Binding of the Catalytic Subunit of Protein Phosphatase-1 to the Ryanodine-Sensitive Calcium Release Channel Protein†

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Received September 4, 1998; Revised Manuscript Received October 12, 1998

ABSTRACT: A number of studies have reported that the activity of the ryanodine-sensitive calcium release channel (ryanodine receptor) in the junctional sarcoplasmic reticulum of skeletal and cardiac muscle can be modulated by protein phosphorylation−dephosphorylation through activation of endogenous protein kinases and/or by addition of exogenous protein kinases and protein phosphatases. In this study, we have investigated the possibility that protein phosphatase-1 (PP1) is targeted to the junctional sarcoplasmic reticulum by the direct isolation of PP1-binding proteins on PP1−Sepharose affinity columns. The results show that the ryanodine receptor of both skeletal and cardiac muscle bind to this affinity support, and are released at supraphysiological salt concentrations in a relatively pure state. Reciprocal experiments demonstrated that PP1 binds to the immobilized muscle ryanodine receptor. The direct binding of PP1 to the ryanodine receptor was supported by the finding that tryptic fragments of the receptor were retained on PP1−Sepharose. The ability of PP1 to dephosphorylate the ryanodine receptor that was phosphorylated by protein kinase A was also demonstrated. These studies show that PP1 is targeted to the junctional sarcoplasmic reticulum by binding to the ryanodine receptor, and provide a biochemical basis for the possibility that PP1 may play a role in the regulation of calcium flux via protein phosphorylation−dephosphorylation mechanisms.

Protein phosphatase-1 is the prototype for one of the four major classes of serine/threonine protein phosphatases. Its activity is involved in control of muscle glycogen metabolism, as well as in a number of important cellular processes, including mitosis (*1*−*5*). The catalytic subunit of protein phosphatase-1 (PP1) is a 37 kDa protein which is highly conserved in evolution and has been extensively characterized. The current paradigm for the multifunctional nature of PP1 is that it forms a number of heterodimers with a diverse group of targeting subunits that serve to bind PP1 and target it to the vicinity of its substrates (*6*). This targeting hypothesis was developed through the characterization of the glycogen and myosin binding PP1 subunits (*7*, *8*). Recent studies have provided additional support for the targeting hypothesis through the discovery of a surprisingly large number of mammalian PP1 binding proteins (reviewed in refs *9* and *10*), as well as studies in yeast which revealed more than a dozen genes encoding PP1 binding proteins (*11*). The diversity and number of PP1 binding subunits can be explained in part by the discovery of a PP1 binding motif (*9*) and the crystal structure of a PP1−peptide complex which reveals a hydrophobic site for the binding of the hexapeptide sequence RRVSFA (*10*).

In this study, we have utilized affinity chromatography to investigate the physical interaction of protein phosphatase-1 with elements of the skeletal muscle triad junctional assembly. The isolation of well-characterized membrane vesicle preparations highly enriched in triads (the terminal cisternae−T-tubule junctions) has allowed the biochemical characterization of its major membrane proteins. A key element of excitation−contraction coupling in mammalian muscle is the ryanodine-sensitive calcium release channel (RyR) of the terminal cisternae (TC) of the sarcoplasmic reticulum. The RyR was first identified as the principal protein of the junctional foot structure (*12*) which appears to maintain the apposition of the TC and the transverse (T-) tubule invaginations of the sarcolemma. It was subsequently identified as the channel through which Ca^{2+} sequestered in the TC is released to activate contraction (*13*−*15*). The gating of the RyR calcium channel is regulated by the voltage-sensitive calcium channel of the T-tubule, the dihydropyridine receptor (*16*−*18*).

Skeletal muscle RyR (RyR1) is a homotetramer consisting of a large monomer of 5037 amino acid residues with a molecular mass of 565 kDa (*19*). The cardiac RyR (RyR2) (*20*) and brain RyR (RyR3) (*21*) isoforms are homotetramers of similar size but are transcribed from separate genes. Current models of the RyR propose that the first 80% of

† Supported by NIH Grants DK18512 (E.Y.C.L.) and AR43355 (A.H.C.).

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10.1021/bi982144k CCC: $15.00
Published on Web 11/25/1998

each subunit resides in the cytoplasm and the last 1000 amino acids form a stalk region and multiple transmembrane helices. The isolated tetramer appears as a stalked quatrefoil structure under high-resolution electron microscopy (*22*). Phosphorylation of the foot protein was observed several years before it was identified as the ryanodine-sensitive calcium release channel of the SR (*23*). A number of lipid bilayer studies using skeletal and cardiac isoforms of the RyR have shown that endogenous protein kinases and protein phosphatases, including PP1, can modulate calcium ion channel activity (*24*, *25*). The original experiments indicated that the RyR could be phosphorylated by exogenous PKA and by an endogenous membrane-associated kinase. A membrane-bound form of Ca^{2+}−calmodulin-dependent kinase II has been implicated (*26*), but the endogenous kinase does not absolutely require Ca^{2+} (*27*). A strong indication that this phosphorylation in skeletal muscle may occur is that RyR is a primary target of the membrane-associated Ca^{2+}-independent kinase which can utilize the ATP generated by triad associated glycolysis (*28*). In this study, we provide biochemical evidence for the targeting of protein phosphatase-1 to the junctional sarcoplasmic reticulum by binding directly to the ryanodine receptor.

EXPERIMENTAL PROCEDURES

Isolation of Skeletal Muscle Triads and Ryanodine Radiolabeling. Terminal cisternae or triads were isolated from rabbit back muscle in 250 mM sucrose and 3 mM histidine (pH 7.3) by differential and isopycnic sucrose gradient centrifugation as described by Caswell et al. (*29*). All solutions contained protease inhibitors (200 μM phenylmethanesulfonyl fluoride, 1 μM leupeptin, and 1 μM pepstatin) unless specifically stated. Triads were routinely resuspended at 6−10 mg of protein/mL in 1 M NaCl, 20 mM MOPS [3-(*N*-morpholino)propanesulfonic acid], 2.5 mM ATP, 1 mM $CaCl_2$, and 3.2 nM [^{3}H]ryanodine (New England Nuclear, 3.7 TBq/mmol) and incubated for 1 h at 20 °C. Unbound ryanodine was removed by centrifugation (60 min at 100000*g*).

