

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

Jürgen Hain,\* Sati Nath,† Martin Mayrleitner,† Sidney Fleischer,† and Hansgeorg Schindler\*

\*Institute for Biophysics, University of Linz, A-4040 Linz, Austria, and †Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37235 USA

**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  $\mu\text{M}$  free  $\text{Ca}^{2+}$ ,  $\pm 0.5$  mM ATP and  $\pm 2.6$  mM free  $\text{Mg}^{2+}$ . Sequential dephosphorylation and rephosphorylation rendered the CRC sensitive and insensitive to block by  $\text{Mg}^{2+}$ , respectively. Channel recovery from  $\text{Mg}^{2+}$  block was obtained by exogenous protein kinase A (PKA) or by  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CalPK II). Somewhat different characteristics were observed with the two kinases, suggesting two different states of phosphorylation. Channel block by  $\text{Mg}^{2+}$  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  $\text{Mg}^{2+}$ . Thus, these two states were interconvertible by treatment with exogenously added protein kinases and phosphatases. Endogenous  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase (end CalPK) had an opposite action to exogenous CalPK II. Previously, dephosphorylated channels using PPT ( $\text{Mg}^{2+}$  absent) were blocked in the closed state by action of endogenous CalPK. This block was removed to normal activity by the action of either PPT or by exogenous CalPK 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  $\text{Mg}^{2+}$ ). 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,  $[\text{Ca}^{2+}]_i$  (Fleischer and Inui, 1989). A rise in  $[\text{Ca}^{2+}]_i$  triggers muscle contraction. The  $[\text{Ca}^{2+}]_i$  must again be lowered to enable the muscle to relax. In skeletal muscle and heart, the sarcoplasmic reticulum serves a key role in  $\text{Ca}^{2+}$  uptake, storage, and release. The macroscopic phenomenology in excitation-contraction coupling in skeletal muscle is referred to as depolarization-induced  $\text{Ca}^{2+}$  release. That is, essentially all of the  $\text{Ca}^{2+}$  to be mobilized for muscle contraction derives from the sarcoplasmic reticulum, the intracellular store of  $\text{Ca}^{2+}$ . 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  $\text{Ca}^{2+}$  from the lumen of the terminal cisternae (Fleischer and Inui, 1989).

The machinery involved in the release of  $\text{Ca}^{2+}$  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  $\text{IP}_3$  receptors (for reviews, see Fleischer and Inui, 1989; Berridge, 1993; Ferris and Snyder, 1992). These receptors

Received for publication 25 March 1994 and in final form 11 August 1994.

Address reprint requests to H. Schindler, Institute for Biophysics University of Linz, Altenbergerstr. 69, 4040 Linz, Austria. Tel.: 43-732-2468-9269; Fax: 43-732-2468-822 or S. Fleischer, Dept. of Molecular Biology, Vanderbilt University, Box 1820 Station B, Nashville, TN 37235. Tel.: 615-322-2132, Fax: 615-343-6833, E-mail: fleiscs@ctrvax.vanderbilt.edu.

**Abbreviations used:**  $[\text{Ca}^{2+}]_i$ , intracellular free  $\text{Ca}^{2+}$  ion concentration; CalPK,  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase; CalPK II, CalPK type II; CRC, calcium release channel; end CalPK, endogenous  $\text{Ca}^{2+}$ /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.

© 1994 by the Biophysical Society

0006-3495/94/11/1823/11 \$2.00

serve a vital role in calcium mobilization in most, if not all, eukaryotic cells. Some cells have been found to contain both IP<sub>3</sub> 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<sup>2+</sup> 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<sup>2+</sup> 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<sup>2+</sup>/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 Varsanyi, 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 <sup>32</sup>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  $\mu$ l 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  $\mu$ g) (provided by Dr. Jackie Corbin) for 5 min in the phosphorylation buffer containing 300  $\mu$ M [<sup>32</sup>P] ATP (NEN), 25 mM MOPS/pH 7.0, 5 mM MgCl<sub>2</sub>, 10 mM NaF, 1 mM EGTA and 1 mM CaCl<sub>2</sub>. Protein phosphorylation with Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CalPK II) (0.13  $\mu$ g) was performed under similar conditions using 1.5 mM CaCl<sub>2</sub> instead of 1 mM CaCl<sub>2</sub>, and Calmodulin (2  $\mu$ g) was added additionally.

The reaction was stopped after 5 min by adding 25  $\mu$ l of SDS dissociation buffer (1% SDS, 5%  $\beta$ -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 <sup>32</sup>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 <sup>32</sup>P/CRC was calculated by dividing the P<sup>32</sup> 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  $\mu$ m PTFE-teflon sheet with boundary inaccuracies less than 1  $\mu$ m, 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 diphtanoyl-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)<sub>2</sub>/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  $\mu$ l of the vesicle suspension (3.5  $\mu$ g total protein) was applied near the bilayer. Fusion was monitored by Cl<sup>-</sup>-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<sub>2</sub> was added, which reduced free Ca<sup>2+</sup> concentration to  $0.15 \pm 0.05$   $\mu$ M in all experiments as measured by a Ca<sup>2+</sup> 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  $\mu$ M

free  $\text{Ca}^{2+}$  adjusted by the Orion  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Mg}^{2+}$  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,  $\text{Mg}^{2+}$ , protein kinases, and protein phosphatases are specified in the figures. Additions of ATP and  $\text{Mg}^{2+}$  refer always to additions of 0.5 mM ATP-Tris and 3 mM  $\text{MgCl}_2$ . Free  $\text{Mg}^{2+}$ , 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  $\mu\text{l}$ ) 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.

