Phosphorylation Modulates the Function of the Calcium Release Channel of Sarcoplasmic Reticulum from Skeletal Muscle

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ABSTRACT The modulation of the calcium release channel (CRC) by protein kinases and phosphatases was studied. For this purpose, we have developed a microsyringe applicator to achieve sequential and multiple treatments with highly purified kinases and phosphatases applied directly at the bilayer surface. Terminal cisternae vesicles of sarcoplasmic reticulum from rabbit fast twitch skeletal muscle were fused to planar lipid bilayers, and single-channel currents were measured at zero holding potential, at 0.15 μM free Ca^{2+}, ±0.5 mM ATP and ±2.6 mM free Mg^{2+}. Sequential dephosphorylation and rephosphorylation rendered the CRC sensitive and insensitive to block by Mg^{2+}, respectively. Channel recovery from Mg^{2+} block was obtained by exogenous protein kinase A (PKA) or by Ca^{2+}/calmodulin-dependent protein kinase II (CaIPK II). Somewhat different characteristics were observed with the two kinases, suggesting two different states of phosphorylation. Channel block by Mg^{2+} was restored by dephosphorylation using protein phosphatase 1 (PPT1). Before application of protein kinases or phosphatases, channels were found to be "dephosphorylated" (inactive) in 60%, and "phosphorylated" (active) in 40% of 51 single-channel experiments based on the criterion of sensitivity to block by Mg^{2+}. Thus, these two states were interconvertable by treatment with exogenously added protein kinases and phosphatases. Endogenous Ca^{2+}/calmodulin-dependent protein kinase (end CaIPK) had an opposite action to exogenous CaIPK II. Previously, dephosphorylated channels using PPT (Mg^{2+} absent) were blocked in the closed state by action of endogenous CaIPK. This block was removed to normal activity by the action of either PPT or by exogenous CaIPK II. Our findings are consistent with a physiological role for phosphorylation/dephosphorylation in the modulation of the calcium release channel of sarcoplasmic reticulum from skeletal muscle. A corollary of our studies is that only the phosphorylated channel is active under physiological conditions (mM Mg^{2+}). Our studies suggest that phosphorylation can be at more than one site and, depending on the site, can have different functional consequences on the CRC.

INTRODUCTION

Muscle contraction and relaxation are regulated by the intrafiber calcium ion concentration, $[Ca^{2+}]_i$ (Fleischer and Inui, 1989). A rise in $[Ca^{2+}]_i$ triggers muscle contraction. The $[Ca^{2+}]_i$ must again be lowered to enable the muscle to relax. In skeletal muscle and heart, the sarcoplasmic reticulum serves a key role in Ca^{2+} uptake, storage, and release. The macroscopic phenomenology in excitation-contraction coupling in skeletal muscle is referred to as depolarization-induced Ca^{2+} release. That is, essentially all of the Ca^{2+} to be mobilized for muscle contraction derives from the sarcoplasmic reticulum, the intracellular store of Ca^{2+}. The action potential at the plasmalemma spreads longitudinally along the length of the fiber and transversely to within the fiber by way of the transverse tubules, invaginations from the plasma membrane. The transverse tubules are junctionally associated with the terminal cisternae, a specialized region of the sarcoplasmic reticulum by way of the "foot structures" (Franzini-Armstrong and Nunzi, 1983). This intracellular junction is referred to as the triad junction. It is across the triad junction that depolarization of the transverse tubule is coupled to the release of Ca^{2+} from the lumen of the terminal cisternae (Fleischer and Inui, 1989).

The machinery involved in the release of Ca^{2+} from the sarcoplasmic reticulum has been defined in molecular terms with the isolation and characterization of the calcium release channel of sarcoplasmic reticulum, also referred to as the ryanodine receptor (Fleischer and Inui, 1989; Inui et al., 1987a, b; Lai et al., 1988; Smith et al., 1988; Hymel et al., 1988). The ryanodine receptors in skeletal muscle and heart have been identified morphologically as the foot structures (Inui et al., 1987a, b; Lai et al., 1988). The three-dimensional structure of the ryanodine receptor from skeletal muscle SR has been determined by image enhancement techniques of electron micrographs. The receptor has fourfold symmetry and is the largest channel structure known (Wagenknecht et al., 1989).

The intracellular calcium release channels are a new class of channels characterized by their large size and fourfold symmetry. There are two main types, the ryanodine and the IP_3 receptors (for reviews, see Fleischer and Inui, 1989; Berridge, 1993; Ferris and Snyder, 1992). These receptors

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Abbreviations used: $[Ca^{2+}]_i$, intracellular free Ca^{2+} ion concentration; CaIPK, Ca^{2+}/calmodulin-dependent protein kinase; CaIPK II, CaIPK type II; CRC, calcium release channel; end CaIPK, endogenous Ca^{2+}/calmodulin-dependent protein kinase; E-C coupling, excitation-contraction coupling; p_o, open probability; PKA, protein kinase A; PPT, potato acid phosphatase; PPT1, protein phosphatase 1; SR, sarcoplasmic reticulum; TC, terminal cisternae.

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serve a vital role in calcium mobilization in most, if not all, eukaryotic cells. Some cells have been found to contain both IP_3 and ryanodine receptors in a single cell type (Walton et al., 1991; Kijima et al., 1993).

How does the depolarization of the transverse tubule in skeletal muscle transduce the release of Ca^{2+} via the calcium release channel of SR, from the lumen of the terminal cisternae of sarcoplasmic reticulum? Two different receptors are known to be involved. The dihydropyridine receptor in the transverse tubule (Adams et al., 1990; Glossman and Striessnig, 1988; Catterall, 1988) appears to serve as a voltage sensor (Rios and Brum, 1987) responding to the excitation of the transverse tubule, and the ryanodine receptor, which then becomes activated, and Ca^{2+} is released from the lumen of the sarcoplasmic reticulum. The precise nature of the coupling in excitation-contraction coupling is largely unknown and represents the next level of knowledge that needs to be elucidated.

Phosphorylation/dephosphorylation by way of protein kinases and phosphatases represents a common motif of modulation of cell function in intracellular signaling. The dihydropyridine receptor is a well studied example (Reuter, 1983; Tsien et al., 1986). Recent reports have indicated that the ryanodine receptor may also be modulated in this way. If so, this could represent an important dimension in E-C coupling. The cardiac ryanodine receptor has been reported to contain a unique phosphorylation site (Ser 2809), which is phosphorylated with Ca^{2+}/calmodulin-dependent protein kinase, thereby activating the calcium release channel (Witcher et al., 1991). Phosphorylation by exogenously added, cAMP-dependent protein kinase to SR containing the cardiac ryanodine receptor was found to increase ryanodine binding by 30% (Takasago et al., 1991). By contrast with heart, phosphorylation of the skeletal muscle ryanodine receptor by endogenous CalPK was detected, but it seemed to lack a functional consequence on the gating of the calcium release channel (Chu et al., 1990). A recent report describes the inactivation of the calcium release channel by phosphorylation of the SR by endogenous CalPK, which could be reactivated by the action of added phosphatase (Wang and Best, 1992); see also Morii et al. (1987). Yet another report describes the activation of the ryanodine receptor by an endogenous kinase activated by ATP. (Herrmann-Frank and Varsànyi, 1993).The combination of the reported studies reflects significant complexity. What are the underlying principles? Does the literature imply that the skeletal muscle ryanodine receptor is modulated differently from the cardiac receptor? Are the diametrically opposite actions of endogenous and exogenous CalPK in skeletal muscle SR real, or could they be referable to subtle differences in experimental conditions as carried out in different laboratories? We carried out a detailed study on the phosphorylation/dephosphorylation of the skeletal muscle ryanodine receptor that provides novel insights into the modulation of the channel. Preliminary reports have appeared (Hymel et al., 1989; Hain et al., 1993, 1994).

