Activation of the Ca^{2+} Release Channel of Skeletal Muscle Sarcoplasmic Reticulum by Palmitoyl Carnitine

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ABSTRACT Studies of [^{3}H]ryanodine binding, $^{45}Ca^{2+}$ efflux, and single channel recordings in planar bilayers indicated that the fatty acid metabolite palmitoyl carnitine produced a direct stimulation of the Ca^{2+} release channel (ryanodine receptor) of rabbit and pig skeletal muscle junctional sarcoplasmic reticulum. At a concentration of 50 μM, palmitoyl carnitine (*a*) stimulated [^{3}H]ryanodine binding 1.6-fold in a competitive manner at all pCa in the range 6 to 3; (*b*) released approximately 65% (30 nmol) of passively loaded $^{45}Ca^{2+}$/mg protein; and (*c*) increased 7-fold the open probability of Ca^{2+} release channels incorporated into planar bilayers. Neither carnitine nor palmitic acid could reproduce the effect of palmitoyl carnitine on [^{3}H]ryanodine binding, $^{45}Ca^{2+}$ release, or channel open probability. $^{45}Ca^{2+}$ release was induced by several long-chain acyl carnitines (C_{14}, C_{16}, C_{18}) and acyl coenzyme A derivatives (C_{12}, C_{14}, C_{16}), but not by the short-chain derivative C_8 or by free saturated fatty acids of chain length C_8 to C_{18}, at room temperature or 36°C. This newly identified interaction of esterified fatty acids and ryanodine receptors may represent a pathway by which metabolism of skeletal muscle could influence intracellular Ca^{2+} and may be responsible for the pathophysiology of disorders of β-oxidation such as carnitine palmitoyl transferase II deficiency.

INTRODUCTION

Lipids, fatty acids, and related metabolites participate in signal transduction pathways that stimulate the release of Ca^{2+} from intracellular stores in a variety of tissues. A well-known cascade is initiated by the breakdown of phosphatidylinositol bisphosphate into inositol 1,4,5-trisphosphate and diacylglycerol (Berridge and Irvine, 1989). Other Ca^{2+}-mobilizing lipid metabolites including arachidonic acid, unsaturated fatty acids, or sphingosine derivatives mobilize Ca^{2+} from intracellular stores but operate independently of the inositol 1,4,5-trisphosphate signaling pathway (Wolf et al., 1986; Chow and Jondal, 1990; Ghosh et al., 1990). Muscle and brain cells have intracellular Ca^{2+} channels sensitive to caffeine and to the muscle-paralyzing alkaloid ryanodine (McPherson et al., 1991). In skeletal and cardiac muscle, ryanodine receptors are abundant in the junctional sarcoplasmic reticulum (Block et al., 1988) and release Ca^{2+} during excitation-contraction coupling in response to cell membrane depolarization (Valdivia et al., 1992*b*). Ryanodine receptors are activated by Ca^{2+} at physiological concentrations and are modulated by a variety of ligands that affect Ca^{2+}-induced Ca^{2+} release such as Mg^{2+}, adenine nucleotides, and caffeine (Ogawa and Ebashi, 1976; Meissner, 1984; Sumbilla and Inesi, 1987; Calviello and Chiesi, 1989; Valdivia et al., 1992*a*). This modulation of ryanodine receptors by intracellular ligands has been critical for understanding the deregulation of intracellular Ca^{2+} that occurs in metabolic diseases of skeletal muscle such as malignant hyperthermia (Fujii et al., 1991; Valdivia et al., 1991*b*).

The intracellular fatty acid metabolite palmitoyl carnitine is a major energy donor for β-oxidation, and its transport into the mitochondrial matrix is facilitated by carnitine palmitoyl transferase II (Woeltje et al., 1990). A possible role of palmitoyl carnitine in the control of intracellular Ca^{2+} was suggested by studies of the autosomal recessive disorder of energy metabolism known as palmitoyl transferase II deficiency (DiMauro and DiMauro, 1973; Hoppel et al., 1980; Demaugre et al., 1988; Haeckel et al., 1990). This is a disorder primarily affecting skeletal muscle and is characterized by the elevation of cellular and plasma levels of palmitoyl carnitine due to a low activity of the mitochondrial transport enzyme (DiMauro and DiMauro, 1973; Demaugre et al., 1988; Haeckel et al., 1990). A molecular defect in palmitoyl transferase II linked to this disorder was recently described (Taroni et al., 1992). Individuals with palmitoyl transferase II deficiency experience recurrent muscle pains, paralysis, muscle necrosis, and myoglobinuria triggered by exercise, cold, or fever (Demaugre et al., 1988). Since muscle wasting is prominent in several muscle disorders and can occur by Ca^{2+} toxicity upon elevation of myoplasmic Ca^{2+} (Martonosi, 1989), we investigated the possibility that palmitoyl carnitine and related metabolites changed the Ca^{2+} permeability of sarcoplasmic reticulum, the primary Ca^{2+} storage site of skeletal muscle cells. Here we show that palmitoyl carnitine and related long-chain esterified fatty acids caused a dramatic increase in the Ca^{2+} permeability of skeletal muscle sarcoplasmic reticulum by a direct activation of the Ca^{2+} release channel formed by the ryanodine receptor. We thus propose that a severe accumulation of palmitoyl carnitine in skeletal muscle, such as that occurring in carnitine palmitoyl transferase II deficiency, leads to an increase of myoplasmic Ca^{2+}, which in turn triggers muscle destruction.

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MATERIALS AND METHODS

Preparation of junctional sarcoplasmic reticulum

Pig soleus muscle or rabbit back and leg white muscle were dissected from anesthetized animals following procedures approved by the University of Wisconsin Research Animal Resources Committee. Soleus muscle was chosen for its ease of dissection and minimal animal bleeding. Preparations occasionally made from pig paraspinous (deep back) muscles yielded a similar receptor density and similar pharmacological properties. Heavy sarcoplasmic reticulum was prepared in both cases as described for rabbit skeletal muscle (Valdivia et al., 1992*a*). Sucrose density-purified membranes sedimenting between 35% and 40% sucrose were used in all experiments. Membranes were stored in 0.3 M sucrose, 0.1 M KCl, and 5 mM sodium *N'*-bis-2-ethanesulfonic acid (Na-PIPES) (pH 6.8) at −80°C.

Binding of [^{3}H]ryanodine to sarcoplasmic reticulum

Assays were carried out as described (Valdivia et al., 1991*a*,*b*). Duplicate samples (40 μg protein each) were incubated for 120 min at 36°C in 0.1 ml of 7 nM [^{3}H]ryanodine, 0.2 M KCl, 10 mM Na-PIPES (pH 7.2), and 1 mM EGTA plus $CaCl_2$ to set free Ca^{2+} in the range of pCa 7 to 3. Free Ca^{2+} was calculated by a computer program that used the stability constants of Fabiato (1988). Samples were filtered onto glass fiber filters (Whatman GF/B or GF/C) and washed twice with 5 ml of cold distilled water. The specific binding was calculated as the difference between the binding in the absence (total binding) and in the presence (nonspecific binding) of 10 μM cold ryanodine. Specific [^{3}H]ryanodine binding in the rabbit and porcine preparations under optimal conditions (1 M KCl, 100 μM Ca^{2+}, 5 mM ATP, 100 nM [^{3}H]ryanodine, 10 mM Na-PIPES, pH 8.5) were 15.5 ± 2.3 and 11.6 ± 2 pmol/mg protein, respectively. Control [^{3}H]ryanodine binding without metabolite was always made in the presence of an equal concentration of methanol, typically 1% (v/v). Methanol had no effect on [^{3}H]ryanodine binding up to a concentration of 5% (v/v). Protein concentration was determined by the Bradford method with a Bio-Rad kit (Richmond, CA).