$\text{Ca}^{2+}$ /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- $\mu\text{l}$  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  $\mu\text{g}/\text{ml}$  CalPK II, 50  $\mu\text{g}/\text{ml}$  calmodulin, 0.5 mM ATP-Tris, 3 mM  $\text{MgCl}_2$ , 1 mM EGTA, 1 mM  $\text{CaCl}_2$  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  $\mu\text{l}$  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  $\mu\text{l}$  of solution (18 units) was added. For syringe application, it was present

in 3 mM  $\text{MgCl}_2$ , 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/ $\mu\text{l}$ ).

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- $\mu\text{l}$  aliquots of solution containing 2  $\mu\text{M}$  PPT1, 0.2 mM  $\text{MnCl}_2$ , 3 mM  $\text{MgCl}_2$ , 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  $(\text{NH}_4)_2\text{SO}_4$ , 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  $\mu\text{l}$ ). For bath application, 65  $\mu\text{l}$  (5 units) was added to the *cis* buffer and for tube application PPT was present in 3 mM  $\text{MgCl}_2$ , 0.5 mM ATP-Tris, 1 mM EGTA, 115 mM Tris/250 mM HEPES (pH 7.4) at 0.01 unit PPT/ $\mu\text{l}$ .

## 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  $\text{Mg}^{2+}$  was present ("state" index --); 2) 0.5 mM ATP was present, but no  $\text{Mg}^{2+}$  (index +-); and 3) both 0.5 mM ATP and 2.6 mM free  $\text{Mg}^{2+}$  were present (index ++). The free  $\text{Ca}^{2+}$  concentration was  $0.15 \pm 0.05 \mu\text{M}$  in all experiments (cf. Materials and Methods). The observed currents were referable to calcium release channels (CRC) as judged from unitary current values of  $4.3 \pm 0.2$  pA from reversal potentials of  $\approx +40$  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  $\text{Mg}^{2+}$  fell into two distinct classes for each of the two TC preparations. In 31 experiments, the channels were blocked by  $\text{Mg}^{2+}$ ; that is, the open probability was reduced from  $0.65 \pm 0.11$  to below 0.004 (see Table 2, states  $d_{\text{init}}^{+-}$  and  $d_{\text{init}}^{++}$ ). 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  $\text{Mg}^{2+}$  was observed but a reduction of  $p_o$  from  $0.67 \pm 0.08$  to  $0.33 \pm 0.09$  (see Table 2, states  $p_{\text{init}}^{+-}$  and  $p_{\text{init}}^{++}$ , and two examples in Figs. 3 and 4). For both classes of observations ( $d_{\text{init}}$  and  $p_{\text{init}}$ ), similar  $p_o$  values were found before  $\text{Mg}^{2+}$  addition (see  $p_{\text{init}}^{+-}$  and  $d_{\text{init}}^{+-}$  in Table 2).

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

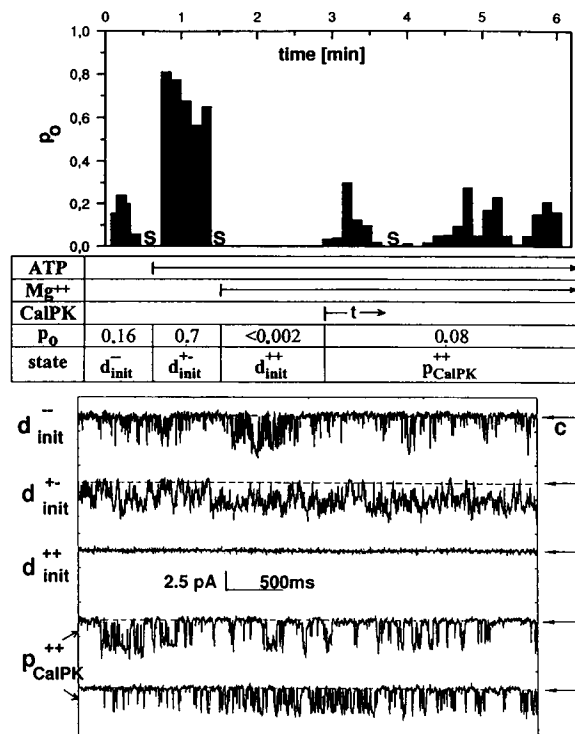


FIGURE 1 Phosphorylation by CalPK II removes channel block by  $Mg^{2+}$ . Channel activity, initially sensitive to block by  $Mg^{2+}$  (see state  $d_{init}^{+-}$  and  $d_{init}^{++}$ ) recovers from  $Mg^{2+}$  sensitivity by application of CalPK II (state  $p_{CalPK}^{++}$ ). For this and all other 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  $\mu$ l of solution to the membrane. Upon removal of the syringe, the 1  $\mu$ l 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.