MATERIALS AND METHODS

Preparation of terminal cisternae vesicles (TC vesicles) of SR

Terminal cisternae of sarcoplasmic reticulum were isolated from fast twitch skeletal muscle from New Zealand white rabbits as previously described (Saito et al., 1984). Protein was measured according to the method of Lowry et al. (1951) using bovine serum albumin as standard. The amount of ryanodine receptor in the terminal cisternae was measured by ryanodine binding isotherms (B_{max}) according to McGrew et al. (1989). This binding value was used to calculate the stoichiometry of ^{32}P phosphorylation/ryanodine receptor using protein kinases (see below).

Stoichiometry of phosphorylation of CRC in terminal cisternae vesicles

Phosphorylation with protein kinases was carried out at room temperature in 50 μl of assay volume using conditions similar to Witcher et al. (1991), but optimized to achieve higher phosphorylation stoichiometry. Among a number of differences in our protocol, the 10 mM NaF is especially important to inhibit phosphatase activity. Terminal cisternae of sarcoplasmic reticulum (Saito et al., 1984) (1 mg/ml) were phosphorylated with catalytic subunit of PKA (0.42 μg) (provided by Dr. Jackie Corbin) for 5 min in the phosphorylation buffer containing 300 μM [^{32}P] ATP (NEN), 25 mM MOPS/pH 7.0, 5 mM $MgCl_2$, 10 mM NaF, 1 mM EGTA and 1 mM $CaCl_2$.Protein phosphorylation with Ca^{2+}/calmodulin-dependent protein kinase II (CalPK II) (0.13 μg) was performed under similar conditions using 1.5 mM $CaCl_2$ instead of 1 mM $CaCl_2$, and Calmodulin (2 μg) was added additionally.

The reaction was stopped after 5 min by adding 25 μl of SDS dissociation buffer (1% SDS, 5% β-mercaptoethanol, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0). The entire sample was then subjected to SDS-PAGE in 6% gels, 1.5 mm thickness (Laemmli, 1970), followed by autoradiography, to identify radioactive ryanodine receptor protomer, using Kodak X-Omat AR film after the gels had been stained with Coomassie Brilliant Blue. The amount of ^{32}P incorporation into the skeletal muscle ryanodine receptor protomer was determined by counting the radioactivity of the gel bands containing the phosphorylated band referable to the CRC protomer. The molar ratio of ^{32}P/CRC was calculated by dividing the P^{32} phosphorylation (pmoles) by the equivalent amount of CRC as determined from the measured B_{max} of ryanodine binding. The latter was determined for each TC preparation (~25 pmol/mg protein).

Planar bilayer measurements

TC vesicles were fused with Mueller-Rudin planar lipid bilayers following the protocol described by Smith et al. (1986) with minor modifications. Planar bilayers were formed across a 0.25-mm hole in a 6 μm PTFE-teflon sheet with boundary inaccuracies less than 1 μm, which rendered bilayer thinning fast and reproducible and gave high bilayer stability in time and against voltage. The lipid mixture applied was phosphatidylethanolamine, phosphatidylserine (both from Bovine brain), and synthetic diphytanoyl-phosphatidylcholine (all from Avanti Polar Lipids, Inc., Alabaster, AL) in a weight ratio of 5:3:2, dissolved in decane at 50 mg/ml. During bilayer formation, *cis* and *trans* chambers contained 53 mM $Ca(OH)_2$/250 mM HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid], pH 7.4. For fusion 200 mM choline-Cl was added to the *cis* chamber, and 10 μl of the vesicle suspension (3.5 μg total protein) was applied near the bilayer. Fusion was monitored by Cl^--specific currents. After the first fusion event, the *cis* chamber was stirred and perfused for 4 min at 3 ml/min with 115 mM Tris/250 mM HEPES, pH 7.4. Then 1 mM EGTA and $CaCl_2$ was added, which reduced free Ca^{2+} concentration to 0.15 ± 0.05 μM in all experiments as measured by a Ca^{2+} electrode (Orion SA720) after each experiment. The chambers always contained 1.3 ml of solution. All additions or treatments in this study start from this condition of *cis*-solution, and perfusion during experiments mimic this initial condition by perfusion for 4 min at 3 ml/min with 115 mM Tris/250 mM HEPES, pH 7.4, 1 mM EGTA, and 0.15 μM

free Ca^{2+} adjusted by the Orion Ca^{2+} electrode. Electrical contact was made by Ag/AgCl electrodes via Agar-bridges (1% Agar in 1 M KCl). Voltage is expressed as the voltage applied to the *cis* chamber. The presented Ca^{2+} channel currents, all observed at 0 mV holding potential, therefore, are negative and are shown as downward deflections. The voltage signal across the feedback resistance (10^{10} ohms) of the current-measuring operational amplifier was filtered at 1 kHz and stored on a pulse-code-modulated audio tape recorder modified to accept DC signals. For fast acquisition and analysis of single-channel data, the data were transferred to a hard disk of a personal computer, at a sampling rate of normally 1 or 10 kHz if required for resolution, and analyzed using programs AXOTAPE and PCLAMP, version 5.5, from Axon Instruments. Channel open probabilities (p_o values) were determined for consecutive 8-s periods of single-channel traces from areas of Gaussian distribution with best fit to peaks of amplitude histograms. Mean currents and unitary channel currents were determined from maxima of Gaussian distribution at best fit. The p_o values for channels blocked by Mg^{2+} were too low to be determined from Gaussian fits to amplitude histograms; they were estimated from basal widths of all negative current spikes reaching at least half-single-channel current (resolvable channel events were rare) and, therefore, are given as an upper limit. For presentation of single-channel traces, the data were filtered at 300 Hz.

Additions to the *cis* chamber

Times of additions of ATP, Mg^{2+}, protein kinases, and protein phosphatases are specified in the figures. Additions of ATP and Mg^{2+} refer always to additions of 0.5 mM ATP-Tris and 3 mM $MgCl_2$. Free Mg^{2+}, in the presence of ATP, was 2.6 mM as calculated from Robertson and Potter (1984). Kinases and/or phosphatases were applied either by adding to the *cis* chamber or directly to the surface of the bilayer.

Microsyringe application of reagents directly to the bilayer interface

The syringe, of inner diameter 0.85 mm, is adjusted with its end to the membrane (center to center) at a distance of 0.15 mm. It can be removed for refilling and accurately replaced to the same position (see also Chadwick et al., 1992). The microsyringe delivers a small volume (1 μl) of enzyme or reagent directly to the bilayer interface. After treatment, the reagent is separated away from the bilayer interface by removal of the microsyringe and stirring (>1000-fold dilution). The next reagent can then be added by microsyringe, which can be accurately repositioned at the bilayer surface. In this way, the channel system can be treated sequentially and repeatedly with enzymes that have different or opposite action. This approach represents a new and powerful approach to study channel modulation. In our study, the action of highly purified kinase can be reversed by highly purified phosphatase in a cyclical manner.

Ca^{2+}/Calmodulin-dependent protein kinase II (CalPK II) (Schulman, 1984), kindly provided by Dr. Howard Schulman (Department of Pharmacology, Stanford University School of Medicine, Stanford, CA) or by Dr.Thomas R. Soderling (Vollum Institute, Oregon Health Science University, Portland, OR) (Brickley et al., 1990) was applied in 1-μl aliquots directly to the membrane via the microsyringe. Both kinases are highly purified; the latter is prepared by recombinant DNA technology. The applied solution contained 7.5 μg/ml CalPK II, 50 μg/ml calmodulin, 0.5 mM ATP-Tris, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM $CaCl_2$ in 115 mM Tris/250 mM HEPES buffer at pH 7.4. Time intervals of application are shown by arrows in the figures (arrows with "t" refer to tube application). At the end of the interval, the syringe was removed and the bath stirred for 20 s to dilute the 1 μl applied into the 1.3 ml *cis* solution.