Purification of RyR. Purification of rabbit muscle RyR was performed by a modification of previous protocols (*13*, *30*). Triads (after [^{3}H]ryanodine labeling) were dissolved in 1 M KCl, 80 mM $KHPO_4$ (pH 7.4), and 2% CHAPS [3-[(cholamidopropyl)dimethlammonio]-1-propanesulfonate]/2% PC (phosphatidylcholine) and centrifuged at 100000*g* for 60 min to remove particulate material. The supernatant was loaded onto a 1.5 cm × 15 cm ceramic hydroxyapatite column (Bio-Rad) at 2.8 mL/min, and the column was sequentially washed with 5 bed volumes of 1 M KCl, 80 mM $KHPO_4$, and 0.2% CHAPS/PC and 5 bed volumes of 80 mM $KHPO_4$ and 0.2% CHAPS/PC (pH 7.4). Elution was effected with 200 mM $KHPO_4$ and 0.2% CHAPS/PC. This protein suspension is essentially free of the SR Ca^{2+} ATPase (which washes through the column) and calsequestrin (which remains bound).

The RyR was further purified by diluting the pooled 200 mM $KHPO_4$ peak (7−10 mL) 3-fold with 0.2% CHAPS/PC and chromatography on a 2 mL heparin−agarose (Sigma) column equilibrated with 0.1 M NaCl, 20 mM MOPS, and 0.2% CHAPS/PC. The column was washed with 10 volumes of the equilibrating buffer. RyR was then eluted with 0.6 M NaCl, 20 mM MOPS, and 0.2% CHAPS/PC (pH 7.4).

Purification of RyR and Immobilization on CH-Sepharose. The RyR obtained by the hydroxyapatite/heparin chromatography protocol is routinely observed to have been partially proteolyzed by endogenous calpain (*31*, *32*) which becomes activated during the [^{3}H]ryanodine labeling and the hydroxyapatite chromatography steps. Therefore, to maximize the content of intact RyR, a modification of the protocol of Lai et al. (*30*) was employed. Rabbit back muscles were rapidly excised and homogenized in 250 mM sucrose, 20 mM MOPS, and 0.2 mM EDTA (pH 7.3) (250 mL/muscle), and "heavy microsomes" (40000*g* for 30 min) were pelleted from the postmitochondrial supernatant (8000*g* for 10 min). The pellet was washed by centrifugation with 0.6 M KCl in 250 mM sucrose and 20 mM MOPS (pH 7.4). The soft fibrous material overlying the pellet was discarded, and the membranes were dissolved in 10−12 mL of 1 M NaCl, 20 mM MOPS, and 2% CHAPS/PC. Particulate material was removed by centrifugation and the dissolved material layered onto six 10 to 30% sucrose gradients in 1 M NaCl, 20 mM MOPS, and 0.2% CHAPS/PC (pH 7.4) and centrifuged in a Beckman SW28 rotor for 18 h at 24 000 rpm. The 30S bands were collected, diluted 10-fold with 20 mM MOPS and 0.2% CHAPS/PC (pH 7.4), and concentrated by heparin−agarose chromatography. The protease inhibitors leupeptin and pepstatin were excluded from the elution medium. The eluate (0.8 mg of protein) was then further purified by HPLC gel filtration on a SEC-250 column (Bio-Rad) equilibrated with IED buffer [50 mM imidazole, 2 mM DTT (dithiothreitol), and 1 mM EDTA (pH 7.4)] and 5% glycerol. The RyR eluting in the column void volume (0.2 mg) was coupled to 3 mL of CH-Sepharose (Pharmacia Biotech) overnight at 4 °C (*34*).

Isolation and Dissolution of Cardiac Dyads. Microsomes were prepared from fresh rat ventricles as described by Brandt et al. (*35*). The ventricles were homogenized in 250 mM sucrose, 20 mM MOPS, 2 mM DTT, and 0.2 mM EDTA (pH 7.4), and the microsome fraction was washed with 250 mM sucrose, 20 mM MOPS, and 25 mM potassium pyrophosphate (pH 7.6). Previous studies demonstrated that >80% of the microsomal protein appeared in the dyad band (T-tubule or junctional SR as indicated by dihydropyridine and ryanodine binding) when the vesicles were centrifuged on isopycnic gradients. To minimize degradation, the pyrophosphate-washed microsomes were directly dissolved in 1 M NaCl, 20 mM MOPS, and 2% CHAPS/PC immediately prior to mixing with the affinity resins.

Immunodot Blotting for RyR. Mouse mAb DG4.84 against rabbit skeletal RyR was produced from a hybridoma originally isolated by Kawamoto et al. (*36*). This mAb immunoprecipitates RyR but does not react in Western blotting. Samples (20 μL) were dot blotted onto nitrocellulose membranes. The membranes were blocked with 2% bovine serum albumin, in Tris-buffered saline [200 mM NaCl, 20 mM Tris-HCl (pH 7.4), and 0.1% Tween 20], for 1 h. The membranes were then incubated with mAb DG4.84 (1:1000 dilution in Tris-buffered saline and 2% bovine serum albumin) at 4 °C overnight with gentle agitation followed by a 1:2000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Life Science) for 1 h at room temperature. The dot blots were developed using a chemiluminescence procedure (ECL, Amersham Life Science).

Preparation of Recombinant PP1 Proteins. Recombinant PP1α was prepared as previously described and purified to near homogeneity (*37*−*39*). The PP1γ1, PP1γ2, and PP1δ isoforms were expressed and purified as previously described (*35*). Assays for the phosphorylase phosphatase activities of PP1 were performed using ^{32}P-labeled rabbit muscle phosphorylase *a* (*37*).

Preparation of PP1−*Sepharose.* Coupling of PP1 to CH-Sepharose (Pharmacia-LKB Biotech) was performed as previously described (*34*).

Tryptic Digestion of RyR. RyR purified to the heparin−agarose chromatography step (see above) was used. The reaction mixtures (0.3 mL) contained 100 μg of membrane protein and 11 μg of trypsin (type XIII, Sigma) in IED buffer containing 10% glycerol and 2 mM $MnCl_2$ and were incubated for 2 h at room temperature. The reactions were terminated by addition of 110 μg of soybean trypsin inhibitor in 11 μL. The mixture was diluted to 1.2 mL with IED buffer/10% glycerol/2 mM $MnCl_2$ and mixed with 300 μL of PP1−Sepharose beads on ice for 18 h. The beads were then washed five times (1 mL each) with 50 mM NaCl in IED buffer/10% glycerol/2 mM $MnCl_2$. The beads were extracted with 0.3 mL of 1 M NaCl/IED buffer/10% glycerol/2 mM $MnCl_2$.