$\pm 0.03$  (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  $\mu$ l) 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

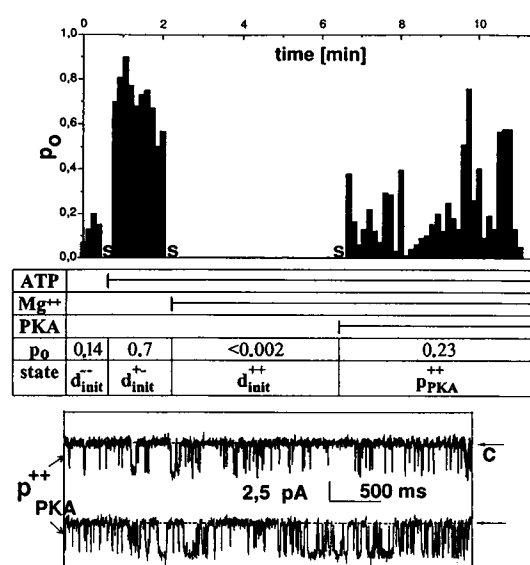


FIGURE 2 Phosphorylation by PKA removes channel block by  $Mg^{2+}$ . Similar protocol to Fig. 1 except that recovery from block by  $Mg^{2+}$  was achieved by application of catalytic subunit of PKA to the bath. The two traces show lowest (top) and average (bottom) activity of the PKA phosphorylated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

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

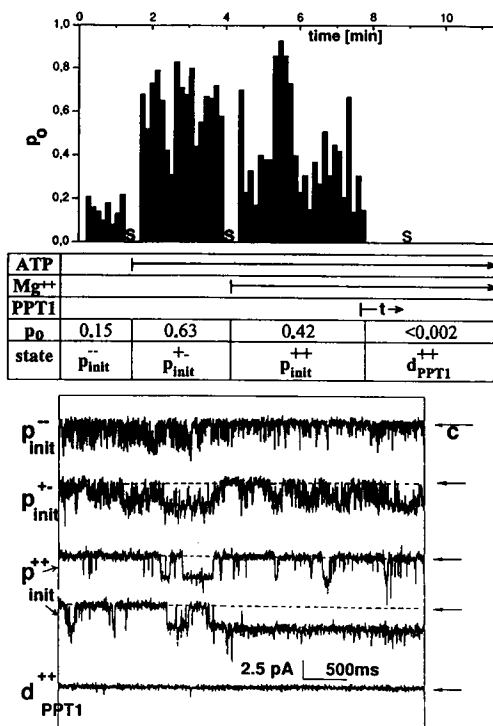


FIGURE 3 Dephosphorylation by PPT1 reinstalls channel block by Mg<sup>2+</sup>. The observed channel was not blocked by Mg<sup>2+</sup> and, therefore, was assumed to be initially phosphorylated (state p<sub>init</sub><sup>-</sup>). Mg<sup>2+</sup> reduces mean open probability (quantitated by p<sub>o</sub>; see also Table 2). Application of protein phosphatase 1 (PPT1) by the microsyringe abolished channel activity.

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

In Fig. 5, initially a number of channels were active (see legend). At least two of these were not blocked by Mg<sup>2+</sup> (see trace for p<sub>init</sub><sup>++</sup>). Dephosphorylation by PPT resulted in Mg<sup>2+</sup> block of all channels. After removal of ATP, Mg<sup>2+</sup>, and PPT by perfusion, the total activity in state d<sub>PPT</sub><sup>-</sup> and d<sub>PPT</sub><sup>+</sup> compared well with those in states x<sub>init</sub><sup>-</sup> and x<sub>init</sub><sup>+</sup>, respectively. Now all channels were closed in response to Mg<sup>2+</sup>, indicating that all channels were dephosphorylated (state d<sub>PPT</sub><sup>++</sup>). Then, application of CalPK II resulted in recovery of channel activity in the presence of Mg<sup>2+</sup>. However, recovery was incomplete (cf. legend to Fig. 5). The two traces for state d<sub>CalPK</sub><sup>++</sup> 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<sup>2+</sup>. Such partial effects (Fig. 5) or changes in the number or mode of activity of channels during the phosphorylation cycle (Fig. 4) were

