vs. *cis* pCa relationships for Mg^{2+}-inhibited channels in the presence and absence of palmitoyl CoA are shown in Fig. 5. In agreement with previous results [5,6], the control data without palmitoyl CoA showed that 5 mM Mg^{2+} totally eliminated the Ca^{2+} dependent activation of the channel in the range of pCa 7 to 4. However, in the presence of 20 μM palmitoyl CoA there was a significant acti-

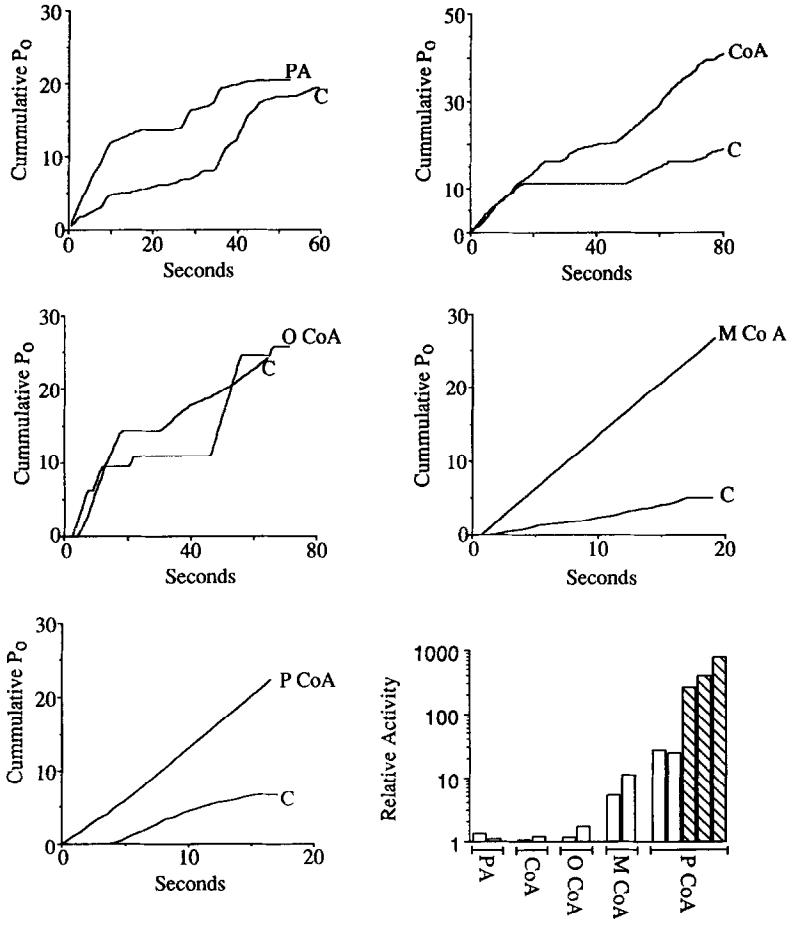

Fig. 3. Specificity of cardiac ryanodine receptor activation by acyl CoA derivatives. Plots of cummulative P_o as a function of time are shown for single channels during control (C) and test periods. Cummulative P_o and recording time were reset to zero after *cis* addition of 50 μM palmitic acid (PA), free CoA (CoA), octanoyl CoA (O CoA), myristoyl CoA (M CoA), or palmitoyl CoA (P CoA). Bottom right panel shows average activity during the test period divided by the average activity during control period. Each bar is a separate recording at ≈ 3 μM *cis* free Ca^{2+} (open bars) or 0.1 μM *cis* free Ca^{2+} (hatched bars).