RESULTS

Binding of RyR to PP1−*Sepharose.* Low levels of phosphorylase phosphatase activity were detected in rabbit skeletal muscle triad (0.44 unit/mg) and rat ventricular microsome preparations (0.2 unit/mg) using [^{32}P]phosphorylase *a* as the substrate. Furthermore, PP1 activity was detected in skeletal muscle RyR preparations (up to 0.5 unit/mg) purified by the hydroxyapatite/heparin−agarose method (Experimental Procedures) even though a protein band with the mobility of PP1 could not be detected by SDS−PAGE. The persistent PP1 activity could simply be a contaminant trapped in the membrane pellets which could be carried through the RyR purification since PP1 binds to heparin−agarose. Alternatively, the PP1 could be part of a fairly strong complex with the RyR, bearing in mind the fact that the RyR purification protocol first calls for membrane dissolution by 1 M KCl and 2% CHAPS/PC.

To determine if there were any PP1 binding proteins in the cardiac dyads, solubilized cardiac dyad protein was subjected to affinity chromatography on PP1−Sepharose. This procedure has been shown to be useful for the isolation of several PP1 binding proteins (*31*). Cardiac membrane preparations that consist of roughly 80% cardiac dyads were prepared from rat ventricles (Experimental Procedures). The dyad proteins were extracted in 1 M NaCl, 20 mM MOPS, and 2% CHAPS/PC, diluted, and chromatographed on a PP1−Sepharose column. The column was then washed with 6 volumes of buffer containing 50 mM NaCl and then eluted with 1 M NaCl in the column buffer. The results show that a single high-molecular mass band was observed on the SDS−PAGE gel (Figure 1A, lane 2) in the 1 M salt eluate, with minor bands at 57 and 46 kDa (Figure 1A, C). Protein sequencing revealed that these two bands were calsequestrin and a partial proteolytic product of calsequestrin. The high-molecular mass polypeptide migrates just slightly faster than muscle RyR (Figure 1B), consistent with its known size (535 kDa) and mobility (*41*). The selectivity of the single step isolation of the cardiac RyR is quite striking considering that the loaded material was essentially the total solubilized protein from washed microsomes (Figure 1A, lane 1). We have repeated these experiments a number of times with consistent results. Control experiments using CH-Sepharose blocked with Tris or with BSA failed to show binding of cardiac RyR.

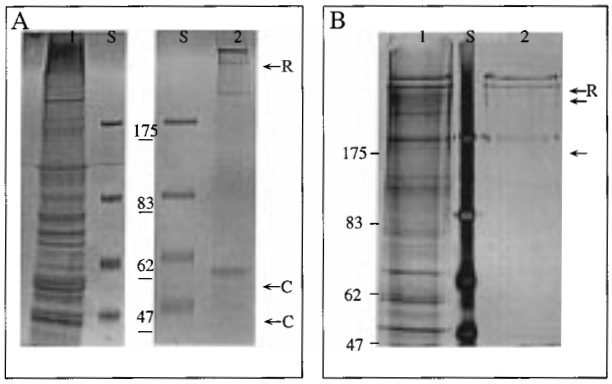

FIGURE 1: Affinity purification of cardiac and skeletal muscle RyR isoforms on PP1−Sepharose. (A) Purification of rat cardiac RyR. The starting material was the heavy microsome fraction which is highly enriched in cardiac dyads. The membrane proteins were solubilized as described in Experimental Procedures. The sample (41 mg of protein in 10 mL) was diluted to 140 mL with IED buffer/10% glycerol/2 mM $MnCl_2$/0.2% CHAPS and loaded onto a 15 mL PP1−Sepharose column at 4 °C. The column was washed with 6 column volumes of IED buffer/50 mM NaCl. The column was then eluted with 1 M NaCl/50 mM IED buffer/0.2% CHAPS at room temperature, and fractions of 1 mL were collected. A total of 420 μg of protein was obtained in the 1 M NaCl eluate. The SDS−PAGE (7.5% acrylamide) of the starting material is shown in lane 1, and that of the pooled 1 M fractions is shown in lane 2. In lanes S are the protein standards with molecular masses as indicated. The gel was stained with Coomassie blue. The arrows denote the position of the 535 kDa cardiac RyR; the bands marked C were found to be degradation products of calsequestrin. (B) Purification of skeletal muscle RyR. The starting material was the [^{3}H]ryanodine-labeled solubilized rabbit skeletal muscle triad protein from which calsequestrin had been removed by hydroxylapatite chromatography. The sample (14 mg, 3 mL) was diluted to a volume of 35 mL in 50 mM IED buffer/10% glycerol/0.2% CHAPS/2 mM $MnCl_2$ and loaded onto the column at 4 °C. The column was washed with 6 column volumes of IED buffer/50 mM NaCl; the eluent was monitored for ^{3}H until this reached background levels. The column was then eluted with 1 M NaCl/50 mM IED buffer/0.2% CHAPS at room temperature, and fractions of 1 mL were collected. The recovery of protein was 0.3 mg in the 1 M NaCl eluate. The diagram shows the SDS−PAGE (7.5% acrylamide) of the partially purified muscle RyR (lane 1) and the 1 M salt fraction (lane 2). In lane S are the protein standards with molecular masses as indicated on the left. The gel was silver-stained. The arrows (R) show the doublet of the 565 kDa RyR, and a third band at ca. 175 kDa.