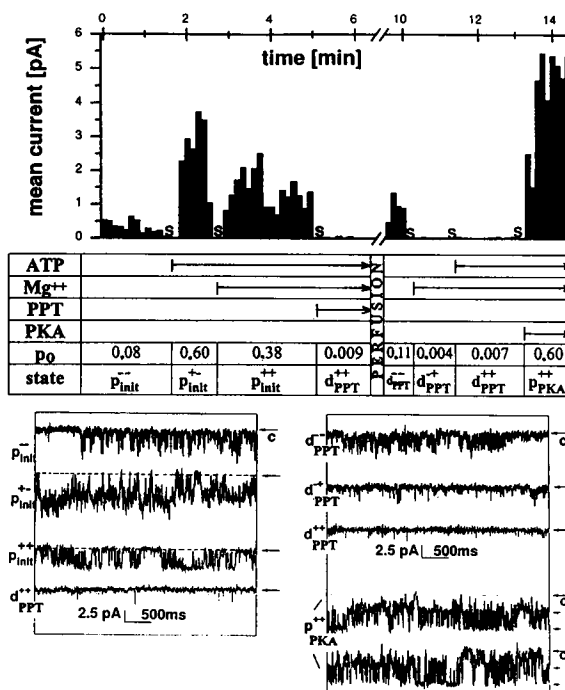


FIGURE 4 Effect of sequential dephosphorylation and rephosphorylation using PPT and PKA. The first four conditions (from state p<sub>init</sub><sup>-</sup> to d<sub>PPT</sub><sup>++</sup>) are the same as in Fig. 3 with comparable changes of activity, except that PPT (acid phosphatase from potato type III) was used. Dephosphorylation reinstalled sensitivity of the channel to block by Mg<sup>2+</sup>, because the activity recovered after perfusion with Mg-free buffer and was again blocked by Mg<sup>2+</sup>, both in the absence and presence of ATP, states d<sub>PPT</sub><sup>+</sup> and d<sub>PPT</sub><sup>++</sup>, respectively, and because this block was reversed by application of PKA (state p<sub>PKA</sub><sup>++</sup>). Before PKA application, all traces were essentially single-channel traces (no overlappings of two channel currents) except for state p<sub>init</sub><sup>++</sup> showing fast unresolved openings to the second level, which were neglected in estimating the p<sub>o</sub> 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<sub>o</sub> = 0.6 ± 0.15, which can be used for better comparison with the p<sub>o</sub> 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<sub>PKA</sub><sup>++</sup>. 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.

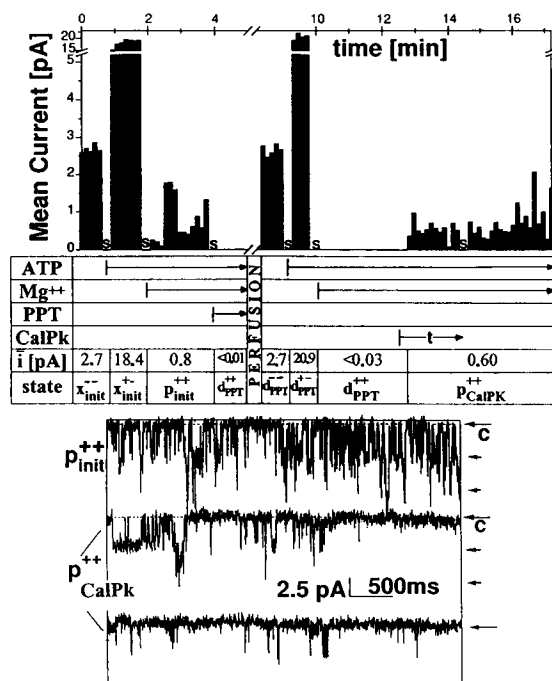
a prominent feature in multichannel experiments. In recognition of these problems, we restricted the quantitation of the observed effects to those 51 experiments in which only one channel was active (Tables 1, 2, and 4). This allowed quantitation of effects by changes of channel open probability p<sub>o</sub> (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<sup>2+</sup> block (d<sub>init</sub><sup>++</sup>). 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<sup>2+</sup> (p<sub>init</sub><sup>++</sup>). 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	$d_{init}^{++}$	$p_{CalPK}^{++}$	$p_{PKA}^{++}$	$p_{init}^{++}$	$d_{PPT1}^{++}$	$d_{PPT}^{++}$	$p_{CalPK}^{++}$	$p_{CalPK}^{++}$
* $p_o$	<0.004	$0.10 \pm 0.02$	$0.23 \pm 0.03$	$0.33 \pm 0.09$	<0.003	<0.009	$0.09 \pm .03$	$0.60 \pm .15$
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  $\pm$  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  $\pm$  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. PPT-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  $0.60 \pm 0.15$  (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 ( $\pm$ ATP, $\pm$ $Mg^{2+}$ )			
	(--)	(+-)	(-+)	(++)
$p_{init}^{++}$	$0.14 \pm 0.05$	$0.67 \pm 0.08$	<0.004	$0.33 \pm 0.09$
$d_{init}^{++}$	$0.11 \pm 0.05$	$0.65 \pm 0.11$	<0.005	<0.004
$d_{PPT}^{++}$	$0.11 \pm .04$	$0.44 \pm 0.25$	<0.004	<0.009

\*  $p_o$  values are given as mean  $\pm$  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	$1.94 \pm 0.1$ (7 in triplicate)
CalPK II	$0.89 \pm 0.08$ (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  $\mu$ l 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

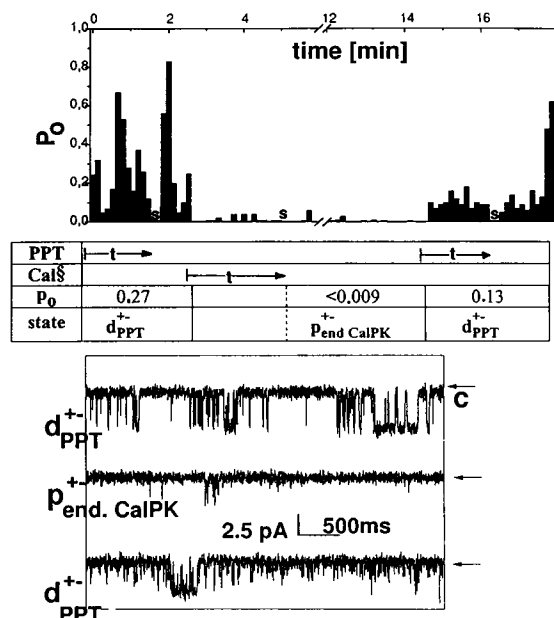


FIGURE 6 Channel block by activation of endogenous CalPK and recovery by protein phosphatase PPT. PPT was applied to activity in the absence of  $Mg^{2+}$  with ATP present (index + -). Then CalPK activation solution, indicated by Cal§ (composition as for CalPK II application but without CalPK II, cf. 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.