Protein kinase A (PKA) catalytic subunit, purified to near homogeneity (kindly provided by Dr. Jackie Corbin, Vanderbilt University, School of Medicine, Nashville, TN (Flockhart and Corbin, 1984)) was either applied to the bath (available in sufficient amounts) or via the microsyringe. For bath application of PKA, the catalytic subunit was dissolved in 115 mM Tris/250 mM HEPES, pH 7.4, with 6 mg/ml dithiothreitol at 0.05 mg protein/ml and 10 μl of solution (18 units) was added. For syringe application, it was present in 3 mM $MgCl_2$, 0.5 mM ATP-Tris, 1 mM EGTA, 3 mg/ml dithiothreitol, 115 mM Tris/250 mM HEPES, pH 7.4, at a concentration of 0.025 mg protein/ml (1 unit/μl).

Protein phosphatase 1 (PPT1) was also highly purified. It was prepared by recombinant DNA technology and kindly provided by Dr. Ernest Lee (Department of Biochemistry, University of Miami School of Medicine, Miami, FL (Zhang et al., 1992)), was applied via the microsyringe in 1-μl aliquots of solution containing 2 μM PPT1, 0.2 mM $MnCl_2$, 3 mM $MgCl_2$, 0.5 mM ATP-Tris, 1 mM EGTA in 115 mM Tris/250 mM HEPES buffer at pH 7.4.

Acid phosphatase from potato Type III, purchased from Sigma Chemical Co. (St. Louis, MO) (0.7 ml, obtained in 3.2 M $(NH_4)_2SO_4$, 1% serum albumin at pH 6.0), was dialyzed for 6 h against 200 ml of solution (2 times) with one buffer change after 3 h containing 115 mM Tris/250 mM HEPES at pH 7.4 (corresponding to one unit PPT in 13 μl). For bath application, 65 μl (5 units) was added to the *cis* buffer and for tube application PPT was present in 3 mM $MgCl_2$, 0.5 mM ATP-Tris, 1 mM EGTA, 115 mM Tris/250 mM HEPES (pH 7.4) at 0.01 unit PPT/μl.

RESULTS

After fusion of TC vesicles and exchange of *cis* solution, membrane currents were monitored at zero holding potential following a standard protocol of additions to the *cis* chamber. The first three conditions were always the same: 1) no ATP or Mg^{2+} was present ("state" index $--$); 2) 0.5 mM ATP was present, but no Mg^{2+} (index $+-$); and 3) both 0.5 mM ATP and 2.6 mM free Mg^{2+} were present (index $++$). The free Ca^{2+} concentration was 0.15 ± 0.05 μM in all experiments (cf. Materials and Methods). The observed currents were referable to calcium release channels (CRC) as judged from unitary current values of 4.3 ± 0.2 pA from reversal potentials of ≈+40 mV, from block of current by ruthenium red or open stabilization by ryanodine, routinely assayed at the end of the experiments.

From more than 100 experiments using two skeletal muscle TC vesicles preparations, we selected those where a single CRC had been observed (51 experiments). Among these, the observed CRC response to Mg^{2+} fell into two distinct classes for each of the two TC preparations. In 31 experiments, the channels were blocked by Mg^{2+}; that is, the open probability was reduced from 0.65 ± 0.11 to below 0.004 (see Table 2, states d_{init}^{+-} and d_{init}^{++}). The states are defined in Fig. 1 legend and below (second paragraph). Typical examples are shown in Figs. 1 and 2. In the other 20 experiments, no block by Mg^{2+} was observed but a reduction of p_o from 0.67 ± 0.08 to 0.33 ± 0.09 (see Table 2, states p_{init}^{+-} and p_{init}^{++}, and two examples in Figs. 3 and 4). For both classes of observations (d_{init} and p_{init}), similar p_o values were found before Mg^{2+} addition (see p_{init}^{+-} and d_{init}^{+-} in Table 2).

Based on this observation of two distinct responses of the Ca^{2+} release channels to Mg^{2+}, we investigated whether this is referable to the state of phosphorylation. Indeed, in experiments where Mg^{2+} blocked activity, application of protein kinases led to recovery of channel activity. Fig. 1 shows reactivation by Ca^{2+}/calmodulin-dependent kinase II (CalPK II). In 7 out of 10 experiments at identical conditions as in Fig. 1, we observed channel recovery from Mg^{2+} block by CalPK II to p_o values of 0.10 ± 0.02 (see Table 1). Similarly, phosphorylation by protein kinase A (PKA) removed channel block by Mg^{2+} in 8 out of 11 attempts to p_o values of 0.23

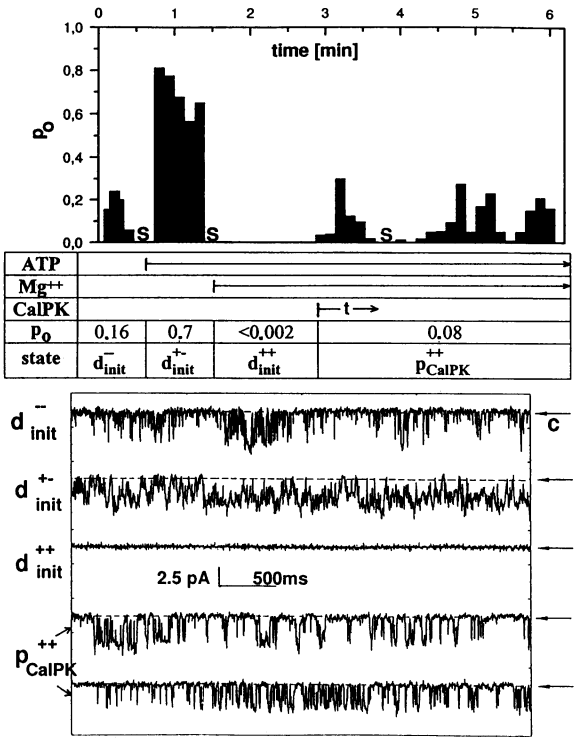

FIGURE 1 Phosphorylation by CalPK II removes channel block by Mg^{2+}. Channel activity, initially sensitive to block by Mg^{2+} (see state d^{+-}_{init} and d^{++}_{init}) recovers from Mg^{2+} sensitivity by application of CalPK II (state p^{++}_{CalPK}). For this and all other figures, the following pertain. Holding potential was 0 mV. Changes of channel open probability (p_o) or mean current were determined for consecutive periods of 8 s (cf. Materials and Methods) in response to particular sequences of additions, all to the *cis* side. Additions of ATP and Mg^{2+} were always to final concentrations of 0.5 mM ATP and 3 mM Mg (2.6 mM free Mg^{2+}). "S" refers to stirring for 20 s. The arrows with index "t" indicate tube addition close to vicinity of bilayer and not to the bath (arrows without "t"). This is achieved by using the microsyringe (see Materials and Methods), adjusted for the placement of 1 μl of solution to the membrane. Upon removal of the syringe, the 1 μl of solution is diluted into the 1.3 ml of *cis* solution by stirring (denoted by "s"). See Materials and Methods for composition of applied solutions to the bath, e.g., ATP, Mg^{2+}, CalPK II, etc. "State" symbols: d_{init} and p_{CalPK} refer to initially dephosphorylated and CalPK II-phosphorylated states of the channel, respectively. Upper indices refer to presence (+) or absence (-) of ATP and Mg^{2+}, respectively. Typical activities of the channel observed in the four distinguished "states" are shown by traces, 4 s each and low-pass-filtered at 300 Hz. In this figure the activity, recovered by CalPK application exhibited bursting (see p_o variations in the histogram) where the two traces for p^{++}_{CalPK} show typical activities during bursts.