In the next series of experiments, the ability of PP1−Sepharose to selectively purify the muscle isoform of RyR was tested. However, the same purification was not observed with solubilized muscle triad membrane protein that was found for cardiac RyR. In initial experiments in which the solubilized protein from muscle triad preparations were used, it was observed that that the 1 M eluates contained significant amounts of ATPase and calsequestrin, which are major protein components of the triad membranes, in excess of the muscle RyR bands (not shown). Tests with control PP1−Sepharose blocked with Tris showed that calsequestrin bound

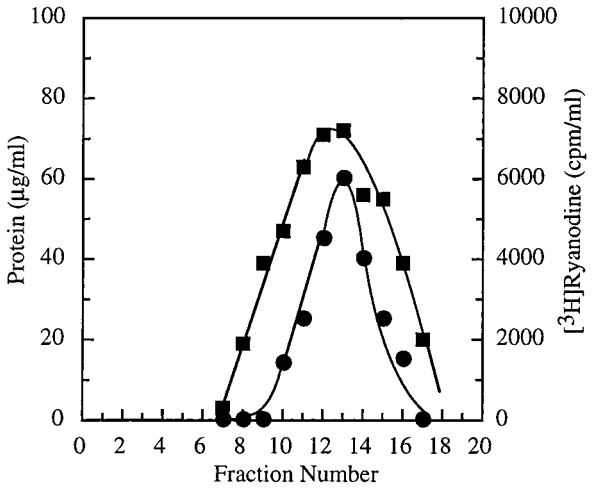

FIGURE 2: Binding of [^{3}H]ryanodine-labeled muscle RyR to PP1−Sepharose. The fractions in the 1 M NaCl eluate for the experiment in which rabbit muscle RyR was chromatographed on PP1−Sepharose (Figure 1) were analyzed for protein content (●) and radioactivity (■).

strongly to the control columns when buffers containing Mn^{2+} were used. Mn^{2+} ions were added because of the dependence of recombinant PP1 on this ion for activity (*37*). We have observed that the presence of divalent cations (calcium) leads to precipitation of calsequestrin from the solubilized triad protein, and this suggested that the effects observed could be due to either precipitation of calsequestrin on the column or its interaction with the support in the presence of divalent cations.

To further test whether muscle RyR binds to PP1−Sepharose, the muscle triad proteins were subjected to an initial chromatography experiment with hydroxyapatite chromatography (Experimental Procedures) which is known to remove >90% of the ATPase and calsequestrin (*40*). When such preparations were chromatographed on PP1−Sepharose, muscle RyR could be reproducibly purified as shown in the experiment in Figure 1B. The only bands present in the 1 M salt eluate were intact RyR (R) and bands at 410 and 170 kDa which correspond to the positions of the first calpain cleavage products of RyR (*28*). (The high-molecular mass band migrated with the same mobility as muscle RyR purified by conventional methods.) About 14 mg of protein was loaded onto the column, and 300 μg of protein was recovered. In the experiment shown in Figure 1B, the rabbit muscle triad membrane fraction had been labeled with [^{3}H]-ryanodine. This was done to confirm the identity of the bound protein as RyR. It was found that the elution of tritium label corresponded to the elution of the protein in the 1 M eluate (Figure 2). This shows that the bound 565 kDa protein retains its functional ability to bind ryanodine.

The fractions from the PP1−Sepharose chromatography of skeletal muscle RyR shown in Figure 2 were also monitored by immunodot blotting with a monoclonal antibody (DG4.84) against muscle RyR. (This antibody is conformation specific; i.e., it does not recognize denatured RyR in Western blots.) The results of this analysis are shown in Figure 3A. It can be seen that the column was overloaded as much of the immunoreactive material passed directly through the column. After washing with 50 mM NaCl until the immunoreactive material was undetectable, the addition of 1 M salt released additional immunoreactive material. The

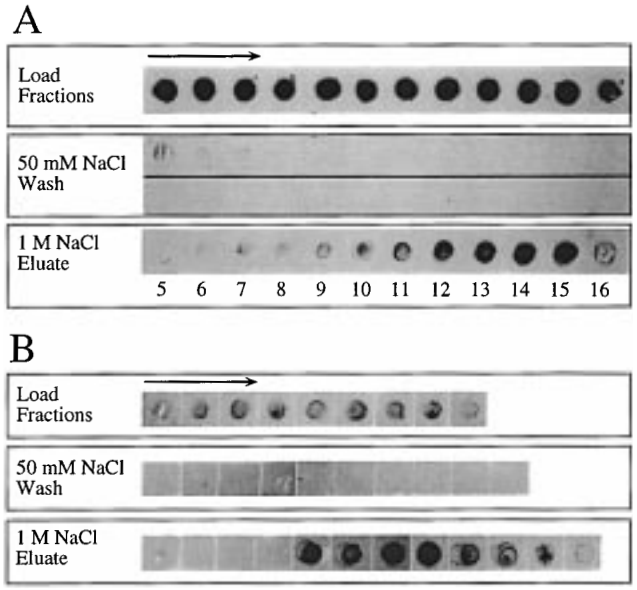

FIGURE 3: Immunochemical identification of the protein bound to PP1−Sepharose as the ryanodine receptor. (A) The fractions from the affinity chromatography of rabbit muscle RyR on PP1−Sepharose (Figures 1 and 2) were analyzed by dot blotting with a polyclonal antibody against the rabbit muscle RyR protein (Experimental Procedures). The fractions in the first row are those obtained on loading of the sample. The dot blots of the 50 M NaCl wash are shown in the second row, and the fractions 5−16 of the 1 M salt elution (cf. Figure 2) are shown in the third row. (B) The unbound fractions from panel A were combined and dialyzed against IED buffer, and a sample (10 mL, approximately 25% of the original volume) was rechromatographed on the PP1−Sepharose column under identical conditions as described for the experiment shown in Figure 2. The fractions were then analyzed for the RyR receptor by immunodot blotting as described for panel A.

distribution pattern of immunoreactive material in the 1 M salt eluate corresponded to the distributions of total protein and [^{3}H]ryanodine (cf. Figure 2). Since the column was overloaded, it could be argued that the bound material represents a subpopulation of the RyR protein. To exclude this possibility, a fraction of the material which originally passed directly through the PP1−Sepharose column was rechromatographed. The results (Figure 3B) show that there is an almost total retention of the immunoreactive material. This analysis, coupled with the presence of [^{3}H]ryanodine in the 1 M NaCl eluates, indicates that the binding is not due to the binding of denatured RyR to PP1.

A sample of the ^{3}H-labeled RyR was subjected to HPLC gel filtration (Figure 4), and the elution was followed by radioactivity and dot blotting. The [^{3}H]ryanodine eluted close to the void volume with a peak at fraction 8 (Figure 4A). Immunodot blots confirmed that this same fraction was the peak of immunoreactivity with antibody DG4.84 (Figure 4B). When a sample of the protein eluted in the 1 M salt fraction from PP1−Sepharose chromatography (Figure 2) was run on the same column, it too eluted in fraction 8, showing that the PP1-bound material was not degraded (Figure 4C).