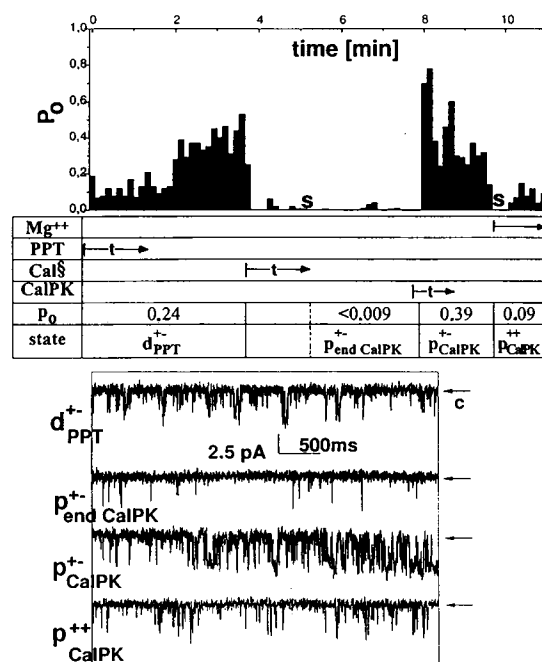


FIGURE 7 Exogenous CalPK II removes block by endogenous CalPK. The protocol was the same as in Fig. 6 except that recovery from block (state  $p_{end\ CalPK}^{+}$ ) was achieved not by dephosphorylation as in Fig. 6 but by apparent further phosphorylation by exogenously added CalPK II. A final addition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

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  $\mu$ M calmodulin and about 10  $\mu$ M 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  $4.3 \pm 0.2$  to  $3.5 \pm 0.2$  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

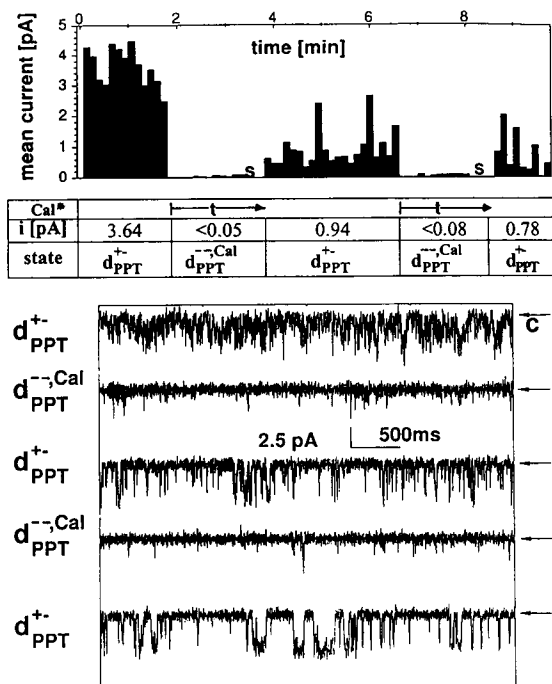


FIGURE 8 Channel block by calmodulin binding is reversible. Solution Cal\*, applied by the microsyringe, was the same as solution Cal§ used in Figs. 6 and 7 except for the absence of both ATP and Mg. Block by calmodulin, indicated by arrow with "t," and recovery of activity after syringe removal and 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<sup>2+</sup> (2.6 mM)