$^{45}Ca^{2+}$ loading of sarcoplasmic reticulum vesicles

Loading of $^{45}Ca^{2+}$ was described previously (Valdivia et al., 1992*a*). Samples of porcine or rabbit sarcoplasmic reticulum (~2.5 mg protein) were incubated in 1 ml of 5 mM $^{45}CaCl_2$ (~7500 cpm/nmol), 150 mM KCl, and 50 mM MES (2-[*N*-morpholino]ethanesulfonic acid) titrated with Tris to pH 7.2 at room temperature for 2 to 3 h. The total $^{45}Ca^{2+}$ content averaged 58 ± 11 nmol/mg protein in seven preparations from rabbit and 32 ± 6 nmol/mg protein in four preparations from porcine sarcoplasmic reticulum. $^{45}Ca^{2+}$ content was determined by a 50-fold dilution of 20 μl of $^{45}Ca^{2+}$-loaded sarcoplasmic reticulum into 150 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 μM ruthenium red, and 50 mM MES-Tris (pH 7.5) followed by a rinse of filters with 3 ml of the same solution. Background $^{45}Ca^{2+}$ was evaluated in each experiment and was subtracted from each filter. To measure $^{45}Ca^{2+}$ background, duplicate samples were diluted 50-fold in the "Mg^{2+}" solution described below containing 10 μM Ca^{2+} ionophore A23187. Background $^{45}Ca^{2+}$ was ≤1 nmol $^{45}Ca^{2+}$/filter/mg protein. Release was initiated by dilution of 20 μl of the $^{45}Ca^{2+}$-loaded sarcoplasmic reticulum (~50 μg protein) into 1 ml of "Mg^{2+}" solution consisting of 5 mM $MgCl_2$, 1 mM EGTA, 150 mM KCl, and 50 mM MES-Tris (pH 7.2) (see Figs. 4*B*, 5*B*, 7*B*, and 9). Metabolites were added to "Mg^{2+}" solution from stocks prepared in methanol. The same concentration of methanol (≤1%) was always added to controls. Methanol had no effect on $^{45}Ca^{2+}$ efflux up to a concentration of 5% (v/v). The diluted sarcoplasmic reticulum was incubated for 5 s and spread onto a 0.8-μm nitrocellulose filter (Millipore, Danvers, MA). Filters were rinsed under a mild vacuum with 3 ml of 150 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 μM ruthenium red, and 50 mM MES-Tris (pH 7.5). Controls were made to ensure that palmitoyl carnitine did not cause loss of sarcoplasmic reticulum vesicles from nitrocellulose filters.

Rapid filtration of extravesicular $^{45}Ca^{2+}$ passively loaded into sarcoplasmic reticulum vesicles

Rapid filtration experiments (see Figs. 4*A* and 8) were performed as described previously (Valdivia et al., 1992*a*) using a Dupont apparatus (Biologic Instruments, Echirolles, France). Rabbit sarcoplasmic reticulum passively loaded with 5 mM $^{45}Ca^{2+}$ as described above was used in all experiments. Prior to rapid filtration, the extravesicular $^{45}CaCl_2$ was lowered to approximately 7 nM by dilution of 20 μl of the $^{45}Ca^{2+}$-loaded material (50 μg protein) into 1 ml of 5 mM $MgCl_2$, 1 mM EGTA, 150 mM KCl, 50 mM MES-Tris (pH 7.5). The diluted sarcoplasmic reticulum was immediately spread onto the surface of a wet 0.8-μm nitrocellulose filter (Millipore, Danvers, MA), and the rapid filtration was then executed. Filtrations were carried out at constant rate of 4 ml/s. In Fig. 8, the filtration solution was labeled Mg^{2+} (described above), PC + Mg^{2+} (5 mM $MgCl_2$, 1 mM EGTA, 50 μM palmitoyl carnitine, 150 mM KCl, 50 mM MES-Tris, pH 7.5); Ca^{2+} (6.25 mM EGTA, 6.23 mM $CaCl_2$, 150 mM KCl, 50 mM MES-Tris, pH 7.5); or ATP (6.25 mM EGTA, 6.23 mM $CaCl_2$, 1 mM ATP, 150 mM KCl, 50 mM MES-Tris, pH 7.5). The calculated free Ca^{2+} of the "Mg^{2+}" solution was 0.2 nM (assuming a contaminant $CaCl_2$ of 3.5 μM) and that of the "Ca^{2+}" solution was 10 μM. Following rapid filtration, filters were rinsed under a mild vacuum with 3 ml of 150 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 μM ruthenium red, and 50 mM MES-Tris (pH 7.5).

Reversibility of palmitoyl carnitine stimulation of [^{3}H]ryanodine binding and $^{45}Ca^{2+}$ release

To establish whether the stimulation of [^{3}H]ryanodine binding by palmitoyl carnitine was reversible, 800 μg of rabbit sarcoplasmic reticulum was diluted into 2 ml of 0.2 M KCl, 1 mM EGTA, 1.25 mM $CaCl_2$ (10 μM free Ca^{2+}), and 10 mM Na-PIPES (pH 7.2). The suspension was divided into two samples and processed side by side. A control sample was incubated for 30 min at room temperature with 5 μl of methanol (final concentration, 0.5% v/v), whereas the test sample was incubated with 5 μl of stock 10 mM palmitoyl carnitine dissolved in methanol (final concentration, 50 μM palmitoyl carnitine). At the end of the incubation period, 200 μl was removed from each sample, and [^{3}H]ryanodine binding assays were carried out as described above in 0.1 ml of 7 nM [^{3}H]ryanodine, 0.2 M KCl, 1 mM EGTA, 1.25 mM $CaCl_2$, and 10 mM Na-PIPES (pH 7.2) in the absence (control sample) or presence (test sample) of 50 μM palmitoyl carnitine. Binding activities measured in duplicate in control and test samples averaged 0.46 ± 0.1 and 0.95 ± 0.21 pmol/mg, respectively. The higher binding of the test sample verified the stimulation by 50 μM palmitoyl carnitine. To remove palmitoyl carnitine, the remaining control and test samples were centrifuged at 10,000 rpm for 10 min in a benchtop centrifuge, the supernatant was discarded, and the pellet was resuspended in 0.8 ml of 0.2 M KCl, 10 mM Na-PIPES (pH 7.2). This centrifugation and resuspension was repeated three times. Control and test samples washed in this manner were assayed for [^{3}H]ryanodine binding activity as described above. Following the wash, binding activities of control and test samples were 0.39 ± 0.1 and 0.34 ± 0.09 pmol/mg, respectively. The absence of a higher binding in the test sample verified that the stimulation by 50 μM palmitoyl carnitine was reversible. The loss of binding activity of the control sample following the wash was due to a rundown and loss of protein during the wash cycles.

To establish whether the stimulation of $^{45}Ca^{2+}$ release by palmitoyl carnitine was reversible, samples were actively loaded with $^{45}Ca^{2+}$ by the incubation of ~90 μg of rabbit sarcoplasmic reticulum in 100 μl of 150 mM K^+-gluconate, 5 mM Mg^{2+}-acetate, 100 μM Ca^{2+}-acetate, 1 mM MgATP, and 20 mM MES-Tris (pH 6.8) for 3 min at 37°C. Release was initiated by dilution into 1 ml of 150 mM K^+-gluconate, 5 mM Mg^{2+}-acetate, and 20 mM MES-Tris (pH 6.8) without (control) or with (test) 50 μM palmitoyl carnitine. Diluted samples were immediately filtered and washed as described above. $^{45}Ca^{2+}$ retained in the sarcoplasmic reticulum samples was 56.5 ± 2 and 16 ± 1.1 nmol/mg, respectively, in control and test samples, each repeated three times. The loss of $^{45}Ca^{2+}$ from the test samples reflected the stimulation of $^{45}Ca^{2+}$ efflux by 50 μM palmitoyl carnitine. Reversibility was determined by the incubation of 800 μg of sarcoplasmic reticulum in 1 ml of 150 mM K^+-gluconate, 5 mM Mg^{2+}-acetate, 20 mM MES-Tris (pH 6.8)

without (control sample) or with (test sample) 50 μM palmitoyl carnitine for 30 min at room temperature. Control and test samples were centrifuged for 10 min at 10,000 rpm and resuspended by vortex in 2 ml of the same buffer without palmitoyl carnitine. Centrifugation and resuspension was repeated three times. Control and test samples washed in this manner were assayed for $^{45}Ca^{2+}$ uptake as described above. At the end of the uptake period, control and test samples were diluted 10-fold in K^+-gluconate buffer without palmitoyl carnitine. Following the wash, $^{45}Ca^{2+}$ uptake of control and test samples were 86 ± 2 and 97 ± 10 nmol/mg, respectively. The absence of a difference in retained $^{45}Ca^{2+}$ in control and test samples indicated the reversibility of the stimulation by palmitoyl carnitine. The higher uptake of both samples was likely due to a selective loss during the wash cycles of light sarcoplasmic reticulum, which has a comparatively lower internal volume than the heavier sarcoplasmic reticulum.

Planar bilayer recording of Ca^{2+} release channels

Planar bilayer formation and recording were described previously (Coronado et al., 1992). Bilayers were composed of equal concentrations of brain phosphatidylethanolamine and phosphatidylserine dissolved in decane (Aldrich Chemical Co., Milwaukee, WI) at 20 mg/ml. Porcine sarcoplasmic reticulum was used in all recordings. Protein (100 to 200 μg) was added to the *cis* (cytosolic) solution composed of 250 mM CsCl and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid titrated with Tris to pH 7.2. The *trans* (lumenal) solution was the same, except that the CsCl was 50 mM. Recordings were routinely made at +20 mV, which is close to the equilibrium potential for anionic channels in the sarcoplasmic reticulum preparation (Coronado et al., 1992). Anion-selective channels were not present in any of the recordings of this study. The contaminant-free Ca^{2+} of the *cis* chamber was in the range of 1 to 3.6 μM and was measured by Ca^{2+} electrode. Recordings were filtered through a low-pass Bessel (Frequency Devices, Haverhill, MA) at 1 kHz and digitized at 4 kHz. Metabolites were added from stock solutions in methanol, except in the case of free carnitine, which was added in distilled water. A concentration of 1% (v/v) methanol, the highest concentration present in any experiment, had no effect on open channel probability.