In all of the experiments shown thus far, RyR binding to PP1 occurred in hypotonic media and elution was usually effected with 1 M salt. These data leave open the question of whether RyR binds to PP1 at physiological ionic strengths. RyR was labeled with ^{125}I and chromatographed on PP1−Sepharose. The column was washed with 50 mM NaCl, and

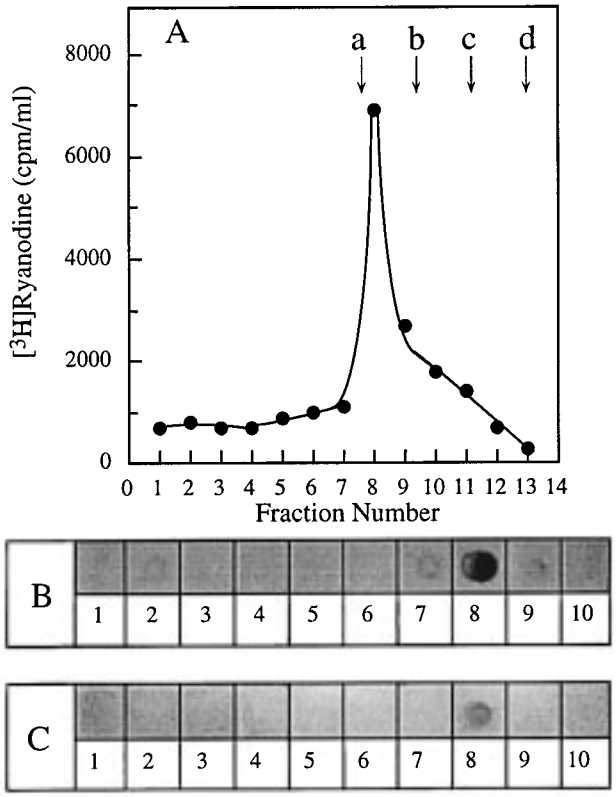

FIGURE 4: HPLC gel permeation chromatography of RyR. (A) [^{3}H]-Ryanodine-labeled RyR was chromatographed on a Bio-Rad SEC-250 HPLC column (Experimental Procedures). The sample (200 μL, 0.8 mg) was passed through a 0.45 μm filter and then injected into the SEC-250 column. Fractions of 0.5 mL were collected at a flow rate of 1 mL/min. Samples (10 μL) were counted for tritium content. The arrows denote the positions of protein standards run on the same column. These were (a) thyroglobulin (670 000 Da), (b) alcohol dehydrogenase) (158 000 Da), (c) chicken ovalbumin (44 000 Da), and (d) horse myoglobin (17 000 Da). (B) Immunodot blots of the column fractions from panel A were performed as described in Experimental Procedures. (C) The [^{3}H]ryanodine-labeled RyR obtained in the 1 M NaCl eluate from PP1−Sepharose chromatography (Figure 2) was concentrated by centrifugation through a Centricon 100 filter and chromatographed as described for panel A.

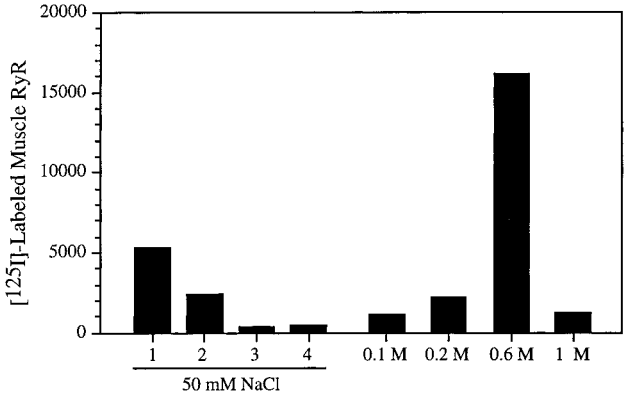

FIGURE 5: Concentration dependence of salt elution of RyR from PP1−Sepharose. RyR (500 ng) purified via heparin−agarose chromatography was incubated for 30 min at 4 °C with 0.5 mCi of Na^{125}I (New England Nuclear, 629 Gbq/mg) in an Iodogen (Pierce) coated tube. Free ^{125}I was removed by a Sephedex G-50 spin column and the labeled RyR diluted 50-fold into IED containing 0.2% CHAPS/PC. A sample (0.5 mL) was incubated with an equal volume of PP1−Sepharose with end-over-end mixing overnight at 4 °C. The gel material was washed four times with 50 mM NaCl in IED/0.2% CHAPS/PC and then with increasing concentrations of NaCl in IED/0.2% CHAPS/PC as indicated.

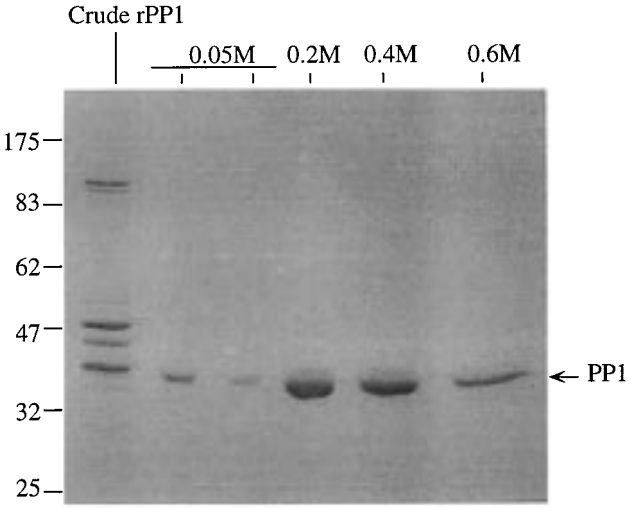

FIGURE 6: Binding of recombinant PP1 to immobilized RyR. Rabbit muscle RyR was coupled to Sepharose as described in Experimental Procedures. A partially purified recombinant rabbit muscle PP1 preparation (2 mg of protein) obtained after DEAE-Sepharose chromatography as described by Zhang et al. (*37*) was chromatographed on a RyR−Sepharose column (3 mL) in 50 mM IED buffer. The column was washed with 20 mL of 50 mM NaCl/IED buffer and then stepwise with 0.2, 0.4, and 0.6 M NaCl in IED buffer as indicated. Fractions of 0.5 mL were collected. The diagram shows the SDS−PAGE results of the first and last of the 50 mM NaCl washes, and of the peak fractions of the 0.2, 0.4, and 0.6 M NaCl salt eluates. The positions of the protein standards are marked on the left in kilodaltons. A total of 0.070 mg of protein was recovered in the salt washes.

then eluted in a stepwise fashion with increasing salt concentrations (Figure 5). Most of the bound RyR remained bound at 0.2 M NaCl but was released at 0.6 M NaCl.