state	*i [pA] for (+ATP, ±Mg <sup>2+</sup> )	
	(+/-)	(++)
d <sub>init</sub>	4.3 ± 0.1	Channel closed
d <sub>PPT</sub>	4.3 ± 0.2	
d <sub>PPT1</sub>	4.4 ± 0.3	
p <sub>init</sub>	4.4 ± 0.3	3.6 ± 0.1
p <sub>PKA</sub>	4.1 ± 0.1	3.4 ± 0.3
p <sub>CalPK</sub>	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<sup>2+</sup> 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<sup>2+</sup> (~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<sup>2+</sup> block by exogenous PKA or CalPK II. It has been recognized for some time that Mg<sup>2+</sup> inhibits Ca<sup>2+</sup> release from SR (Meissner et al., 1986). Thus, it remained a paradox as to how Ca<sup>2+</sup> can be released from the CRC under physiological Mg<sup>2+</sup> concentration. The free Mg<sup>2+</sup> concentration in heart and skeletal muscle myoplasm is approximately 1 mM (see review by Romani and Scarpa, 1992). The free Mg<sup>2+</sup> concentration in the cell does not appreciably change, unlike Ca<sup>2+</sup>, 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<sup>2+</sup>, 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<sup>2+</sup> 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<sub>PKA</sub><sup>++</sup>) or CalPK II (state p<sub>CalPK</sub><sup>++</sup>). Also, dependence on the preceding dephosphorylated states (d<sub>init</sub><sup>++</sup> or d<sub>PPT</sub><sup>++</sup>) 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 pre-treated 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<sub>init</sub><sup>++</sup> → d<sub>PPT</sub><sup>++</sup> → p<sub>PKA</sub><sup>++</sup> or p<sub>CalPK</sub><sup>++</sup>). For recovery of the block by Mg<sup>2+</sup>, 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  $\sim 2.6$  mM  $Mg^{2+}$  may be incomplete at high channel activation conditions, i.e., reduced to 25% of maximal activity at 2  $\mu$ M 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 Varsanyi (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 Varsanyi, 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 ( $\sim 4$  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  $\beta$ -adrenergic agonists stimulate phosphorylation of the ryanodine receptor by PKA. It had already been noted 25 years ago (Allen and Blinks, 1978) that  $\beta$ -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  $\beta$ -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 ( $Mg^{2+}$  ( $\sim 1$  mM) (Romani and Scarpa, 1992), ATP (several mM) (Godt and Manghan, 1988), and the resting level of free  $Ca^{2+}$ ,  $\sim 0.1$   $\mu M$  (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  $\beta$ -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 modu-

latory 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.

These studies were supported in part by grants S45/03 and 07 from the Austrian Research Funds (to J. Hain and H. Schindler) and by National Institutes of Health grant HL32711 and the Muscular Dystrophy Association (to S. Fleischer). S. Nath is recipient of a National Research Service Award from National Institutes of Health. M. Mayrleitner is the recipient of a Schroedinger Fellowship of the Austrian Science Foundation. We are pleased to acknowledge the initial input to these studies by Dr. Lin Hymel (Hymel et al., 1989).

## REFERENCES

- Adams, B. A., T. Tanabe, A. Mikami, S. Numa, and K. G. Beam. 1990. Intramembrane charge movement restored in dysgenic skeletal muscle by injection of dihydropyridine receptor cDNAs. *Nature*. 346: 569–372.
- Allen, D. G., and J. R. Blinks. 1978. Calcium transients in aequorin-injected frog cardiac muscle. *Nature*. 273:509–513.
- Anderson, K., F. A. Lai, Q.-Y. Liu, E. Rousseau, H. P. Erickson, and G. Meissner. 1989. Structural and functional characterization of the purified cardiac ryanodine receptor- $Ca^{2+}$  release channel complex. *J. Biol. Chem.* 264:1329–1335.
- Berridge, M. J. 1993. Inositol trisphosphate and calcium signalling. *Nature*. 361:315–325.
- Blatter, L. A., and J. R. Blinks. 1991. Simultaneous measurement of  $Ca^{2+}$  in muscle with Ca electrodes and aequorin: diffusible cytoplasmic constituent reduces  $Ca^{2+}$ -independent luminescence of aequorin. *J. Gen. Physiol.* 98:1141–1160.
- Brickley, D. A., R. J. Colbran, Y. L. Fong, and T. R. Soderling. 1990. Expression and characterization of the alpha-subunit of  $Ca^{2+}$ /calmodulin dependent kinase II using the baculovirus expression system. *Biochem. Biophys. Res. Commun.* 173:578–584.
- Catterall, W. A. 1988. Structure and function of voltage-sensitive ion channels. *Science*. 242:50–61.
- Chadwick, C. C., A. P. Timmerman, A. Saito, M. Mayrleitner, H. Schindler, and S. Fleischer. 1992. Structural and functional characterization of an inositol polyphosphate receptor from cerebellum. *J. Biol. Chem.* 267:3473–3481.
- Chu, A., C. Sumbilla, G. Inesi, S. D. Jay, and K. P. Campbell. 1990. Specific association of calmodulin-dependent protein kinase and related substrates with the junctional sarcoplasmic reticulum of skeletal muscle. *Biochemistry*. 29:5899–5905.
- Fleischer, S., and M. Inui. 1989. Biochemistry and biophysics of excitation-contraction coupling. *Annu. Rev. Biophys. Biophys. Chem.* 18:333–364.
- Ferris, C. D., and S. H. Snyder. 1992. Inositol 1,4,5-trisphosphate-activated calcium channels. *Annu. Rev. Physiol.* 54:469–488.