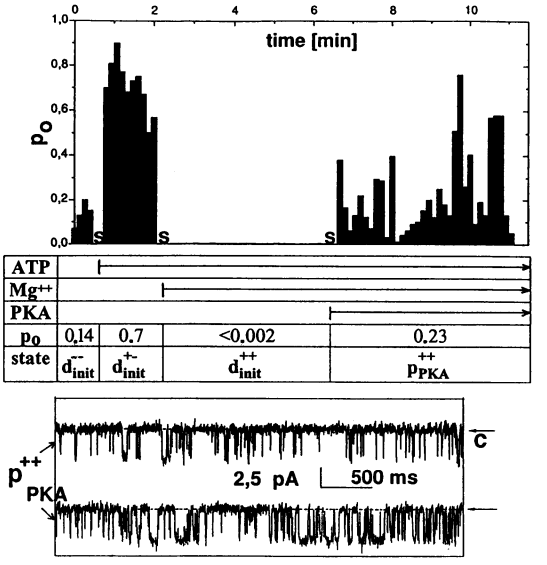

FIGURE 2 Phosphorylation by PKA removes channel block by Mg^{2+}. Similar protocol to Fig. 1 except that recovery from block by Mg^{2+} was achieved by application of catalytic subunit of PKA to the bath. The two traces show lowest (*top*) and average (*bottom*) activity of the PKA phosphorylated state p^{++}_{PKA}. For states d^{--}_{init} and d^{+-}_{init} channel traces were indistinguishable from those in Fig. 1 and, therefore, not included.

$\pm$ 0.03 (see Table 1). One example is shown in Fig. 2. Single-channel traces before PKA application were similar to those in Fig. 1. PKA led to channel activity with a p_o value of 0.23 (Fig. 2). The two traces for state p^{++}_{PKA} (*bottom*) show minimal activity (*top*) and average activity (*bottom*) observed in this experiment. Application of CalPK II solution (1 μl) applied directly to the membrane via microsyringe was sufficient; it would have required 1000 times more reagent when added to the bath. PKA, and PPT available in larger quantities, could be added directly to the bath or via the microsyringe. Similar results were observed by either mode of application. In the unsuccessful attempts, three for each kinase (see Table 1), CRC reactivation occurred either delayed or to significantly lower p_o values. No reactivation at all was observed in only one experiment when using CalPK II, probably because of misalignment of the microsyringe used for direct application to the membrane.

Because channel open probability qualitatively and persistently changed upon application of either kinases, it can be inferred that phosphorylation of some sites took place (denoted by state index p_{PKA} or p_{CalPk}) and that these sites were dephosphorylated before application of the kinases (denoted by state index d_{init}). This assignment was further supported by the complementary study of channels that initially were not blocked by Mg^{2+}. Induction of block was found by treatment with phosphatases. Figs. 3 and 4 show examples of this inhibition. Both phosphatases available to us, PPT1 and acid phosphatase from potato (abbreviated by PPT), gave similar results, summarized in Table 1. p_o values dropped from 0.33 $\pm$ 0.09 to <0.03 in six out of eight attempts using PPT1 and to <0.009 in seven out of eight attempts using PPT. The state before phosphatase application is denoted by the index p_{init}, because there are sites that were phosphorylated before they were dephosphorylated by either PPT (index d_{PPT}) or PPT1 (index d_{PPT1}).

Thus, phosphorylation and dephosphorylation interconvert the two states with respect to the Mg^{2+} block, and this justifies the assignments made for the initial states with regard to being phosphorylated (p_{init}) or dephosphorylated (d_{init}). These studies indicate that it is the phosphorylated channel that is active in the presence of Mg^{2+}.

Interconvertability of the two states was further reinforced by cyclic dephosphorylation/rephosphorylation; see Table 1 and the experiments shown in Figs. 4 and 5. In Fig. 4, the

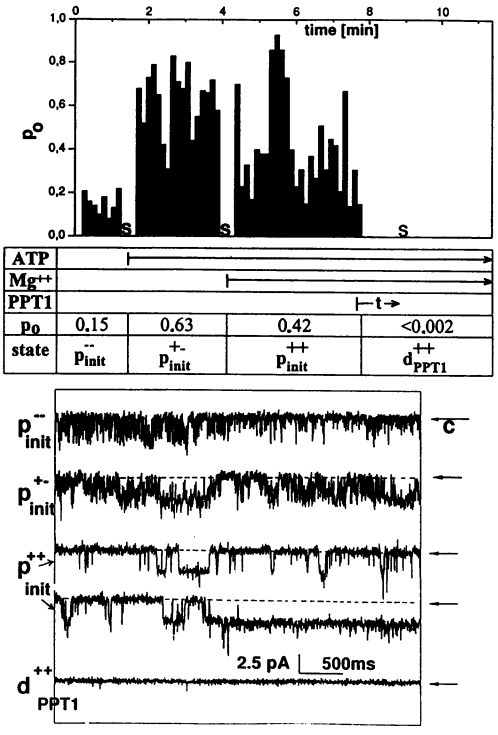

FIGURE 3 Dephosphorylation by PPT1 reinstalls channel block by Mg^{2+}. The observed channel was not blocked by Mg^{2+} and, therefore, was assumed to be initially phosphorylated (state p_{init}). Mg^{2+} reduces mean open probability (quantitated by p_o; see also Table 2). Application of protein phosphatase 1 (PPT1) by the microsyringe abolished channel activity.

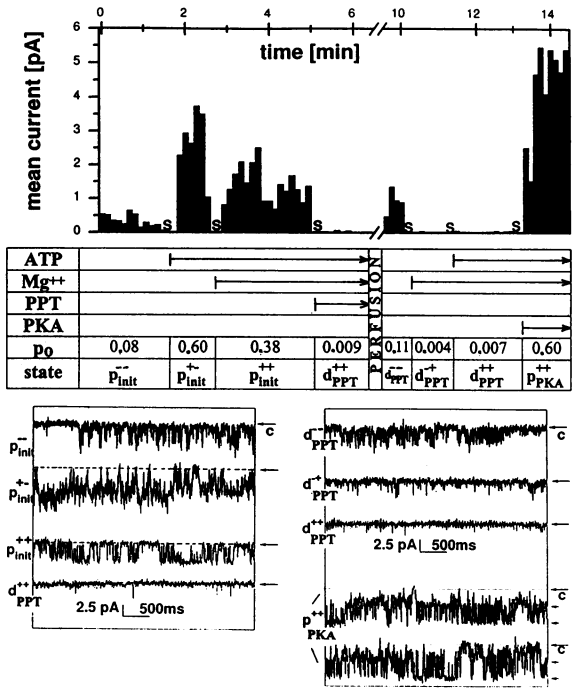

FIGURE 4 Effect of sequential dephosphorylation and rephosphorylation using PPT and PKA. The first four conditions (from state p_{init}^{--} to d_{PPT}^{++}) are the same as in Fig. 3 with comparable changes of activity, except that PPT (acid phosphatase from potato type III) was used. Dephosphorylation reinstalled sensitivity of the channel to block by Mg^{2+}, because the activity recovered after perfusion with Mg-free buffer and was again blocked by Mg^{2+}, both in the absence and presence of ATP, states d_{PPT}^{-+} and d_{PPT}^{++}, respectively, and because this block was reversed by application of PKA (state p_{PKA}^{++}). Before PKA application, all traces were essentially single-channel traces (no overlappings of two channel currents) except for state p_{init}^{+-} showing fast unresolved openings to the second level, which were neglected in estimating the p_o value of ~0.6 for the mainly active channel. After PKA application two channels were active. The population of levels fitted a binomial distribution with $p_o = 0.6 \pm 0.15$, which can be used for better comparison with the p_o values of the previous states. However, these two channels do not act fully independently as evident from some synchronous opening and closing events of the two channels, especially seen in the lower trace for state p_{PKA}^{++}. For this reason, the multichannel traces in Figs. 4 and 5 cannot readily be quantitated. They do show that the ensemble of multichannels responds qualitatively to cyclical activation by kinases and inactivation by phosphatases.

channel activity showed initially no block by Mg^{2+} but only the characteristic reduction of activity from p_{init}^{+-} to p_{init}^{++} (cf. Table 2). PPT induced Mg^{2+} block (state d_{PPT}^{++}) upon which PPT, Mg^{2+}, and ATP were removed by perfusion. This led to recovery of some activity (state d_{PPT}^{--} in Fig. 4). Mg^{2+} addition also blocked this activity in the absence of ATP (state d_{PPT}^{-+}, usually not included in the standard protocol, i.e., indices $--$, $+-$, $++$) even though 0.15 μM Ca^{2+} was present. The channel stayed blocked after ATP addition (state d_{PPT}^{++}). Application of PKA fully released the block by Mg^{2+} (state p_{PKA}^{++}).