Chemicals

Palmitoyl-D,L-carnitine chloride, palmitoyl coenzyme A (free acid), L-carnitine hydrochloride, palmitic acid (sodium salt), other acyl carnitines, acyl coenzyme A, and free fatty acids (octanoyl C_8:0; lauroyl C_{12}:0; myristoyl C_{14}:0; stearoyl C_{18}:0) were from Sigma Chemical Co. (St. Louis, MO). Stock solutions of metabolites (except free carnitine, which was prepared in distilled water) were prepared in 100% methanol and kept at room temperature in dark vials under N_2. Bovine brain phosphotidylethanolamine (PE) and phosphatidylserine (PS) were from Avanti Polar Lipids (Birmingham, AL). Na_2ATP was purchased from Sigma Chemical Co. Ruthenium red (Ruthenium III chloride oxide) and CsCl (Johnson Matthey Materials Technology, Danvers, MA) were purchased from Alpha Products (Andover, MA). [^{3}H]Ryanodine (60 Ci/mmol) and $^{45}Ca^{2+}$ (1 Ci/mmol) were from Du Pont–New England Nuclear (Wilmington, DE).

RESULTS

Stimulation of [^{3}H]ryanodine binding by palmitoyl carnitine

Ryanodine is an alkaloid that binds with nanomolar affinity to the open conformational state of the Ca^{2+} channel formed by a ryanodine receptor tetramer (Carrol et al., 1991; Pessah and Zimanyi, 1991). The number of high-affinity [^{3}H]ryanodine binding sites increases in the presence of ligands that selectively open the channel (Valdivia et al., 1992*b*). We exploited this property of the ryanodine binding site to test whether palmitoyl carnitine could interact directly with ryanodine receptors under the assumption that, by stimulating gating, palmitoyl carnitine would increase [^{3}H]ryanodine binding. Fig. 1 shows the effect of palmitoyl carnitine (labeled PC), palmitic acid (labeled PA), or free carnitine (labeled C) on [^{3}H]ryanodine binding to porcine sarcoplasmic reticulum. Specific binding in a reference solution at 36°C without metabolites was 0.48 ± 0.1 pmol/mg. The reference solution was composed of 7 nM [^{3}H]ryanodine, 0.2 M KCl, 10 μM free Ca^{2+}, and 10 mM Na-PIPES (pH 7.2) and was chosen to ensure that only a small number of receptors would be tagged such that changes in [^{3}H]ryanodine binding produced by the modulator in either direction (stimulation or inhibition) would be noticeable. The total number of [^{3}H]-ryanodine binding sites estimated at optimal salt concentration (1 M KCl), Ca^{2+} (100 μM $CaCl_2$), adenine nucleotide (5 mM ATP), and saturating [^{3}H]ryanodine concentration (100 nM) was 6.3 ± 0.5 pmol/mg at pH 7.2 and 11.6 ± 2 pmol/mg at optimal pH (pH 8.5). These values agreed well with previous reports in porcine sarcoplasmic reticulum in similar solutions (Hawkes et al., 1992). Palmitoyl carnitine in the range of 5 to 50 μM produced an almost linear stimulation of [^{3}H]ryanodine binding that reached 0.76 ± 0.12 pmol/mg at 50 μM, the highest concentration tested. Neither palmitic acid nor free carnitine affected [^{3}H]ryanodine binding, indicating that the stimulatory effect of palmitoyl carnitine was specific for the esterified fatty acid. The stimulation of [^{3}H]ryanodine binding was fully reversible, as demonstrated by binding experiments in palmitoyl carnitine-pretreated sarcoplasmic reticulum (see Materials and Methods). Fig. 2 shows that stimulation of [^{3}H]ryanodine binding by palmitoyl carnitine was observed at all Ca^{2+} concentrations in the pCa range 6 to 3 and was largest at 100 μM free Ca^{2+}. The Hill plots in Fig. 2*B* indicated the absence of changes in Hill coefficients in the presence of palmitoyl carnitine in both the stimulatory (n_H = 0.8) and inhibitory

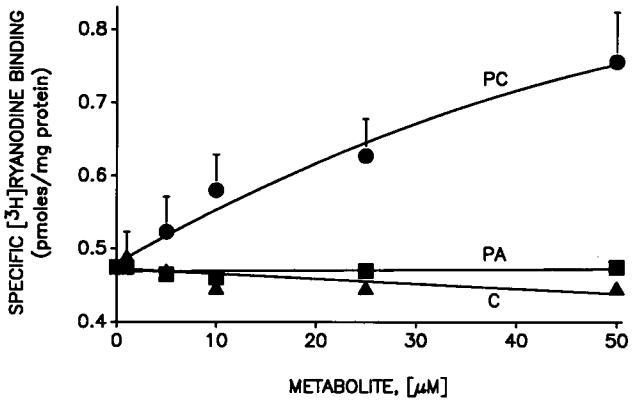

FIGURE 1 Stimulation of [^{3}H]ryanodine binding by palmitoyl carnitine. Effect of palmitoyl carnitine (*PC*, ●), free carnitine (*C*, ▲), and palmitic acid (*PA*, ■) on the specific [^{3}H]ryanodine binding to porcine skeletal muscle sarcoplasmic reticulum. Duplicate samples (40 μg each) were incubated in 0.1 ml of 7 nM [^{3}H]ryanodine, 0.2 M KCl, 10 μM Ca^{2+}, and 10 mM Na-PIPES (pH 7.2) at 36°C for 120 min in the presence of indicated concentrations of PC (mean and SD of three determinations); PA (mean of two determinations); C (mean of two determinations).

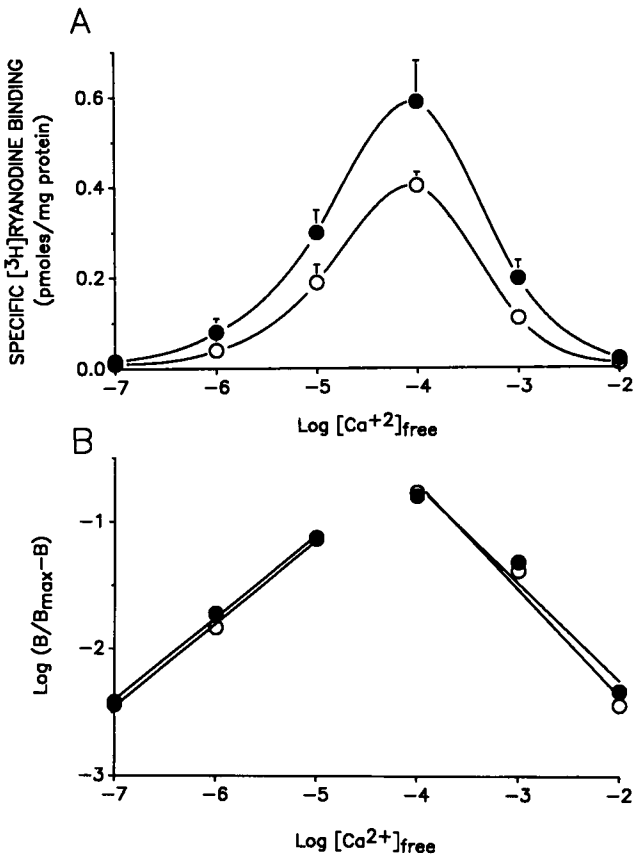

FIGURE 2 Ca^{2+} dependence of palmitoyl carnitine stimulation of [^{3}H]-ryanodine binding. (*A*) Duplicate samples (40 μg each) of procine skeletal muscle junctional sarcoplasmic reticulum were incubated with 7nM [^{3}H]-ryanodine at 36°C for 120 min in reference solution containing 1 mM EGTA and $CaCl_2$ necessary to reach specified free Ca^{2+}. Specific binding in the absence (○) and presence (●) of 50 μM palmitoyl carnitine. (*B*) Hill representation of the same data. Mean and SD (*A*) or mean without SD (*B*) of three determinations.

(n_H = 1.1) phases of the Ca^{2+} dependence of the ryanodine receptor. This Ca^{2+} dependence was similar to that produced by ATP (Pessah and Zimanyi, 1991) but differed from that produced by caffeine, since stimulation of [^{3}H]ryanodine binding by the latter compound is observed only at submicromolar Ca^{2+} (Valdivia et al., 1991*b*).