- Flockhart, D. A., and J. D. Corbin. 1984. Brain Receptor Methodologies, Part A. P. J. Marangos, P. J. Marangos, I. C. Campbell, and R. M. Cohen, editors. Academic Press, New York. 209–215.
- Franzini-Armstrong, C., and G. Nunzi. 1983. Junctional feet and particles in the triads of a fast-twitch muscle fibre. *J. Muscle Res. Cell Motil.* 4:233–252.
- Glossman, H., and J. Striessnig. 1988. Calcium channels. *Vitam Horm.* 44: 155–328.
- Godt, R. E., and D. W. Manghan. 1988. On the composition of the cytosol of relaxed skeletal muscle of the frog. [Review] *Am. J. Physiol.* 254: C591–C604.
- Hain, J., S. Nath, A. Sonleitner, S. Fleischer, and H. Schindler. 1994. Activation and inactivation of the calcium release channel (CRC) of sarcoplasmic reticulum (SR) from skeletal muscle and heart. *Biophys. J.* 65:225a. (Abstr.)
- Hain, J., H. Schindler, S. Nath, and S. Fleischer. 1993. Phosphorylation of the skeletal muscle calcium release channel removes block by magnesium ions. *Biophys. J.* 64:151a. (Abstr.)
- Harkins, A. B., N. Kurebayashi, and S. M. Baylor. 1993. Resting myoplasmic free calcium in frog skeletal muscle fibers estimated with fluo-3. *Biophys. J.* 65:865–881.
- Herrmann-Frank, A., and M. Varsanyi. 1993. Enhancement of  $\text{Ca}^{2+}$  release channel activity by phosphorylation of the skeletal muscle ryanodine receptor. *FEBS Lett.* 332:237–242.
- Hymel, L., M. Inui, S. Fleischer, and H. Schindler. 1988. Purified ryanodine receptor of skeletal muscle forms  $\text{Ca}^{2+}$ -activated oligomeric  $\text{Ca}^{2+}$  channels in planar bilayers. *Proc. Natl. Acad. Sci. USA.* 85:441–445.
- Hymel, L., H. Schindler, S.-D. Yang, M. Inui, M., S. Reif, and S. Fleischer. 1989. Protein kinase/phosphatase modulation of purified skeletal muscle calcium release channel activity in planar bilayers. *Biophys. J.* 55:307a. (Abstr.)
- Inui, M., A. Saito, and S. Fleischer. 1987a. Purification of the ryanodine receptor and identity with feet structures of junctional terminal cisternae of sarcoplasmic reticulum from fast skeletal muscle. *J. Biol. Chem.* 262: 1740–1747.
- Inui, M., A. Saito, and S. Fleischer. 1987b. Purification of the ryanodine receptor (RyR) and identity with feet structures of junctional terminal cisternae (JTC) of sarcoplasmic reticulum (SR). *J. Biol. Chem.* 262:15637–15642.
- Inui, M., S. Wang, A. Saito, and S. Fleischer. 1988. Characterization of junctional and longitudinal sarcoplasmic reticulum from heart muscle. *J. Biol. Chem.* 263:10843–10850.
- Kijima, Y., A. Saito, T. L. Jetton, M. A. Magnuson, and S. Fleischer. 1993. Different intracellular localization of inositol 1,4,5-tris phosphate and ryanodine receptors in cardiomyocytes. *J. Biol. Chem.* 268:3499–3506.
- Laemmli, U. K. 1970. Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685.
- Lai, F. A., H. P. Erickson, E. Rousseau, Q.-Y. Liu, and G. Meissner. 1988. Purification and reconstitution of the calcium release channel from skeletal muscle. *Nature.* 331:315–319.
- Leddy, J. J., B. J. Murphy, Qu-Yi, J.-P. Doucet, C. Pratt, and B. S. Tuana. 1993. A 60-kDa polypeptide of skeletal-muscle sarcoplasmic reticulum is a calmodulin-dependent protein kinase that associates with and phosphorylates several membrane proteins. *Biochem. J.* 295:849–856.
- Lindsay, A. R. G., and A. J. Williams. 1991. Functional characterization of the ryanodine receptor purified from sheep cardiac muscle sarcoplasmic reticulum. *Biochim. Biophys. Acta.* 1064:89–102.
- Liu, Q.-Y., F. A. Lai, E. Rousseau, R. V. Jones, and G. Meissner. 1989. Multiple conductance states of the purified calcium release channel complex from skeletal sarcoplasmic reticulum. *Biophys. J.* 55:415–424.
- Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.
- McGrew, S. G., C. Wollenben, P. Siegl, M. Inui, and S. Fleischer. 1989. Positive cooperativity of ryanodine binding to the calcium release channel of SR from heart and skeletal muscle. *Biochemistry.* 28:1686–1691.
- Meissner, G., E. Darling, and J. Eveleth. 1986. Kinetics of rapid  $\text{Ca}^{2+}$  release by sarcoplasmic reticulum: effects of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and adenine nucleotides. *Biochemistry.* 25:236–244.
- Morii, H., H. Takisawa, and T. Yamamoto. 1987. A possible role of protein phosphorylation in the inactivation of a  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release channel from skeletal muscle sarcoplasmic reticulum. *J. Biochem.* 102:263–271.
- Morii, H., and Y. Tonomura. 1983. The gating behavior of a channel for  $\text{Ca}^{2+}$  induced  $\text{Ca}^{2+}$  release in fragmented sarcoplasmic reticulum. *J. Biochem. (Tokyo).* 93:1271–1285.