In Fig. 5, initially a number of channels were active (see legend). At least two of these were not blocked by Mg^{2+} (see trace for p_{init}^{++}). Dephosphorylation by PPT resulted in Mg^{2+} block of all channels. After removal of ATP, Mg^{2+}, and PPT by perfusion, the total activity in state d_{PPT}^{--} and d_{PPT}^{+-} compared well with those in states x_{init}^{--} and x_{init}^{+-}, respectively. Now all channels were closed in response to Mg^{2+}, indicating that all channels were dephosphorylated (state d_{PPT}^{++}). Then, application of CalPK II resulted in recovery of channel activity in the presence of Mg^{2+}. However, recovery was incomplete (cf. legend to Fig. 5). The two traces for state d_{CalPK}^{++} in Fig. 5 show typical activity (*top*) and lowest activity (*bottom*). Apparently, there are channels that are not reactivated by CalPK II in the presence of Mg^{2+}. Such partial effects (Fig. 5) or changes in the number or mode of activity of channels during the phosphorylation cycle (Fig. 4) were a prominent feature in multichannel experiments. In recognition of these problems, we restricted the quantitation of the observed effects to those 51 experiments in which only one channel was active (Tables 1, 2, and 4). This allowed quantitation of effects by changes of channel open probability p_o (data in Tables 1 and 2). Most of the data in Table 1 have been addressed already. In summary, in 31 out of 51 experiments, channels were initially sensitive to Mg^{2+} block (d_{init}^{++}). They could be reactivated by either CalPK II or by PKA in 7 out of 10 and in 8 out of 11 experiments, respectively. In 20 out of 51 experiments, channels were initially insensitive to block by Mg^{2+} (p_{init}^{++}). Treatment by PPT1 or by PPT abolished this activity in six out of eight and in seven out of eight experiments, respectively. In a few experiments, PPT treatment was followed by application of CalPK II or

TABLE 1 Channel open probability (p_o) for different phosphorylated states in the presence of 2.6 mM free Mg^{2+} and 0.5 mM ATP (index++)

State	d^{++}_{init}	p^{++}_{CalPK}	$^{\ddagger}p^{++}_{PKA}$	p^{++}_{init}	d^{++}_{PPT1}	d^{++}_{PPT}	p^{++}_{CalPK}	p^{++}_{CalPK}
$*P_o$	<0.004	0.10 ± 0.02	0.23 ± 0.03	0.33 ± 0.09	<0.003	<0.009	0.09 ± .03	0.60 ± .15
Observations	31	7	8	20	6	7	2	3
(total)	(51)	(10)	(11)	(51)	(8)	(8)	(3)	(3)

* All data are from single-channel experiments. The number of observations refer to the number of membranes that showed changes of the p_o values, out of all membranes studied (total). For states p (phosphorylated), p_o values are given as mean ± SD of the p_o values of the number of observations, or as upper limits for the residual activities in dephosphorylated states (d).

‡ The mean ± SDs for p^{++}_{PKA} treatment includes four microsyringe applications and four bath applications, which were used, in part, to validate that the two modes of treatment give essentially the same results.

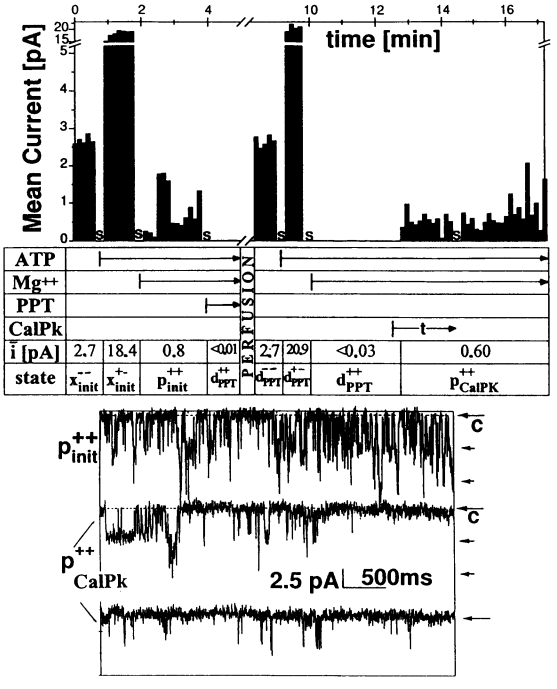

FIGURE 5 Effect of sequential dephosphorylation and rephosphorylation using PPT and CalPK II on multichannel activity. Initially, a number of channels (perhaps 6) were active. After Mg^{2+} application, at least two channels were active, which means that by our operational definition, these channels were initially phosphorylated. The state x_{init} is meant to indicate a mixture of Mg-insensitive (phosphorylated) and Mg^{2+} sensitive (dephosphorylated) channels. PTT-blocked channels were reactivated to initial activity by perfusing out Mg^{2+}, which again became blocked by Mg^{2+} addition. Exogenously added CalPK II led to partial recovery of channel activity. The two traces for p^{++}_{CalPK} show typical activity observed with two channel events (*top*) and lowest activity (*bottom*).

PKA. This led to recovery of activity in two out of three and in three out of three experiments, respectively. It led to activation of two channels with about equal p_o values of 0.60 ± 0.15 (see traces in Fig. 4 and legend).

Evidence that the ryanodine receptor is phosphorylatable in the terminal cisternae vesicles, both by PKA and CalPK II, is provided in Table 3. We found that the stoichiometric values were dependent on the buffer conditions used for phosphorylation. The conditions (Witcher et al., 1991) were adjusted to give higher phosphate incorporation and perhaps

TABLE 2 Dependence of channel open probability p_o on presence/absence of ATP and Mg^{2+} for three different phosphorylation states

State	$*p_o$ for (±ATP, ±Mg^{2+}) (−−)	(+−)	(−+)	(++)
p_{init}	0.14 ± 0.05	0.67 ± 0.08	<0.004	0.33 ± 0.09
d_{init}	0.11 ± 0.05	0.65 ± 0.11	<0.005	<0.004
d_{PPT}	0.11 ± .04	0.44 ± 0.25	<0.004	<0.009

* p_o values are given as mean ± SD or, at low p_o values, as upper limits. At least three independent experiments.

are not yet fully optimized. Further, the stoichiometric ratios represent minimal values because the vesicles had not been pretreated with phosphatases to remove endogenous phosphorus in the receptor or the terminal cisternae vesicles, especially because the bilayer data show that a significant fraction of the sites available are already phosphorylated. The stoichiometric ratios given in Table 3 obtained in the test tube may not be identical to those resulting from microsyringe application of the protein kinases directly to the membrane. They do show that the ryanodine receptor from skeletal muscle can be phosphorylated under these conditions.