In experiments shown Fig. 3*A*, fixed concentrations of palmitoyl carnitine (50 μM) and Ca^{2+} (10 μM) stimulated [^{3}H]ryanodine binding when the alkaloid was varied in the range of 1 to 60 nM. A double reciprocal representation (Fig. 3*B*) indicated a linear relationship in which the slope was decreased by the metabolite. The fact that the x-intercepts in the presence and absence of palmitoyl carnitine were nonidentical suggested a noncompetitive bisubstrate type of reaction between palmitoyl carnitine and ryanodine. The parallel lines in the Hill representation of Fig. 3*C* indicated the absence of changes in Hill coefficients (n_H = 1.2). Finally, the Scatchard analysis in the absence and presence of palmitoyl carnitine (Fig. 3*D*) resulted in a significant difference in B_{max} (1.6 ± 0.2 versus 2.8 ± 0.3 pmol/mg, respectively) but not in K_d (16 ± 1.8 versus 19.7 ± 2.1 nM, respectively). Thus the main effect of palmitoyl carnintine was to increase the number of high affinity binding sites available for [^{3}H]-ryanodine without changes in cooperativity of the interaction of Ca^{2+} or ryanodine. These data and those of Fig. 2 are consistent with a Ca^{2+}-independent, noncompetitive stimulation of [^{3}H]ryanodine binding and suggested a direct effect of palmitoyl carnitine on channel gating.

Palmitoyl carnitine-induced release of $^{45}Ca^{2+}$ from sarcoplasmic reticulum

To determine if the observed changes in [^{3}H]ryanodine binding activity affected the Ca^{2+} permeability of sarcoplasmic reticulum, we measured the $^{45}Ca^{2+}$ content of sarcoplasmic reticulum exposed to palmitoyl carnitine. In Fig. 4*A*, $^{45}Ca^{2+}$ content as a function of time was measured by rapid filtration in a reference solution (labeled Mg^{2+}) containing 5 mM $MgCl_2$ and 1 mM EGTA to set the free Ca^{2+} to ≤5 nM. Nanomolar free Ca^{2+} and millimolar Mg^{2+} were chosen so that $^{45}Ca^{2+}$ release in the absence of palmitoyl carnitine would be minimal (Meissner 1984; Valdivia et al., 1992*a*). The results indicated that Ca^{2+} release in the reference solution was no more than 12% of the original content during the first 9 s. However, when 50 μM palmitoyl carnitine was added to the reference solution (labeled PC + Mg^{2+}), $^{45}Ca^{2+}$ content decreased in time and settled at approximately 25% of the original content after 5 s of exposure. Thus the metabolite induced a massive release of the stored Ca^{2+} on a relatively slow time scale. $^{45}Ca^{2+}$ release on the time scale of milliseconds was also investigated and is described in Fig. 8. To determine the size of the Ca^{2+} pool mobilized by palmitoyl carnitine we focused on the steady-state $^{45}Ca^{2+}$ content after prolonged exposure to the metabolite. This was done in Fig. 4*B*, where we simplified the efflux protocol by performing filtrations manually. $^{45}Ca^{2+}$ content was measured by the dilution of a small aliquot of rabbit sarcoplasmic reticulum passively loaded with $^{45}Ca^{2+}$ in the reference solution described above supplemented with the indicated concentration of palmitoyl carnitine (labeled PC), palmitic acid (labeled PA), or free carnitine (labeled C). Following dilution, vesicles were retained on nitrocellulose filters and washed as described in Materials and Methods. The time of exposure of vesicles to the metabolite from the moment of dilution to the moment of filtration and wash was timed at a minimum of 5 s and was never more than 10 s. Dose-response curves obtained in this manner in rabbit sarcoplasmic reticulum are shown in Fig. 4*B*. A similar result in the porcine preparation is shown in Fig. 7. The $^{45}Ca^{2+}$ content in the absence of metabolites was 35 ± 4 nmol/mg (n = 7). The lowest concentration of palmitoyl carnitine tested (5 μM) reduced the $^{45}Ca^{2+}$ content to 32 ± 4 nmol/mg (n = 7), a difference that was statistically significant to a value $t \geq t_{0.1}$. The highest concentrations tested (50 μM) reduced the $^{45}Ca^{2+}$ content to 12 ± 4 nmol/mg (~66% release). The palmitoyl carnitine concentration that released 50% of the $^{45}Ca^{2+}$ content in 5 s was approximately 16 μM. In contrast, neither free fatty acid nor free carnitine in the same concentration range affected

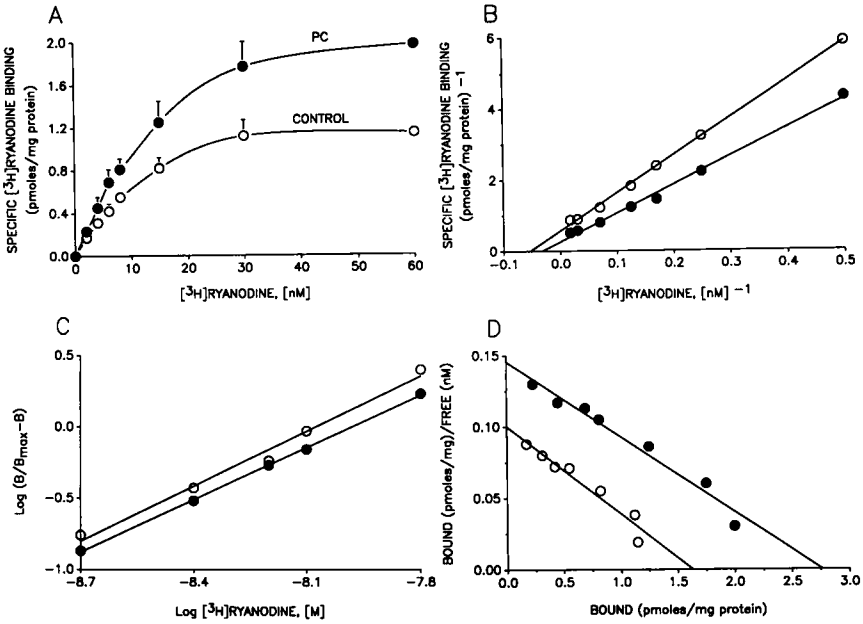

FIGURE 3 Scatachard analysis of palmitoyl carnitine stimulation of [^{3}H]ryanodine binding. (*A*) Duplicate samples of porcine skeletal muscle junctional sarcoplasmic reticulum (40 μg each) were incubated for 120 min at 36°C in reference solution with indicated concentrations of [^{3}H]ryanodine in the absence (○, *CONTROL*) and presence (●, *PC*) of 50 μM palmitoyl carnitine. (*B*) Double reciprocal representation of the same data. Fitted lines extrapolated to K_d values of 18 and 33 nM, respectively, for control binding and binding in the presence of palmitoyl carnitine. (*C*) Hill representation of the same data. Slope of the fitted lines was 1.2. (*D*) Scatchard representation of the same data yielded apparent B_{max} and K_d values of 1.6 ± 0.2 pmol/mg and 16.0 ± 1.8 nM for control binding and 2.8 ± 0.3 pmol/mg and 19.7 ± 2.1 nM for binding in the presence of palmitoyl carnitine. Mean and SD (*A*) or mean without SD (*B, C, D*) of four determinations.

the $^{45}Ca^{2+}$ content. Thus, palmitoyl carnitine and not the free fatty acid nor the esterifying group alone increased the passive Ca^{2+} permeability of the sarcoplasmic reticulum.

Although palmitoyl carnitine affected both [^{3}H]ryanodine binding and $^{45}Ca^{2+}$ efflux in the same concentration range, these results did not establish that Ca^{2+} efflux occurred by activation of the Ca^{2+} release channel. In Fig. 5, we addressed this question by comparing the effect of well-known activators and inhibitors of Ca^{2+} release channels on the palmitoyl carnitine-stimulated [^{3}H]ryanodine binding (Fig. 5 *A*) and the palmitoyl carnitine-induced $^{45}Ca^{2+}$ efflux (Fig. 5*B*). In Fig. 5*A*, the control corresponded to the nonstimulated [^{3}H]ryanodine binding at pCa 5, similar to that shown in Fig. 1. In agreement with previous results (Carrol et al., 1991; Pessah and Zimanyi, 1991; Valdivia et al., 1992) we found that activators of the channel such as caffeine (30 mM), adenine nucleotides (e.g., adenylyl (β,γ-methylene)diphosphate (AMP-PCP), 3 mM), KCl (0.7 M), or alkaline pH (pH 8.5) stimulated [^{3}H]ryanodine binding, whereas blockers such as Mg^{2+} (1 mM), ruthenium red (10 μM), millimolar Ca^{2+} (1 mM), or near-acid pH (pH 6) inhibited [^{3}H]ryanodine binding. Palmitoyl carnitine (50 μM) in the presence of each of the activators (except pH 8.5) increased [^{3}H]ryanodine binding by an amount equal to or greater than that produced by palmitoyl carnitine in the reference solution (labeled control). The lack of stimulation by palmitoyl carnitine at alkaline pH could be due to a saturation of the number of binding sites, since alkaline pH may have increased to nearly unity the open probability of the entire population of Ca^{2+} release channels (Ma et al., 1988). On the other hand, palmitoyl carnitine was not able to stimulate [^{3}H]ryanodine binding in the presence of inhibitors such as ruthenium red, millimolar Ca^{2+}, or pH 6. We noticed, however, that in the presence of Mg^{2+} there was a small, yet statistically significant stimulation. Hence, with the exception of millimolar Mg^{2+}, extreme ligand conditions that generate a strong closure of the channel abolished the stimulatory effect of palmitoyl carnitine. These results strengthened the view that the interaction of palmitoyl carnitine with ryanodine receptors, much like that of caffeine and adenine nucleotides, occurred by modulation of Ca^{2+} release channel gating.