- Reuter, H. 1983. Calcium channel modulation by neurotransmitters, enzymes and drugs. [Review] *Nature.* 301:569–574.
- Rios, E., and G. Brum. 1987. Involvement of dihydropyridine receptors in excitation-contraction coupling in skeletal muscle. *Nature.* 325:717–720.
- Robertson, S. P., and J. D. Potter. 1984. Methods in Pharmacology. A. Schwartz, editor. Plenum Press, New York. 5:63–75.
- Romani, A., and A. Scarpa. 1992. Regulation of cell magnesium. *Arch. Biochem. Biophys.* 298:1–12.
- Rousseau, E., J. Pinkos, and D. Savaria. 1992. Functional sensitivity of the native skeletal  $\text{Ca}(2+)$ -release channel to divalent cations and the Mg-ATP complex. *Can. J. Physiol. Pharmacol.* 70:394–402.
- Saito, A., S. Seiler, A. Chu, and S. Fleischer. 1984. Preparation and morphology of sarcoplasmic reticulum terminal cisternae from rabbit skeletal muscle. *J. Cell Biol.* 99:875–885.
- Schulman, H. 1984. Phosphorylation of microtubule-associated proteins by a  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase. *J. Cell Biol.* 99:11–19.
- Smith, J. S., R. Coronado, and G. Meissner. 1986. Single channel measurements of the calcium release channel from skeletal muscle sarcoplasmic reticulum: activation by  $\text{Ca}^{2+}$  and ATP and modulation by  $\text{Mg}^{2+}$ . *J. Gen. Physiol.* 88:573–588.
- Smith, J. S., T. Imagawa, J. Ma, M. Fill, K. P. Campbell, and R. Coronado. 1988. Purified ryanodine receptor from rabbit skeletal muscle is the calcium-release channel of sarcoplasmic reticulum. *J. Gen. Physiol.* 93:1–26.
- Somlyo, A. V., H. Gonzalez-Serratos, H. Shuman, G. McClellan, and A. P. Somlyo. 1981. Calcium release and ionic changes in the sarcoplasmic reticulum of tetanized muscle: an electron-probe study. *J. Cell Biol.* 90:577–594.
- Suko, J., I. Maurer-Fogy, B. Plank, O. Bertel, W. Wyskovsky, M. Hehenegger, and G. Hellman. 1993. Phosphorylation of serine 2843 in ryanodine receptor-calcium release channel of skeletal muscle by cAMP-, cGMP- and CaM-dependent protein kinase. *Biochim. Biophys. Acta.* 1175:193–206.
- Takasago, T., T. Imagawa, K. Furukawa, T. Oguruso, and M. Shigekawa. 1991. Regulation of the cardiac ryanodine receptor by protein kinase-dependent phosphorylation. *J. Biochem. (Tokyo).* 109:163–170.
- Takasago, T., T. Imagawa, and M. Shigekawa. 1989. Phosphorylation of the cardiac ryanodine receptor by cAMP-dependent protein kinase. *J. Biochem. (Tokyo).* 106:872–877.
- Timerman, A. P., E. Ogunbunmi, E. Freund, A. Wiederrecht, A. Marks, and S. Fleischer. 1993. The calcium release channel of sarcoplasmic reticulum is modulated by FK-506 binding protein: dissociation and reconstitution of FKBP-12 to the calcium release channel of skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* 268:22992–22999.
- Tsien, R., B. P. Bean, P. Hess, J. Lansman, B. Nilius, and M. Nowicky. 1986. Mechanisms of calcium channel modulation by beta-adrenergic agents and dihydropyridine calcium agonists. [Review] *J. Mol. Cell Cardiol.* 18:691–710.
- Wagenknecht, T., R. Grassucci, J. Frank, A. Saito, M. Inui, and S. Fleischer. 1989. Three-dimensional architecture of the calcium channel/foot structure of sarcoplasmic reticulum. *Nature.* 338:167–170.
- Walton, P. D., J. A. Airey, J. L. Sutko, C. F. Beck, G. A. Mignery, T. C. Sudhof, T. J. Deerinck, and M. H. Ellisman. 1991. Ryanodine and inositol trisphosphate receptors coexist in avian cerebellar Purkinje neurons. *J. Cell Biol.* 113:1145–1157.
- Wang, J., and P. M. Best. 1992. Inactivation of the sarcoplasmic reticulum calcium channel by protein kinase. *Nature.* 359:739–741.
- Wier, W. G. 1992.  $[\text{Ca}^{2+}]$  transients during excitation-contraction coupling of mammalian heart. In *The Heart and Cardiovascular System*, 2nd ed. H. A. Fozzard E. Haber, R. B. Jennings, A. M. Katz, H. E. Morgan, editors. Raven Press, New York. 1223–1248.
- Witcher, D. R., R. J. Kovacs, D. C. Cefali, and L. R. Jones. 1991. Unique phosphorylation site on the cardiac ryanodine receptor regulates calcium channel activity. *J. Biol. Chem.* 266:11144–11152.
- Yoshida, A., M. Takahashi, T. Imagawa, M. Shigekawa, H. Takisawa, and T. Nakamura. 1992. Phosphorylation of ryanodine receptors in rate myocytes during beta-adrenergic stimulation. *J. Biochem.* 111:186–190.
- Zhang, Z., G. Bai, S. Deans-Zirattu, M. F. Browner, and E. Y. C. Lee. 1992. Expression of the catalytic subunit of phosphatase phosphatase (protein phosphatase-1) in *Escherichia coli*. *J. Biol. Chem.* 267:1484–1490.