In view of reports that endogenous Ca^{2+}/Calmodulin-dependent kinase (end CalPK), which copurifies with the terminal cisternae of SR (Chu et al., 1990), may be involved in CRC inactivation (Wang and Best, 1992), we tried to activate endogenous CalPK, which may still be associated with

TABLE 3 Stoichiometry of phosphorylation of CRC in terminal cisternae vesicles

Protein kinase	Phosphorylation stoichiometry (phosphorus/CRC)
PKA	1.94 ± 0.1 (7 in triplicate)
CalPK II	0.89 ± 0.08 (8 in triplicate)

Terminal cisternae of skeletal muscle sarcoplasmic reticulum were treated with protein kinases as described in Materials and Methods. The stoichiometry was obtained by measuring incorporation of ^{32}P from [^{32}P]-ATP into terminal cisternae, separating an aliquot by SDS-PAGE, and measuring the ^{32}P radioactivity in the high molecular weight band referable to the ryanodine receptor protomer. This value was divided by the measured amount of high affinity ryanodine binding (one ryanodine per CRC) per aliquot. The ryanodine binding (B_{max}) was determined from ryanodine binding isotherms on the terminal cisternae (McGrew et al., 1989).

CRC in planar bilayers after fusion of TC vesicles. Obviously, such experiments should start with active CRC in the dephosphorylated state, which we achieved by application of PPT in the absence of Mg^{2+} (see state d^{+-}_{PPT} in Figs. 6 and 7). The solution for activating endogenous CalPK (contains calmodulin, Mg^{2+}, ATP, but no CalPK II) was then applied by the microsyringe, which abolished channel activities. This block is expected from the presence of Mg^{2+} and calmodulin in the activation solution. The effect of block by Mg^{2+} and calmodulin is reversible by dilution (see Figs. 4 and 5 for Mg^{2+} and Fig. 8 for calmodulin). The persisting block in Figs. 6 and 7 after removal of the microsyringe and dilution of the activation solution by stirring, therefore, is not attributed to block by Mg^{2+} or by calmodulin, but caused by the activity of endogenous CalPK. Further evidence that this block results from phosphorylation by end CalPK was obtained by applying phosphatase PPT as shown in Fig. 6. This led to the recovery of the channel to an activity similar to that observed before in state d^{+-}_{PPT}. Recovery from block was also obtained by exogenous action of CalPK II added to the membrane as shown in Fig. 7. Upon Mg^{2+} addition, the activity revealed the typical p_o value found for application of CalPK II alone (cf. Table 1). Persisting channel block after dilution of the 1 μl phosphorylation buffer applied by the microsyringe was observed in five out of nine attempts. In the four failures, the activity was recovered upon dilution. This indicates that functional coassociation of end CalPK and the

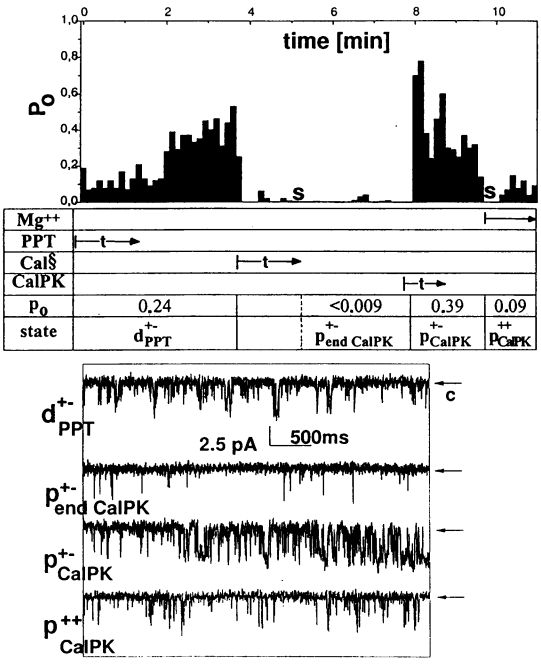

FIGURE 7 Exogenous CalPK II removes block by endogenous CalPK. The protocol was the same as in Fig. 6 except that recovery from block (state $p^{+-}_{endCalPK}$) was achieved not by dephosphorylation as in Fig. 6 but by apparent further phosphorylation by exogenously added CalPK II. A final addition of Mg^{2+} reduced p_o to 0.09, a typical value found in state p^{++}_{CalPK} (cf. Table 1).

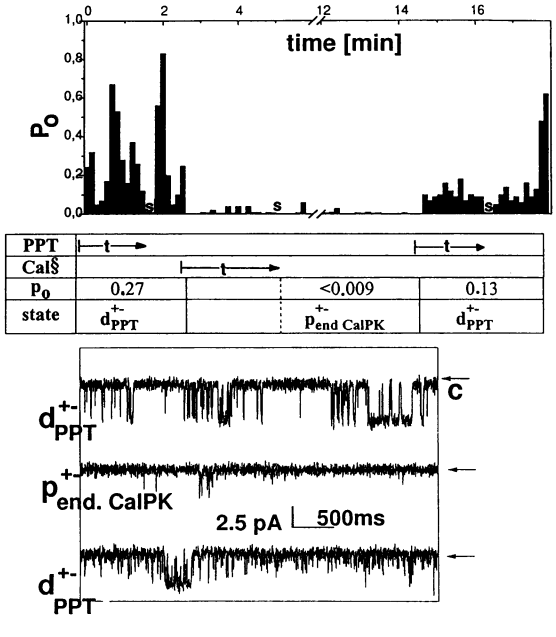

FIGURE 6 Channel block by activation of endogenous CalPK and recovery by protein phosphatase PPT. PPT was applied to activity in the absence of Mg^{2+} with ATP present (index +−). Then CalPK activation solution, indicated by Cal§ (composition as for CalPK II application but without CalPK II, cf. Materials and Methods), induced block of channel activity that persisted after microsyringe removal and stirring, which is attributed to activation of end CalPK. A second application of PPT removed this block to comparable channel activity (see first and last channel trace), providing evidence for previous phosphorylation by end CalPK. This entire experiment was repeated twice with essentially the same results.

CRC structure did not always survive the purification/reconstitution procedures. In Fig. 8, the starting condition is the same as in Figs. 6 and 7 (state d^{+-}_{PPT}), but the solution applied by the microsyringe was devoid of ATP and Mg^{2+} (albeit containing 3 μM calmodulin and about 10 μM free Ca^{2+} in HEPES/Tris buffer). The block, observed two times in this experiment (indicated by the arrows marked with "t"), is attributed to block by direct interaction of calmodulin with the channel. Dilution of calmodulin by syringe removal and stirring led to recovery of activity. This was found in four out of four independent experiments at conditions of Fig. 8. In two experiments recovery was only partial, as in Fig. 8, for reasons not investigated further, whereas in the two other experiments recovery was complete.

Inspection of unitary currents in the different states yielded the results summarized in Table 4. They fell into two distinct classes. In the absence of Mg^{2+}, unitary currents were essentially the same in all states within experimental error. Addition of Mg^{2+} reduced unitary current from 4.3 ± 0.2 to 3.5 ± 0.2 pA. The latter value is equal for all three phosphorylated states (unitary current for dephosphorylated states in the presence of Mg^{2+} was not determinable because of lack of resolvable activity). This change of unitary current at zero holding potential is likely caused by Mg^{2+} permeation from *cis* to *trans* in competition with Ca^{2+} permeation from *trans* to *cis*, in accordance with the report of an approximately equal permeation of Mg^{2+} and Ca^{2+} (Smith et al., 1988). An exchange of Mg^{2+} for Ca^{2+} in terminal cisternae is also supported by electron probe analysis studies of ultrathin sections