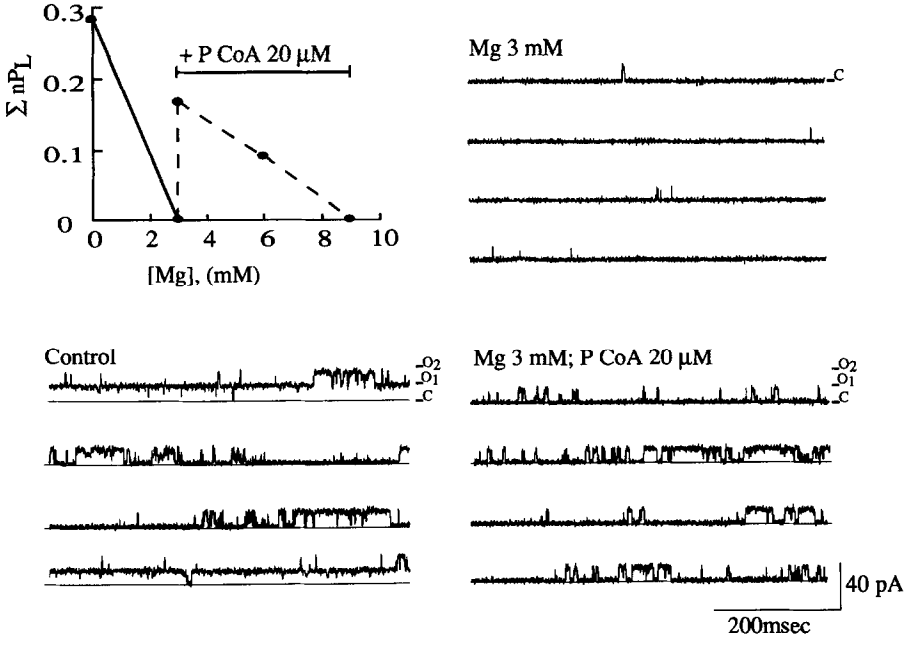

Fig. 4. Removal of Mg^{2+} inhibition of cardiac ryanodine receptor by palmitoyl CoA. Recordings are from the same bilayer at 0 mV and *cis* pCa 6 without EGTA plus indicated *cis* concentrations of total $MgCl_2$ and palmitoyl CoA. Dose–response curve is for the same bilayer recording.

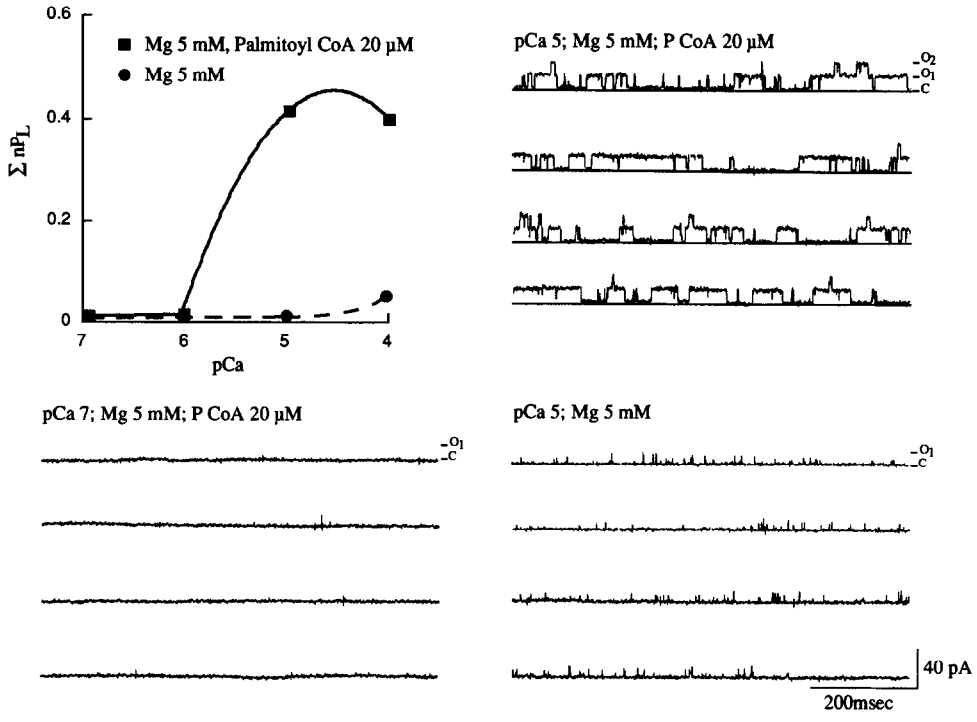

Fig. 5. Ca^{2+} dependence of cardiac ryanodine receptor in the presence of Mg^{2+} and palmitoyl CoA. Recordings are from the same bilayer at 0 mV and indicated *cis* pCa plus 5 mM free Mg^{2+} and 20 μM palmitoyl CoA. Each dose–response curve corresponds to a separate bilayer recording.