The effect of activators and inhibitors of the Ca^{2+} release channel on the $^{45}Ca^{2+}$ efflux stimulated by palmitoyl carnitine were also consistent with this hypothesis. In Fig. 5*B*, 100% release corresponds to the $^{45}Ca^{2+}$ released by the ionophore A23187 as described in Materials and Methods. We designated as control the $^{45}Ca^{2+}$ efflux induced by the dilution of porcine sarcoplasmic reticulum into a reference solution with a fixed free Ca^{2+} of ~10 μM. This resulted in a ~50% reduction of $^{45}Ca^{2+}$ content at the end of a 5-s incubation period followed by manual filtration. Two features suggested that this efflux was mediated by a Ca^{2+}-dependent activation of ryanodine receptors. First, the open bars of Fig. 5*B* show that $^{45}Ca^{2+}$ release in the reference solution plus 20 μM ruthenium red (labeled RUTH. RED) or in the presence of 5 mM Mg^{2+} without added Ca^{2+} (labeled Mg^{2+}) was substantially reduced, to no more than ~8% of the total $^{45}Ca^{2+}$ content. Second, as will be described later (Fig. 8), ATP dramatically increased the rate of $^{45}Ca^{2+}$ release measured on a millisecond time scale. Both the inhibition by ruthenium red and Mg^{2+} and the increase in the efflux rate by ATP are hallmarks of Ca^{2+}-induced Ca^{2+} release mediated by ryanodine receptors. The filled bars of Fig. 5*B* show that palmitoyl carnitine produced a small decrease in $^{45}Ca^{2+}$ content (increase in $^{45}Ca^{2+}$ release) when added to the control medium but had no effect on the presence of ATP or caffeine. We interpreted this result to indicate that palmitoyl carnitine released $^{45}Ca^{2+}$ from the same pool as micromolar Ca^{2+}, caffeine, and ATP. Thus at sufficiently long times, the loss of $^{45}Ca^{2+}$ content produced by a single ligand, i.e., ATP or caf-

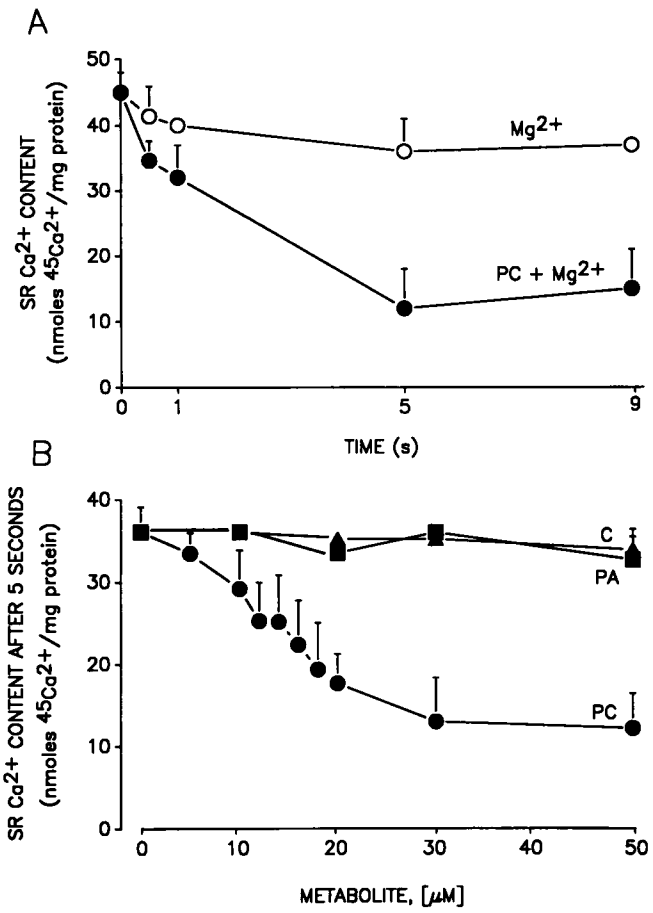

FIGURE 4 $^{45}Ca^{2+}$ content of sarcoplasmic reticulum exposed to various concentrations of palmitoyl carnitine. (*A*) Rapid filtration of $^{45}Ca^{2+}$ passively loaded into rabbit skeletal muscle sarcoplasmic reticulum was performed for the indicated times in duplicate samples each of 50 μg protein. Filtration solutions consisted of 5 mM $MgCl_2$, 1 mM EGTA, 150 mM KCl, 50 mM MES-Tris (pH 7.2) (Mg^{2+}) or 5 mM $MgCl_2$, 1 mM EGTA, 150 mM KCl, 50 mM MES-Tris (pH 7.2) plus 50 μM palmitoyl carnitine (*PC* + Mg^{2+}). (*B*) Duplicate samples (50 μg each) of $^{45}Ca^{2+}$ passively loaded into rabbit skeletal muscle sarcoplasmic reticulum were diluted for 5 s at 36°C in 5 mM $MgCl_2$, 1 mM EGTA, 150 mM KCl, 50 mM MES-Tris (pH 7.2) plus indicated concentrations of palmitoyl carnitine (*PC*, ●), free carnitine (*C*, ▲), palmitic acid (*PA*, ■). (*A*) Mg^{2+}, PC + Mg^{2+} (mean and SD of three to five determinations). (*B*) PC (mean and SD of six to nine determinations), C (mean and SD of three determinations), and PA (mean and SD of 3 determinations).

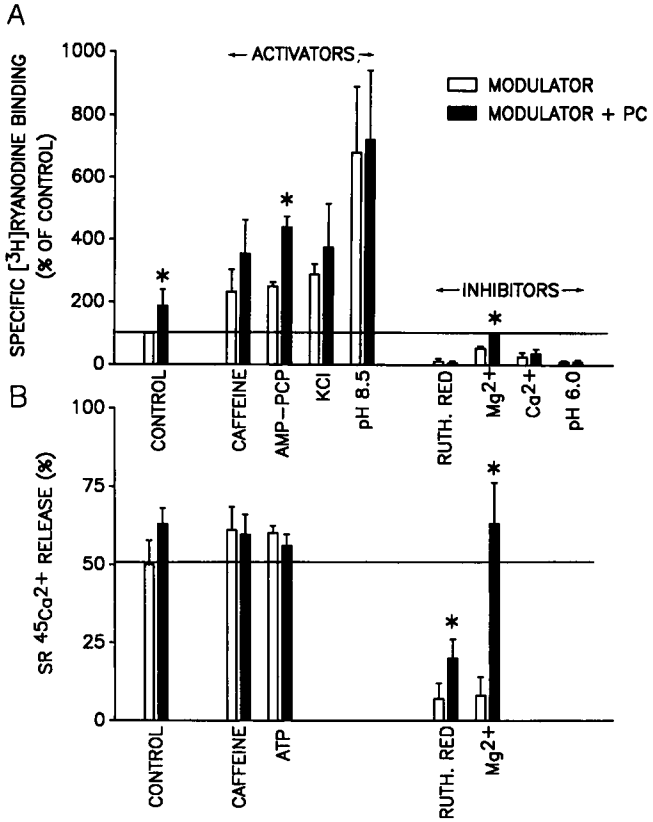

FIGURE 5 Effect of activators and inhibitors of the Ca^{2+} release channel on palmitoyl carnitine-induced activation. (*A*) [^{3}H]ryanodine binding to duplicate samples (40 μg each) of porcine skeletal muscle sarcoplasmic reticulum was assayed in reference solution (0.2 M KCl, 10 mM Na-PIPES, 10 μM Ca^{2+}, pH 7.2, and 7 nM [^{3}H]ryanodine) in the absence (□) or presence (■) of 50 μM palmitoyl carnitine. Reference solution was modified to include activators (caffeine, 30 mM; AMP-PCP, 3 mM; KCl, 0.7 M; pH 8.5) and inhibitors (ruthenium red, 10 μM; Mg^{2+}, 3 mM; pH 6.5) of [^{3}H]ryanodine binding. Control [^{3}H]ryanodine binding labeled 100% was 0.5 pmol/mg protein. (*B*) Duplicate samples (50 μg each) of $^{45}Ca^{2+}$ passively loaded into porcine skeletal muscle sarcoplasmic reticulum were diluted for 5 s at 36°C in solutions without (□) or with (■) 50 μM palmitoyl carnitine. The compositions of solutions were: control, 6.25 mM EGTA, 6.23 mM $CaCl_2$, 150 mM KCl, 50 mM MES-Tris, pH 7.5; caffeine, 6.25 mM EGTA, 6.23 mM $CaCl_2$, 30 mM caffeine, 150 mM KCl, 50 mM MES-Tris, pH 7.5; ATP, 6.25 mM EGTA, 6.23 mM $CaCl_2$, 1 mM ATP, 150 mM KCl, 50 mM MES-Tris, pH 7.5; ruthenium red, 6.25 mM EGTA, 6.23 mM $CaCl_2$, 20 μM ruthenium red, 150 mM KCl, 50 mM MES-Tris, pH 7.5; and Mg^{2+}, 5 mM $MgCl_2$, 1 mM EGTA, 150 mM KCl, 50 mM MES-Tris, pH 7.5. $^{45}Ca^{2+}$ content was 30 ± 4 nmol/mg and was taken as 0% release. Bars in (*A*) correspond to the mean and SD of three determinations. Bars in (*B*) correspond to the mean and SD of three to five determinations. Statistical significance $t > t_{0.025}$ for the difference between the two means (modulator and modulator + PC) is indicated by asterisks.