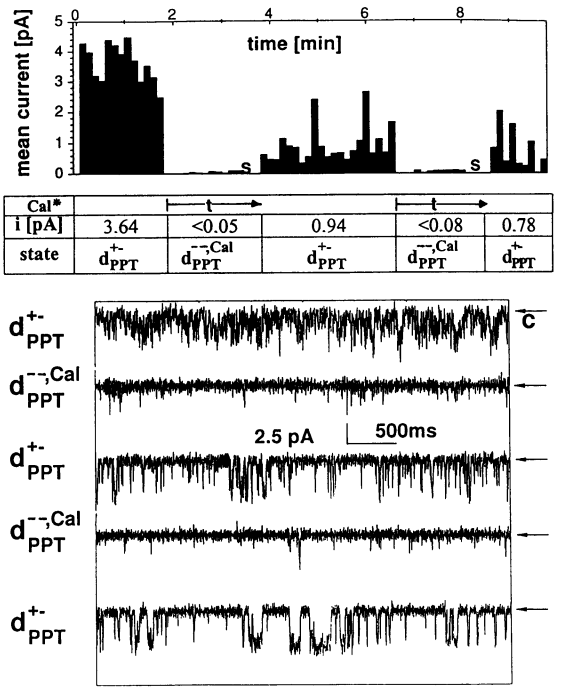

FIGURE 8 Channel block by calmodulin binding is reversible. Solution Cal*, applied by the microsyringe, was the same as solution Cal§ used in Figs. 6 and 7 except for the absence of both ATP and Mg. Block by calmodulin, indicated by arrow with "t," and recovery of activity after syringe removal and stirring was fast and observed twice in this experiment. The traces show typical activities in the sequence of the solution changes.

TABLE 4 Unitary channel currents for different phosphorylation states in absence and presence of free Mg^{2+} (2.6 mM)

	*i [pA] for (+ATP, ±Mg^{2+})	
state	(+−)	(++)
d_{init}	4.3 ± 0.1	Channel closed
d_{PPT}	4.3 ± 0.2	
d_{PPT1}	4.4 ± 0.3	
p_{init}	4.4 ± 0.3	3.6 ± 0.1
p_{PKA}	4.1 ± 0.1	3.4 ± 0.3
p_{CalPK}	4.3 ± 0.2	3.6 ± 0.2
All states	4.3 ± 0.2	3.5 ± 0.2

* Holding potential was 0 mV. Mean ± SD refer to at least three independent experiments.

of muscle (Somlyo et al., 1981). An account on Mg^{2+} permeation under physiologically relevant conditions would require further study.

DISCUSSION

In this study, we analyzed effects of phosphorylation and dephosphorylation on CRC channel activities in planar bilayers after fusion of terminal cisternae of SR from skeletal muscle. Qualitative changes in the open probability p_o of the channel were obtained by application of the protein kinases PKA or CalPK II, and protein phosphatases, PPT or PPT1, as well as by activating endogenous CalPK. There were two prominent findings: 1) activation of the channel at physiological concentrations of free Mg^{2+} (~mM) required phosphorylation by added protein kinases; and 2) inhibition of the channel is achieved by activating membrane bound (endogenous) CalPK.

We will first discuss the removal of Mg^{2+} block by exogenous PKA or CalPK II. It has been recognized for some time that Mg^{2+} inhibits Ca^{2+} release from SR (Meissner et al., 1986). Thus, it remained a paradox as to how Ca^{2+} can be released from the CRC under physiological Mg^{2+} concentration. The free Mg^{2+} concentration in heart and skeletal muscle myoplasm is approximately 1 mM (see review by Romani and Scarpa, 1992). The free Mg^{2+} concentration in the cell does not appreciably change, unlike Ca^{2+}, which acts as a second messenger. There can be little doubt from the studies presented here that phosphorylation and dephosphorylation can modulate the CRC. It is suggestive from these studies that phosphorylation can be at more than one site, on same or different proteins and, depending on the site, the action on the CRC can be different. In the absence of Mg^{2+}, CRC channel activities of dephosphorylated and phosphorylated states were not significantly different (insofar as they were analyzed, cf. Table 2). Recovery from block by Mg^{2+} was obtained by phosphorylation using exogenously added PKA or CalPK II. Open probabilities of recovered channel activities were significantly different when using PKA (state p^{++}_{PKA}) or CalPK II (state p^{++}_{CalPK}). Also, dependence on the preceding dephosphorylated states (d^{++}_{init} or d^{++}_{PPT}) was found for PKA, but not for CalPK II (Table 1). This indicates that PKA and CalPK II result in different states of phosphorylation either at the same sites at different stoichiometries or at different sites. Previous studies found "insignificant" phosphorylation of the CRC from skeletal muscle by protein kinase A and CalPK (Takasago et al., 1989; Witcher et al., 1991; Chu et al., 1990) even though CalPK was found associated with the terminal cisternae (Chu et al., 1990). We find in biochemical studies of skeletal muscle terminal cisternae a stoichiometry of phosphorylation of 0.9 and 1.9 sites per ryanodine homotetramer for CalPK II and PKA, respectively (Table 3). These are minimal values because some of the channels already contain endogenous phosphorylation (initially phosphorylated), i.e., the samples were not pretreated to remove endogenous phosphorylation. In an earlier report, with phosphorylation of the skeletal muscle ryanodine receptors by three different protein kinases including PKA and CalPK, the same serine residue was phosphorylated (Suko et al., 1993), albeit each kinase favors different consensus sequences for interaction. In this regard, other studies indicate a second phosphorylation site for the heart CRC, in addition to that of CalPK (Tasakago et al., 1991). In any case, both sets of phosphorylation sites introduced by PKA and by CalPK II are apparently dephosphorylated by acid phosphatase (PPT) as evident from sequential dephosphorylation and rephosphorylation ($p^{++}_{init} \rightarrow d^{++}_{PPT} \rightarrow p^{++}_{PKA}$ or p^{++}_{CalPK}). For recovery of the block by Mg^{2+}, it was sufficient to rephosphorylate with either protein kinase A or CalPK II.

The isolated SR membrane fractions (terminal cisternae vesicles showed a mixed population of channels with regard to their sensitivity to block by Mg^{2+}. About 60% of the channels are interpreted to be in the dephosphorylated state (d_{init}) and 40% in the phosphorylated state (p_{init}) by the criterion of presence and absence of Mg^{2+} block, and interconversion of these states by protein kinases and phosphatases ($d_{init} \rightarrow p_{PKA}$ or p_{CalPK} and $p_{init} \rightarrow d_{PPT}$ or d_{PPT1}). This finding may explain why in other reports CRC channels were not always found to be blocked by mM Mg^{2+}, especially when purified CRC was incorporated into black lipid membranes (Lai et al., 1988; Smith et al., 1988). However, in the majority of planar bilayer studies that addressed Mg^{2+} effects, efficient channel block was induced by mM free Mg^{2+} in the presence of ATP at low or moderate free Ca^{2+} concentrations (Hymel et al., 1988; Smith et al., 1986; Anderson et al., 1989; Liu et al., 1989; Lindsay and Williams, 1991; Rousseau et al., 1992). Our studies would suggest that mostly dephosphorylated channels were observed in these studies. A more quantitative study on Mg^{2+} block (Smith et al., 1986) suggested that block by ~2.6 mM Mg^{2+} may be incomplete at high channel activation conditions, i.e., reduced to 25% of maximal activity at 2 μM free Ca^{2+} and 3.6 mM AMP-PCP. However, another study indicated retention of the block by mM free Mg^{2+} even at high activation conditions (Rousseau et al., 1992). Such differences may originate from observing channels in different phosphorylation states. The relevant literature with respect to channel characteristics of the ryanodine receptor will have to be reevaluated in terms of the phosphorylation state of the receptor and the Mg concentration that was used.

Ca^{2+} flux studies on SR microsomes have revealed that activation of Ca^{2+} release by adenine nucleotides occurs even in the presence of 5 mM Mg^{2+} (Morii et al., 1983) and that nucleotides render release less sensitive to inhibition by Mg^{2+} (Meissner et al., 1986). This is consistent with our finding of a mixed population of phosphorylated and dephosphorylated channels in the vesicles used. Only one phosphorylated channel per vesicle would be sufficient to render Ca^{2+} flux insensitive to Mg^{2+}.