vation of the channel in the micromolar range of Ca^{2+}. This is also clear from the records taken from the same channel showing that a *cis* solution of pCa 5 containing 5 mM total Mg^{2+} was not capable of activating the channel unless 20 μM palmitoyl CoA was present. From these results we concluded that palmitoyl CoA partially removed the inhibition by Mg^{2+} which resulted in a Ca^{2+}-dependent activation of the ryanodine receptor. It is important to emphasize that since Mg^{2+} and palmitoyl CoA counteracted each other (Fig. 4), a concentration of palmitoyl CoA lower than 20 μM could have produced the same level of channel activation if the free Mg^{2+} was lower than 5 mM.

Contraction of cardiac myocytes involves a Ca^{2+}-dependent activation of ryanodine receptors [1,2]. However cardiac cells, like many other cells, are known to have a myoplasmic free Mg^{2+} concentration in the millimolar range [4] and millimolar Mg^{2+} is a known blocker of Ca^{2+} release channels in isolated SR and planar bilayers [2,5,6,12]. In the present study, 3 mM free Mg^{2+}, with (not shown) or without ATP, effectively blocked ryanodine receptors whereas, Fabiato [13] reported an optimum free Mg^{2+} of $\approx$3 mM for Ca^{2+}-induced Ca^{2+} release in skinned canine cardiac Purkinje cells. How then is the Mg^{2+}-dependent inhibition of the ryanodine receptor seen in isolated preparations overcome in more intact preparations? There are several mechanisms that may serve this purpose, none of which are mutually exclusive. For example, the cytosolic free Mg^{2+} in the vicinity of the cardiac ryanodine receptor may be lowered below the cellular average by active Mg^{2+} removal [14]. Furthermore, a phosphorylation event [15], an associated protein [16], or a cytosolic component may increase the Ca^{2+} sensitivity of the channel in the presence of Mg^{2+}. In the present report, we explored the latter hypothesis and showed that palmitoyl CoA is a potent activator of the cardiac ryanodine receptor that may directly bind to a site in the channel controlling Ca^{2+} dependent gating. Evidence of a direct binding of long-chain acyl carnitine and CoA to skeletal ryanodine receptors has been presented elsewhere [7,8].

There is insufficient information on the cytosolic concentrations of long-chain acyl CoA to predict if cardiac ryanodine receptors would respond to these metabolites in situ. Blood plasma concentrations of long-chain esterified fatty acids of normally fed individuals are in the range of 2 to 4 μM [17]. Cytosolic concentrations may be much higher because the synthesis of acyl CoA is localized primarily to the outer surface of the outer mitochondrial membrane [18]. In cardiac myocytes, Idell-Wenger et al. [19] estimated a cytosolic pool of total CoA in the range of 5 to 22 μM and a total mitochondrial CoA concentration >2 mM. It is plausible that a cytosolic pool of acyl CoA, maintained by this large concentration gradient directed towards the cytoplasm, may be constantly in contact with the SR. Such maintained interaction of ryanodine receptors with acyl CoA may reduce the Mg^{2+} inhibition of the channel and may bring the channel to a threshold for activation by Ca^{2+}. Thus, acyl CoA activation of the cardiac ryanodine receptor could represent a mechanism by which the sensitivity of the channel to myoplasmic Ca^{2+} is controlled within cells. A deregulation of intracellular Ca^{2+} during metabolic stresses in which fatty acid metabolites are elevated could also occur by this mechanism [7,20].

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