Binding of PP1 to RyR−Sepharose. The experiments described above indicated that the RyR could bind to PP1. To support the idea that this is a direct interaction, we performed the converse experiments in which the binding of PP1 to purified RyR immobilized on Sepharose was demonstrated. Recombinant PP1 was expressed in *Escherichia coli* and partially purified on DEAE-Sepharose. This material was chromatographed on RyR−Sepharose (Figure 6). The bulk of the PP1 (loaded in 0.05 M NaCl) was eluted in the 0.2 and 0.4 M NaCl fractions but with some appearing in the 0.6 M NaCl fraction. About 70 μg of near-homogeneous PP1 was recovered from a 3 mL column; i.e., the loading capacity was about 25 μg of PP1/mL of gel. The studies in this work utilized the PP1α isoform of PP1. Three other isoforms are known, PP1γ1, PP1γ2, and PP1δ (*42*). Their sizes are 323, 337, and 327 amino acid residues for 1γ1, 1γ2, and 1δ, respectively, compared to PP1α which has 330 residues. Their primary structures are highly conserved with differences occurring in the 40 N-terminal residues and in the 30 C-terminal residues (*42*). The rat isoforms have been expressed as recombinant proteins (*38*), and these were tested for their ability to bind to RyR−Sepharose. All four isoforms bound to RyR (not shown), showing that the binding is not isoform specific.

Binding of Tryptic Fragments of RyR to PP1−Sepharose. Because of the large size of RyR, it is still conceivable that there is present a small protein which is responsible for the

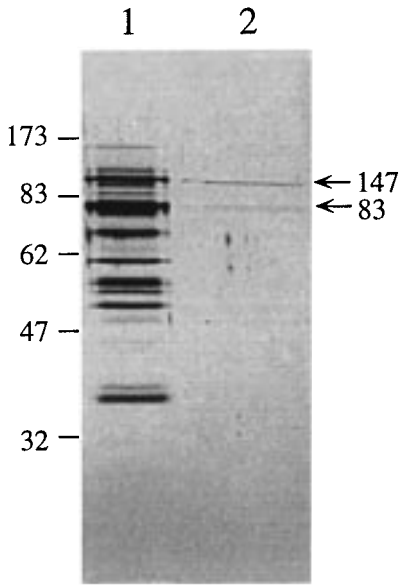

FIGURE 7: Binding of tryptic fragments of RyR on PP1−Sepharose. Rabbit skeletal muscle RyR was purified by hydroxyapatite/heparin−agarose chromatography, and digested with trypsin (Experimental Procedures). The digested RyR was bound to PP1−Sepharose beads, and the bound material was eluted with 1 M NaCl. The trypsinized RyR and the bound material were subjected to SDS−PAGE and silver-stained for protein. Lane 1 shows the trypsinized RyR, and lane 2 shows the polypeptides in the 1 M eluate from PP1−Sepharose. The positions of the protein standards are shown on the left. The positions of the main bound polypeptides are marked by arrows on the left.

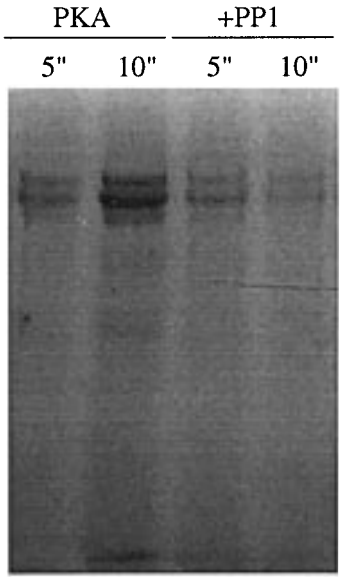

FIGURE 8: Dephosphorylation of RyR by recombinant PP1. Rabbit skeletal muscle RyR was purified by hydroxyapatite/heparin−agarose (Experimental Procedures). The reaction mixtures (60 μL) contained 2 μg of RyR, 16 mM $CaCl_2$, 5 mM $MgCl_2$, 80 μM ATP (10 μCi of [γ-^{32}P]ATP), 0.2 μg of PKA, and 20 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid] (pH 7.0). The reaction mixtures were incubated at 30 °C for 5 and 10 min and subjected to SDS−PAGE and autoradiography. After incubation for 10 min, 1 μg of PP1 was added, and the reactions were analyzed after incubation for an additional 5 and 10 min, respectively (Experimental Procedures). The arrow marks the position of the 565 kDa RyR protein.

interaction of RyR with PP1. For example, a small protein of 12 kDa [such as FK506 binding protein which is a subunit of the RyR (*43*)] would account for less than 2.5% of the mass of RyR, so even if present it would be undetectable in the SDS−PAGE gels in our experiments. The fact that the 170 kDa polypeptide which is a known endogenous calpain product of the RyR is retained by PP1−Sepharose does not address this issue because the RyR proteolytically "nicked" by calpain behaves as an intact tetramer. Muscle RyR purified by hydroxyapatite/heparin−agarose was digested with trypsin, and the reaction mixture (after addition of soybean trypsin inhibitor) was incubated with PP1−Sepharose. The beads were washed and then eluted with 1 M NaCl. The SDS−PAGE analysis of the bound material shows that two polypeptides of 147 and 83 kDa were retained (Figure 7). Tryptic RyR fragments of these sizes have been previously reported (*44*−*48*). The demonstration that these large tryptic RyR fragments bind to PP1−Sepharose strongly suggests that interaction between PP1 and the RyR is direct, since the tryptic digestion would likely either degrade an intermediary binding protein or destroy its interaction with RyR.