In this study, we find profound modulation of the calcium release channel activity by the action of kinases and phosphatases. The microsyringe made possible the experiments described here, which otherwise would have been very difficult to achieve. With this technique, application of enzymes and/or reagents can be made directly to the channel system at the bilayer surface. The same channel system can be treated multiply and with different reagents. When used in conjunction with highly purified enzymes/reagents, the tube syringe approach is stringent in its own right. It represents a powerful new approach to the study of channel modulation. The microsyringe affords a number of advantages: (1) application of reagent directly to the membrane, which does not interfere with the composition of the bath solution; (2) application of small amounts of reagents that otherwise would be prohibitive by bath applications; (3) reagents or enzymes applied to the membrane can readily be diluted (>1000-fold) to assess whether the consequence of application is reversible on dilution, or persistent, indicating covalent modification; (4) these combined attributes make possible multiple sequential treatment and analysis in the same experiment, to study sequential phosphorylation/dephosphorylation using different reagents. We validated experimentally that similar results are obtained by microsyringe and bath addition using PKA (see legend Table 1) and PPT (Fig. 2, bulk application of PPT and Figs. 6 and 7, syringe application of PPT), which were available in larger quantities.

The studies presented definitively show modulation of the ryanodine receptor by action of protein kinases and phosphatases on the terminal cisternae incorporated into planar bilayers. The sites of phosphorylation remain to be ascertained and could be referable in part to polypeptides other than the protomers of the ryanodine receptor (see Leddy et al., 1993). We have carried out similar studies with the purified skeletal muscle ryanodine receptor incorporated into planar bilayers. The purified receptor displays similar cyclical sensitivity to Mg^{2+} block, i.e., sensitivity to Mg^{2+} block is conferred by treatment with PPT1 and relief from Mg^{2+} block is obtained by treatment with protein kinase A (M. Mayrleitner, H. Schindler, and S. Fleischer, unpublished observations (1994)). Even so, it remains to be ascertained whether the FK-506-binding protein, which is tightly associated with the purified ryanodine receptor and appears to modulate its behavior (Timerman et al., 1993), becomes phosphorylated. A complementary approach using a spectrophotometric Ca^{2+}-loading assay of terminal cisternae vesicles, which measures the ensemble behavior of the channels, further supports the conclusions of our study. Treatment of terminal cisternae with protein kinase A results in decreased Ca^{2+} uptake, consistent with enhanced leakage of Ca^{2+} by activation of the ryanodine receptor channels (M. Mayrleitner, B. Chandler, and S. Fleischer, unpublished observations (1994)).

Very little is known about the inactivation of the calcium release channel of SR. An important recent study on frog muscle implicated the involvement of endogenous CalPK in inactivating the CRC (Wang and Best, 1992). Our studies support this finding on mammalian skeletal muscle membrane fractions. Another mode of inhibiting the ryanodine receptor channel, carefully studied by Meissner et al. (1986), showed that calmodulin directly inhibits Ca^{2+} release from terminal cisternae vesicles. In our studies, the dual inhibitory role of calmodulin could be confirmed. In this regard, the microsyringe application was again instrumental in assessing the dual action of calmodulin. Application of calmodulin directly to the membrane caused inactivation of the channel that was readily reversible. The application of calmodulin together with conditions for activating endogenous CalPK caused inactivation that persisted upon dilution of activation solution, whereas reactivation was achieved by either protein phosphatase (PPT) or by exogenously added CalPK (see also Witcher et al., 1991). These findings resolve the apparent contradiction in the literature regarding inhibition of the channel by endogenous CalPK (Wang and Best, 1992) versus activation of the channel by exogenously added CalPK (see

Witcher et al., 1991). Herrmann-Frank and Varsànyi (1993) have recently reported an ATP-stimulated, endogenous kinase-activated calcium release channel in rabbit SR. The channel activation by exogenously added protein kinases described by us undoubtedly reflects an action by an endogenous kinase.

The differential action of exogenous versus endogenous CalPK in our studies may reflect differential localization of the kinases at the triad junction and, hence, phosphorylation at different sites on the receptor. For example, CalPK associated with the junctional face membrane (Chu et al., 1990) would phosphorylate the ryanodine receptor in the proximity of the baseplate. Phosphorylation by kinases at the transverse tubule (Herrmann-Frank and Varsànyi, 1993) would be sterically limited to the transverse tubule face of the receptor. Alternatively, such phosphorylation of the homotetrameric ryanodine receptor could break structural symmetry of the receptor, which may well lead to channel closing, whereas further phosphorylation by exogenously added CalPK reinstalls symmetry leading to open channels. This would be analogous to the effect of ryanodine, which locks the channel in half-open state when one ryanodine molecule is bound (high affinity binding) and closes the channel in case of low affinity binding (~4 ryanodine molecules bound) to the receptor (Inui et al., 1988; McGrew et al., 1989). The possible physiological role of phosphorylation of the CRC is that it may modulate skeletal muscle SR calcium release in excitation-contraction coupling. At the very least, phosphorylation of CRC likely determines the recruitment of the number of active channels. Indeed, recent studies in intact rat cardiomyocytes (Takasago et al., 1991) show that β-adrenergic agonists stimulate phosphorylation of the ryanodine receptor by PKA. It had already been noted 25 years ago (Allen and Blinks, 1978) that β-adrenergic stimulation dramatically increases the rates of the rise and fall of $[Ca^{2+}]_i$ and the maximal level of transient $[Ca^{2+}]_i$. These two studies infer that enhanced Ca^{2+} release by β-adrenergic stimulation is via ryanodine receptor phosphorylation by PKA. Despite strong inferences based on studies with isolated SR (Wang and Best, 1992), there is little direct evidence for involvement of particular phosphorylation/dephosphorylation events in EC-coupling of skeletal muscle or heart in situ. In heart, there are important relevant studies, both with isolated SR (Witcher et al., 1991; Takasago et al., 1991) and in the intact myocyte (Yoshida et al., 1992). Our studies with skeletal muscle provide insights at the level of the isolated terminal cisternae and the CRC in planar bilayers. Approximating physiological conditions (Mg^{2+} (~1 mM) (Romani and Scarpa, 1992), ATP (several mM) (Godt and Manghan, 1988), and the resting level of free Ca^{2+}, ~0,1 μM (Wier, 1992; Harkins et al., 1993; Blatter and Blinks, 1991)), we find the channel completely closed when dephosphorylated and open when phosphorylated by exogenously added PKA or CalPK. The finding of CRC activation by PKA application matches inferences from β-adrenergic stimulation (Yosida et al., 1992; Allen and Blinks, 1978) and, therefore, may be relevant for hormonal modulation of Ca^{2+} release from SR. It remains to be tested whether this activation is only modulatory or is involved also directly in triggering Ca^{2+} release upon depolarization of the transverse tubule via activation of kinases in the contact region between CRC and transverse tubule. As to the inactive resting state of the CRC, there are two ways to inactivate the channel. 1) It can be dephosphorylated. This would be required if phosphorylation is directly involved in triggering release. 2) It can be phosphorylated by endogenous CalPK, perhaps concerted with block from calmodulin binding. In this case, phosphorylation by PKA may be modulatory only. Phosphorylation by end CalPK would have to be reversed during each release cycle. This dephosphorylation might be induced in response to transverse tubule depolarization.

The studies presented are consistent with the modulation of E-C coupling by phosphorylation and dephosphorylation, albeit they reflect complexity in terms of multiple phosphorylation sites with different functional consequences. At the very least, it would appear that phosphorylation mediates recruitment of the number of active channels. It may infer more. Phosphorylation/dephosphorylation may be involved in opening and closing of the channel per each contraction cycle in E-C coupling.

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