feine, should be the same as that produced by two ligands acting on the same pool, i.e., ATP or caffeine plus metabolite. On the other hand, when palmitoyl carnitine was added in the presence of inhibitors, there was a partial inhibition by ruthenium red, but Mg^{2+} was unable to inhibit the release induced by the metabolite. Thus palmitoyl carnitine appeared to override the inhibition of the Ca^{2+} release channel by Mg^{2+} but not that produced by ruthenium red.

Taken together, the [^{3}H]ryanodine binding data of Fig. 5*A* and $^{45}Ca^{2+}$ flux data of Fig. 5*B* suggest that palmitoyl carnitine was unlikely to act as a Ca^{2+} ionophore. If this were the case, the releases induced separately by palmitoyl carnitine and ryanodine receptor activators (ATP, caffeine) should have been additive. Consequently, the percentage of $^{45}Ca^{2+}$ released by palmitoyl carnitine in the presence of activators (when Ca^{2+} release channels are open) should have been much larger than the percentage of $^{45}Ca^{2+}$ released by palmitoyl carnitine in millimolar Mg^{2+} and nanomolar Ca^{2+} (when Ca^{2+} release channels are closed). Instead, the data showed that releases in control solution as well as in the presence of activators, with or without metabolite, were not statistically different. The most likely explanation for the fact that palmitoyl carnitine did not increase $^{45}Ca^{2+}$ release under

conditions in which [^{3}H]ryanodine binding was significantly stimulated, such as in the control solution or in the presence of adenine nucleotide, appears to be that the metabolite specifically activated $^{45}Ca^{2+}$ flux in a subpopulation of sarcoplasmic reticulum vesicles sensitive to ryanodine receptor ligands, comprising approximately 65% of the internal volume.

Opening of Ca^{2+} release channels by palmitoyl carnitine

If the interaction of palmitoyl carnitine and ryanodine receptors resulted in a significant activation of the Ca^{2+} release channel, this should be reflected as an increase in open channel probability. To confirm this hypothesis, we performed recordings of Ca^{2+} release channels in planar bilayers using CsCl as the charge carrier (Coronado et al., 1992). In these studies we used porcine junctional sarcoplasmic reticulum, since vesicle-bilayer fusions occurred more readily in this preparation than in preparations of rabbit skeletal muscle. Recording solutions were composed of 250 mM *cis* (cytosolic) and 50 mM *trans* (lumenal) CsCl buffered to pH 7.2. Continuous records compressed in time before and after *cis* addition of 50 μM palmitoyl carnitine are shown in Fig. 6*A*. In five recordings (see Table 1), open channel probability (P_o) monitored over 80 s increased from 0.028 ± 0.04 during control to 0.201 ± 0.10 in the presence of palmitoyl carnitine. The most significant effect was an increase in the number of open events with a comparatively longer duration (Fig. 6*B*). The increase in activity was quantified in Fig. 6*C* as a graph of cumulative P_o as a function of recording time. In this plot, P_o was averaged continuously every 5 s for a total time of 80 s. Each probability was summed to that of the next interval, and the cumulative sum was plotted as a function of time. Thus, the slope is proportional to the number of open events per unit time in each condition. From this plot it is apparent that palmitoyl carnitine produced a constant activation during the time of exposure, and the activation did not decay with time. In kinetic terms, activation by palmitoyl carnitine resulted in an increase in mean open channel duration caused by a shift from mostly short openings to roughly equal proportion of short and long openings. As shown in Fig. 6*D*, a total number of 1272 events collected during the control period were fit by two exponentials with time constants of 0.7 ms (τ short) and 6 ms (τ long) with a ratio of τ short/τ long of 16.6. In the presence of 50 μM palmitoyl carnitine, a total of 8221 events collected during a similar period were fit by time constants of 1 and 5 ms with a ratio of τ short/τ long of 4.3. Table 1 shows that in two separate control recordings in which activity was monitored

TABLE 1 Increase in open probability of Ca^{2+} release channels of porcine skeletal muscle by palmitoyl carnitine

	Open probability (P_o)	Recording time (s)	n
Control	0.028 ± 0.04	798	5
50 μM palmitoyl carnitine	0.201 ± 0.10	1124	
Control	0.101	184	2
50 μM free carnitine	0.088	215	
Control	0.059	230	2
50 μM palmitic acid	0.054	312	

n is the number of recordings, each composed of a control and a test period. Recording time corresponds to the total time during the n recordings. Open probability corresponds to the mean (±SD) of n values weighed according to the recording time.

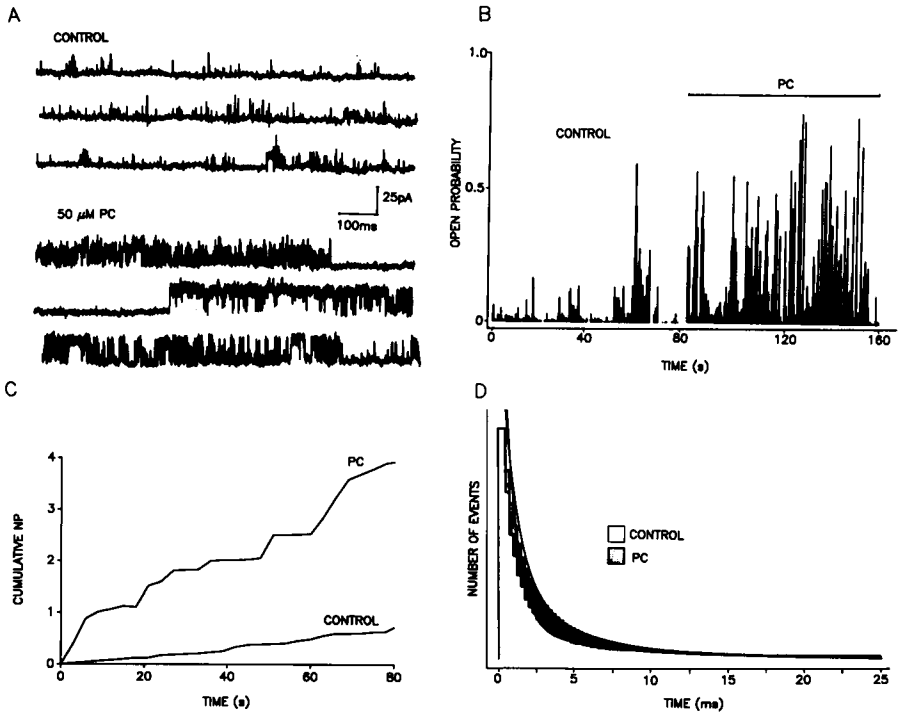

FIGURE 6 Opening of Ca^{2+} release channels by palmitoyl carnitine. (*A*) Recordings from the same channel before and after the *cis* addition of 50 μM palmitoyl carnitine at +20 mV. Openings are shown as upward deflections. (*B*) A total of 160 s of continuous recordings before and after addition of 50 μM palmitoyl carnitine was divided into 250-ms intervals. P_o in each interval is plotted as a bar of length 0 to 1. (*C*) A total of 80 s before and after the addition of 50 μM palmitoyl carnitine were divided into intervals of 3 s. P_o in each interval was added to that of the previous one, and the cumulative sum was plotted as a function of recording time. (*D*) Number of open events (normalized) of different durations is plotted as a function of duration. The number of fitted events was 1272 (control) and 8221 (after 50 μM palmitoyl carnitine). The mean P_o and SD of five determinations are given in Table 1.

before and after *cis* addition of 50 μM palmitic acid, average P_o was 0.06 and 0.05. Similarly, in two recordings before and after *cis* addition of 50 μM carnitine, average P_o was 0.1 and 0.09. Therefore, palmitic acid and carnitine did not significantly alter channel activity, which is consistent with their lack of effect on [^{3}H]ryanodine binding and $^{45}Ca^{2+}$ flux.