Dephosphorylation of Phosphorylated RyR by Recombinant PP1. PP1 (*28*) isolated from rabbit skeletal muscle and PP2A (*49*) have been used to dephosphorylate RyR phosphorylated by PKA, whereas in the experiments reported above, we used recombinant muscle PP1 expressed in *E. coli.* To confirm that recombinant PP1 is functionally able to dephosphorylate RyR, hydroxyapatite/heparin−agarose-purified RyR was phosphorylated with PKA and treated with PP1 (Figure 8). Phosphorylation of RyR with PKA led to phosphorylation of the high-molecular mass doublet, with the smaller band being more heavily phosphorylated. This is consistent with previous observations that the lower band of the doublet (i.e., the first calpain cleavage product) is more readily phosphorylated than intact RyR (*50*). Addition of recombinant PP1 causes dephosphorylation with the label almost completely removed by incubation for 10 min (Figure 8). This experiment shows that the recombinant PP1 is capable of in vitro dephosphorylation of RyR. This is consistent with other observations in which recombinant PP1 has been used to effect functional effects on calcium flux in RyR inserted into bilayer membranes (*25*).

DISCUSSION

The studies presented above demonstrate a direct interaction between PP1 and the ryanodine receptors of rabbit skeletal muscle and rat cardiac muscle, indicating that both the RyR1 and RyR2 isoforms bind to PP1. This interaction between muscle RyR and PP1 was demonstrated in a reciprocal manner using affinity chromatography methods, which also showed that binding persists at salt concentrations that are well above physiological ionic strength. The possibility that a small intermediary targeting protein is involved cannot be completely ruled out because of the large size of the RyR protein. However, this seems unlikely, as tryptic fragments of RyR were shown to bind at supraphysiological ionic strength. These observations support the view that PP1 is targeted to the triad junction, and may play a role in the modulation of calcium flux during excitation−contraction coupling. It is relevant that PP1 activity was found to be associated with both muscle and cardiac RyR during their isolation, indicating that this association may exist in vivo. The PP1 expressed from *E. coli* used in most

of our studies is fully capable of dephosphorylating RyR, as would be expected from a number of studies of bilayer preparations which have shown a decrease in the calcium channel open time on exposure to exogenous PP1, supporting a potential physiological role for PP1 (*49*−*54*).

The finding that PP1 can bind directly to the RyR supports the contention that SR Ca^{2+} release can be modulated by phosphorylation or dephosphorylation in vivo. Bilayer experiments have indicated that channel open times for both the cardiac and skeletal muscle RyR are increased by treatment with protein kinase A and decreased by treatment with PP1 or other phosphatases (*49*−*54*). This effect is consistent with the inotropic response to β-adrenergic agonists, and the increased channel open time could contribute to the decreased time to peak force in ventricular muscle. The studies presented here that indicate that PP1 is targeted by direct binding to RyR find a parallel in that a second serine/threonine protein phosphatase, calcineurin, has been shown to be targeted to RyR through the binding of the immunophilin FKBP12 and to modulate its channel activity (*55*−*57*). Calmodulin is an inhibitor of the channel, and multiple calmodulin binding sites have been reported (*58*−*60*).

This work forms part of a growing paradigm for the targeting of PP1 which generally involves a targeting protein, e.g., to glycogen, myosin, and the endoplasmic reticulum (*6*−*8*). A number of these targeting proteins have been shown to possess a peptide motif that confers binding to a specific site on PP1 (*9*, *10*). This motif carries the signature VXF or VXW preceded by several basic residues, and followed by an acidic residue. Examination of the 5037-amino acid sequence of muscle RyR revealed a number of occurrences of sequences that could be candidates for such a motif, but none of these exhibited a strong correspondence to the motif found in other mammalian proteins, or in the array of peptide sequences that were identified by a random peptide screen (*10*). Further investigations by mutagenesis will be required to make a more precise definition of the PP1 binding site in the RyR sequence.

The architecture of the triad junction has been extensively studied at the biochemical and ultrastructural level, and it has become evident that the elements that control calcium flux at this membrane junction are highly organized and that a number of ancillary regulatory proteins may also be present. As already noted (introductory section), there remain a number of unanswered questions regarding the identity and the targets of the kinases and phosphatases in relation to the control of calcium flux at the triad junction. This could involve the direct phosphorylation or dephosphorylation of RyR itself, or of the ancillary proteins that are part of the supramolecular protein assemblies that form the functional triad junction. Our data provide a further experimental step for the concept that regulatory kinases and phosphatases are targeted to the protein assemblies involved in calcium flux during muscle excitation−contraction coupling. Moreover, there is now growing evidence for the concept of kinase/phosphatase targeting in a number of other cellular systems, some of which involve specialized scaffold or targeting proteins such as the family of AKAP proteins (*61*). Given the specialized architecture of the triad, and the growing evidence that signaling events require the molecular juxtaposition of the interacting proteins, the finding that PP1 is also targeted to RyR is likely to be significant to the control of RyR function.