Fig. 7*A* shows the activation of a Ca^{2+} release channel by various concentrations of palmitoyl carnitine. The metabolite produced a concentration-dependent increase in the number of openings per unit time. In this particular recording there was an 8-fold increase in P_o from 0.03 in the control period without palmitoyl carnitine to 0.25 in the presence of 100 μM palmitoyl carnitine. The effect of palmitoyl carnitine on open probability saturated between 50 and 100 μM. In Fig. 7*B* we compared the dose-dependent increase in open probability

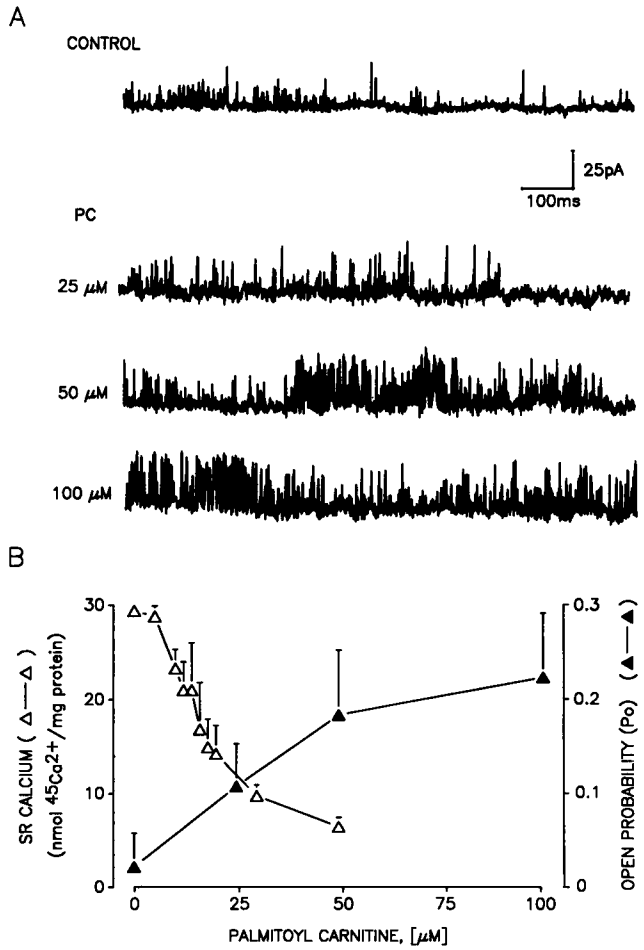

FIGURE 7 Concentration-dependent activation of the skeletal muscle Ca^{2+} release channel by palmitoyl carnitine. (*A*) Single-channel recordings at +20 mV and indicated concentrations of palmitoyl carnitine added to the *cis* solution. Traces are from the same channel before and after addition. (*B*) Open triangles correspond to $^{45}Ca^{2+}$ content of porcine skeletal muscle sarcoplasmic reticulum after exposure to various palmitoyl carnitine concentrations at room temperature 20–22°C. Protocol was the same as in Fig. 4. Filled triangles correspond to P_o of Ca^{2+} release channels from pig sarcoplasmic reticulum at the indicated palmitoyl carnitine concentrations. $^{45}Ca^{2+}$ content (△) corresponds to the mean and SD of three to five determinations. P_o (▲) corresponds to the mean and SD of three determinations.

(open triangles) with the palmitoyl carnitine-induced $^{45}Ca^{2+}$ efflux (filled triangles) in porcine sarcoplasmic reticulum at room temperature (20–22°C). There was a close correspondence between the level of activation of the Ca^{2+} release channel and the increase in sarcoplasmic reticulum Ca^{2+} permeability. This agreement was considered strong evidence that the increase in the sarcoplasmic reticulum Ca^{2+} permeability was generated by a direct opening of the Ca^{2+} release channel by palmitoyl carnitine.

Mechanism of activation of the Ca^{2+} release channel by palmitoyl carnitine

In order to gain insight into the mechanism of channel activation by palmitoyl carnitine, we compared in Fig. 8 the kinetics of Ca^{2+} release by palmitoyl carnitine and that of two ligands that directly open the Ca^{2+} release channel, namely Ca^{2+} and ATP (Ogawa and Ebashi, 1976; Meissner, 1984). The kinetics of release was resolved by rapid filtration, a technique that permits the fast replacement of a reference extravesicular solution with a test solution containing an activator of interest (Valdivia et al., 1992*a*). The reference solution (labeled Mg^{2+}) contained 5 mM $MgCl_2$ plus 1 mM EGTA (~7 nM final free Ca^{2+}) and was chosen to maintain the Ca^{2+} release channel in a closed state (Kawano and Coronado, 1991). Test solutions designed to suddenly open the channel contained 10 μM free Ca^{2+} (labeled Ca^{2+}) or 10 μM free Ca^{2+} plus 1 mM total ATP (labeled ATP). Palmitoyl carnitine (50 μM) was delivered in reference solution (labeled PC + Mg^{2+}) in order to distinguish this activation from that by Ca^{2+} and ATP. The combination of the latter two activators, which open the channel to the highest level (Kawano and Coronado, 1991), resulted in a release of 67 ± 4% of the Ca^{2+} pool in 200 ms with a $t_{1/2}$ of less than 20 ms. Ca^{2+} alone released 49 ± 11% of the pool with a $t_{1/2}$ of approximately 40 ms, whereas Mg^{2+} inhibited release almost entirely (8 ± 10%). Although Ca^{2+} and Ca^{2+} + ATP released

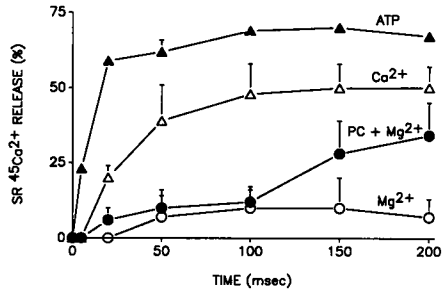

FIGURE 8 Time course of $^{45}Ca^{2+}$ release from sarcoplasmic reticulum induced by 50 μM palmitoyl carnitine. Duplicate samples (50 μg each) of $^{45}Ca^{2+}$ passively loaded into rabbit skeletal muscle sarcoplasmic reticulum were fast-filtered for the indicated time in milliseconds. Filtration solutions were labeled Mg^{2+} (5 mM $MgCl_2$, 1 mM EGTA, 150 mM KCl, 50 mM MES-Tris, pH 7.5); PC + Mg^{2+} (5 mM $MgCl_2$, 1 mM EGTA, 50 μM palmitoyl carnitine, 150 mM KCl, 50 mM MES-Tris, pH 7.5); Ca^{2+} (6.25 mM EGTA, 6.23 mM $CaCl_2$, 150 mM KCl, 50 mM MES-Tris, pH 7.5); and ATP (6.25 mM EGTA, 6.23 mM $CaCl_2$, 1 mM ATP, 150 mM KCl, 50 mM MES-Tris, pH 7.5). Mean and SD of four determinations.

different amounts of stored Ca^{2+} at 200 ms, the releases at 5 s were statistically indistinguishable, in either the presence or absence of palmitoyl carnitine (see Fig. 5*B*). Thus Ca^{2+} release was comprised of fast and slow phases, in agreement with many previous publications (Ikemoto et al., 1985; Calviello and Chiesi, 1989; Meissner, 1984; Brandt et al., 1992). Palmitoyl carnitine produced a release that was much slower than that produced by Ca^{2+} and ATP and had a distinctive lag, in these experiments, of approximately 100 ms. At 200 ms, the release by palmitoyl carnitine was 25 ± 14% or about one-third of the efflux at 5 s (see Fig. 4). These results indicated that the interaction of the ryanodine receptor and palmitoyl carnitine was inherently slow and did not lead to an instantaneous activation of the channel.

An important clue about the steps leading to the activation of the release channel by palmitoyl carnitine was provided by the results of Fig. 9 in which we tested acyl carnitines (labeled AC), acyl coenzyme A (labeled AcoA), and free fatty acids (labeled FA) of saturated hydrocarbon chain length C_8 to C_{18}. In these experiments, $^{45}Ca^{2+}$-loaded sarcoplasmic reticulum was exposed to a constant concentration of metabolite (50 μM) at 36°C, and the $^{45}Ca^{2+}$ content was measured by manual filtration at the end of a 5-s incubation period. The data indicated that long-chain acyl carnitines (C_{14} to C_{18}) but not medium-chain acyl carnitines (C_8 to C_{12}) could induce $^{45}Ca^{2+}$ release. When carnitine was replaced by coenzyme A as the esterifying group, release was enhanced for the medium-chain fatty acid but drastically inhibited for the long-chain fatty acids of the series. Hence the esterifying group may also play a critical role, either by a specific interaction at a receptor site or by changing the solubility or hydrophobicity of the metabolite. The control experiments with free fatty acids were equally significant since none of the free fatty acids in the series could induce $^{45}Ca^{2+}$ release. Hence saturated free fatty acids per se, despite reports to the contrary (Fletcher et al., 1991), do not increase the sarcoplasmic reticulum Ca^{2+} permeability. We also tested the unsaturated oleic (C_{18}:1), linoleic (C_{18}:2), and arachidonic (C_{20}:4) free fatty acids. In our hands, only arachidonic acid (50 μM) induced a Ca^{2+} release significantly different from controls, amounting to 51 ± 9% (n = 4) of the original $^{45}Ca^{2+}$ passively loaded. The results obtained with the carnitine and coenzyme A series suggested that the interaction of palmitoyl carnitine and the ryanodine receptor required a partitioning of palmitoyl carnitine into the membrane phase or into a hydrophobic domain of the receptor since the affinity of the interaction increased as the fatty acid chain length increased. On the other hand, the esterifying group played a critical role in determining the specificity of the interaction for exclusively long-chain length metabolites, as in the case of acyl carnitine derivatives, or exclusively intermediate-chain length metabolites, as in the case of acyl coenzyme A derivatives.

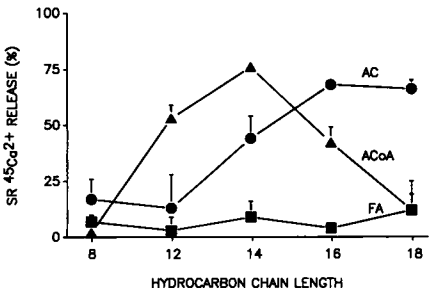

FIGURE 9 $^{45}Ca^{2+}$ content of sarcoplasmic reticulum exposed to acyl carnitines and acyl coenzyme A derivatives of various fatty acid chain lengths. Duplicate samples (50 μg each) of $^{45}Ca^{2+}$ passively loaded into rabbit skeletal muscle sarcoplasmic reticulum were diluted for 5 s at 36°C in 5 mM $MgCl_2$, 1 mM EGTA, 150 mM KCl, 50 mM MES-Tris (pH 7.2) plus 50 μM acyl carnitine (*AC*, ●), acyl coenzyme A (*ACoA*, ▲), or free fatty acid (*FA*, ■). $^{45}Ca^{2+}$ content after rapid filtration for 2 ms in dilution medium in the absence of metabolites was 56 ± 4.5 nmol/mg and was taken as 0% release. Mean and SD of three to seven determinations.

DISCUSSION

The results of this study indicate that palmitoyl carnitine is an activator of the Ca^{2+} channel formed by the ryanodine receptor that directly binds to a site in the channel controlling gating. This conclusion is consistent with earlier findings in which palmitoyl carnitine in the range of 1 to 10 μM reduced the net Ca^{2+} sequestered into skeletal muscle sarcoplasmic reticulum by the Ca^{2+} pump (Messineo et al., 1982). The activation of ryanodine receptors by palmitoyl carnitine did not depend on the animal species and was quantitatively similar in porcine, rabbit, or frog (not shown) skeletal muscle sarcoplasmic reticulum. Although other studies have shown that palmitoyl carnitine inhibits numerous transport proteins, these disturbances occur at metabolite concentrations in the range of 100 to 1000 μM (Corr et al., 1984), which is much higher than those used here to activate the skeletal ryanodine receptor. In recent studies in isolated ventricular myocytes (Meszaros and Pappano, 1990; Wu and Corr, 1992), low concentration of palmitoyl carnitine in the range of 1 to 10 μM produced an inhibition of Ca^{2+} currents, a reduction in the duration of action potentials, and afterdepolarizations resulting in triggered activity that were consistent with Ca^{2+} overloading of cells. The latter could have occurred by an increase in the Ca^{2+} permeability of the cardiac sarcoplasmic reticulum. However, we are not sure at this point if cardiac ryanodine receptors may also be activated by palmitoyl carnitine, inasmuch as we did not detect changes in [^{3}H]ryanodine binding in the heart induced by this metabolite (El-Hayek et al., 1992).

Potential roles of palmitoyl carnitine as a cellular modulator of Ca^{2+} release channels

Palmitoyl carnitine increased the sarcoplasmic reticulum Ca^{2+} permeability in nanomolar free Ca^{2+} and millimolar Mg^{2+}, which are conditions known to prevail in the cytoplasm of resting muscle cells (Maughan and Godt, 1989). This suggests that there is no major impediment for the Ca^{2+}-releasing action of these metabolites in situ. However, there is little information on the intracellular concentrations of

these metabolites that would allow us to compare the sensitivity of the Ca^{2+} release channel to in situ concentrations of acyl carnitines under various metabolic conditions. Plasma concentrations of long-chain acyl carnitines in normal fed individuals are in the range of 2 to 4 μM (Deufel, 1990). Cytosolic concentrations may hence be higher because the synthesis of palmitoyl carnitine is localized primarily to the inner surface of the outer mitochondrial membrane (Murthy et al., 1987; Scholte et al., 1990). It is thus plausible that a cytosolic pool of acyl carnitines may be constantly in contact with the sarcoplasmic reticulum, producing a "chronic" stimulation of ryanodine receptors in situ, albeit to a low open probability.

The possibility that palmitoyl carnitine may always be in contact with the sarcoplasmic reticulum lends itself to several speculations about the potential role of palmitoyl carnitine as a modulator of intracellular Ca^{2+}. For example, a small Ca^{2+} leak from the sarcoplasmic reticulum induced by physiological concentrations of palmitoyl carnitine could influence the resting cytosolic Ca^{2+} and serve to bring the Ca^{2+} release channel to a threshold for activation by voltage or other signals that control the Ca^{2+} permeability of the sarcoplasmic reticulum (Martonosi, 1984). By the same token, palmitoyl carnitine and perhaps other acyl carnitines and acyl coenzyme A derivatives may be utilized by skeletal muscle cells to compensate for the severe Mg^{2+} inhibition of Ca^{2+} release channels (Kawano and Coronado, 1991) which would otherwise leave the intracellular Ca^{2+} stores of skeletal muscle cells unresponsive to cytosolic ligands. It is also of interest to consider roles related to the increase in acyl carnitine production and breakdown that occurs during exercise (Harris and Foster, 1990). For example, an increased sarcoplasmic reticulum Ca^{2+} permeability driven by a sudden increase in palmitoyl carnitine may lead to sarcoplasmic reticulum Ca^{2+} depletion that could contribute to muscle fatigue (O'Brien et al., 1991). Likewise, the increase in acyl carnitine production that occurs in working skeletal muscle could set in motion a positive feedback mechanism whereby the Ca^{2+} released from the sarcoplasmic reticulum by palmitoyl carnitine could activate Ca^{2+}-dependent mitochondrial dehydrogenases (McCormack et al., 1990), which in turn would stimulate β-oxidation and further metabolic demand for palmitoyl carnitine.

Implications for pathophysiology of carnitine palmitoyl transferase II deficiency

Plasma levels of long-chain acyl carnitines are significantly higher in individuals with carnitine palmitoyl transferase II deficiency (Hoppel et al., 1980). In a particular individual, the plasma concentration of long-chain acyl carnitines increased from 14 μM in the fed state to 22 μM under fasting (Hoppel et al., 1980). At these concentrations, our study (Fig. 4) showed that palmitoyl carnitine produced a significant loss (~30–50%) of sarcoplasmic reticulum Ca^{2+}. Thus, in pathologic conditions leading to the accumulation of palmitoyl carnitine, such as in carnitine palmitoyl transferase II deficiency, a severe deregulation of intracellular Ca^{2+} will almost certainly occur. The loss of sarcoplasmic reticulum Ca^{2+} induced by palmitoyl carnitine is an observation that may help to resolve dilemmas first noted in the original descriptions of carnitine myopathy and carnitine palmitoyl transferase II deficient myopathy (DiMauro and DiMauro, 1973; Engel and Angelini, 1973). Both are defects of long-chain fatty acid metabolism, yet as reviewed by Siliprandi et al. (1990), the signs and symptoms are quite different. Progressive muscle weakness, exercise intolerance, and massive accumulation of triglycerides in type I skeletal muscle fibers are characteristic of carnitine-deficient myopathy, whereas the primary features of carnitine palmitoyl transferase II deficiency are recurrent episodes of muscle necrosis, and myoglobinuria induced by fasting, prolonged exercise, and intercurrent illness. We propose that palmitoyl carnitine accumulation in carnitine palmitoyl transferase II deficiency leads to severe deregulation of sarcoplasmic reticulum Ca^{2+} release and consequent muscle destruction under conditions of increased metabolic demand. The latter may not occur in carnitine-deficient myopathy because palmitoyl carnitine is not elevated in this disorder due to a reduction in the muscle levels of free carnitine (Siliprandi et al., 1990).

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