REFERENCES

1. Bollen, M., and Stalmans, W. (1992) *Crit. Rev. Biochem. Mol. Biol. 27*, 227−281.
2. Brautigan, D. L. (1994) *Recent Prog. Horm. Res. 49*, 197−214.
3. Shenolikar, S. (1994) *Annu. Rev. Cell Biol. 10*, 55−96.
4. Shenolikar, S., and Nairn, A. (1991) *Adv. Second Messenger Phosphoprotein Res. 23*, 1−123.
5. Lee, E. Y. C. (1995) *Zool. Stud. 34*, 149−163.
6. Hubbard, M. J., and Cohen, P. (1993) *Trends Biochem. Sci. 18*, 172−177.
7. Stralfors, P., Hiraga, A., and Cohen, P. (1985) *Eur. J. Biochem. 149*, 295−303.
8. Chisholm, A. A., and Cohen, P. (1988) *Biochim. Biophys. Acta 971*, 163−169.
9. Zhao, S., and Lee, E. Y. C. (1997) *J. Biol. Chem. 272*, 28368−28372.
10. Egloff, M. P., Johnson, D. F., Moorhead, G., Cohen, P. T. W., Cohen, P., and Barford, D. (1997) *EMBO J. 16*, 1876−1887.
11. Tu, J., Song, W., and Carlson, M. (1996) *Mol. Cell. Biol. 16*, 4199−4206.
12. Kawamoto, R. M., Brunschwig, J.-P., Kim, K. C., and Caswell, A. H. (1986) *J. Cell Biol. 109*, 1405−1414.
13. Inui, M., Saito, A., and Fleischer, S. (1987) *J. Biol. Chem. 262*, 1740−1747.
14. Imagawa, T., Smith, J. S., Coronado, R., and Campbell, K. P. (1987) *J. Biol. Chem. 262*, 16636−16643.
15. Lai, A. F., Erickson, H. P., Rousseau, E., Liu, Q. Y., and Meissner, G. (1988) *Nature 331*, 315−319.
16. Schneider, M. F., and Chandler, W. K. (1973) *Nature 242*, 244−246.
17. Rios, E., and Brum, G. (1987) *Nature 325*, 717−720.
18. Rios, E., Karhanek, M., Ma, J., and Gonzalez, A. (1993) *J. Gen. Physiol. 102*, 449−482.
19. Takeshima, H., Nishimura, S., Matsumoto, R., Ishida, H., Kanagawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T., and Numa, S. (1989) *Nature 339*, 439−445.
20. Nakai, J., Imagawa, T., Hakamata, Y., Shigekawa, M., Takeshima, H., and Numa, S. (1990) *FEBS Lett. 271*, 169−177.
21. Hakamata, Y., Nakai, J., Takeshima, H., and Imoto, K. (1992) *FEBS Lett. 312*, 229−235.
22. Wagenknecht, T., Grassucci, R., Frank, J., Saito, A., Inui, M., and Fleischer, S. (1989) *Nature 338*, 167−170.
23. Seiler, S., Wegener, A. D., Whang, D. D., Hathaway, D. R., and Jones, L. R. (1984) *J. Biol. Chem. 262*, 12309−12315.
24. Herrmann-Frank, A., and Varsanyi, M. (1993) *FEBS Lett. 332*, 237−224.
25. Hain, J., Nath, S., Mayrleitner, M., Fleischer, S., and Schindler, H. (1994) *Biophys. J. 67*, 1823−1833.
26. Suko, J., Maurer-Fogy, I., Plank, B., Bertel, O., Wyskovsky, W., Hohenegger, M., and Heilmann, G. (1993) *Biochim. Biophys. Acta 1175*, 193−206.
27. Dombradi, V. K., Silberman, S. R., Lee, E. Y. C., Caswell, A. H., and Brandt, N. R. (1984) *Arch. Biochem. Biophys. 230*, 615−630.
28. Han, J.-W., Thieleczek, R., Varsanyi, M., and Heilmeyer, L. M. G., Jr. (1992) *Biochemistry 31*, 377−384.
29. Caswell, A. H., Lau, Y. H., and Brunschwig, J.-P. (1976) *Arch. Biochem. Biophys. 176*, 417−430.
30. Motoike, H., Caswell, A. H., Smilowitz, H. M., and Brandt, N. R. (1994) *J. Muscle Res. Cell Motil. 15*, 493−504.
31. Brandt, N. R., Caswell, A. H., Brandt, T., Brew, K., and Mellgren, R. L. (1992) *J. Membr. Biol. 127*, 35−47.
32. Shoshan-Barmatz, V., Weil, S., Meyer, H., Varsanyi, M., and Heilmeyer, L. M. G. (1994) *J. Membr. Biol. 142*, 281−288.
33. Lai, F. A., Erickson, H. P., Rousseau, E., Liu, Q.-Y., and Meissner, G. (1988) *Nature 331*, 315−319.

34. Zhao, S., Xia, W., and Lee, E. Y. C. (1996) *Arch. Biochem. Biophys. 325*, 82−90.
35. Brandt, N. R., Caswell, A. H., Lewis, C. S. A., Fergusen, D. G., Brandt, T., Brunschwig, J.-P., and Bassett, A. L. (1993) *J. Membr. Biol. 131*, 219−228.
36. Kawamoto, R. M., Brunschwig, J.-P., Kim, K. C., and Caswell, A. H. J. (1986) *Cell Biol. 103*, 1405−1414.
37. Zhang, Z., Bai, G., Deans-Zirattu, S., Browner, M. F., and Lee, E. Y. C. (1992) *J. Biol. Chem. 267*, 1464−1490.
38. Zhang, Z., Bai, G., Shima, S., Zhao, S., Nagao, M., and Lee, E. Y. C. (1993) *Arch. Biochem. Biophys. 303*, 402−406.
39. Zhang, Z., Zhao, S., Deans-Zirattu, S., Bai, G., and Lee, E. Y. C. (1994) *J. Biol. Chem. 269*, 13766−13770.
40. Kim, K. C., Caswell, A. H., Talvenheimo, J. A., and Brandt, N. R. (1990) *Biochemistry 29*, 9281−9289.
41. Otsu, K., Willard, H. F., Khanna, V. K., Zorzato, F., Green, N. M., and MacLennan, D. H. (1990) *J. Biol. Chem. 265*, 3472−3483.
42. Sasaki, K., Shima, H., Kitagawa, Y., Irino, S., Sugimura, T., and Nagao, M. (1990) *Jpn. J. Cancer Res. 81*, 1272−1280.
43. Jayaraman, T., Brillantes, A. M., Timerman, A. P., Fleischer, S., Erdjument-Bromage, H., Temptst, P., and Marks, A. R. (1992) *J. Biol. Chem. 267*, 9474−9477.
44. Chen, S. R. W., Airey, J. A., and MacLennan, D. H. (1993) *J. Biol. Chem. 268*, 22642−22649.
45. Marks, A. R., Fleischer, S., and Tempst, P. (1990) *J. Biol. Chem. 265*, 13143−13149.
46. Meissner, G., Rousseau, E., and Lai, F. A. (1989) *J. Biol. Chem. 264*, 1715−1722.
47. Chu, A., Sumbilla, C., Scales, D., Piazza, A., and Inesi, G. (1988) *Biochemistry 27*, 2977−2833.
48. Shoshan-Barmatz, V., and Zarka, A. (1988) *J. Biol. Chem. 263*, 16772−16779.
49. Herrmann-Frank, A., and Varsanyi, M. (1993) *FEBS Lett. 332*, 2378−2242.
50. Seiler, S., Wegener, A. D., Whang, D. D., Hathaway, D. R., and Jones, L. R. (1984) *J. Biol. Chem. 262*, 12309−12315.
51. Hain, J., Onoue, H., Mayerleitner, M., Fleischer, S., and Schindler, H. (1995) *J. Biol. Chem. 270*, 2074−2081.
52. Mayerleitner, M. R., Chandler, H., Schindler, H., and Fleischer, S. (1995) *Cell Calcium 18*, 197−206.
53. Netticadan, T., Xu, A., and Narayanan, N. (1996) *Arch. Biophys. Biochem. 333*, 368−376.
54. Sonnleitner, A., Fleischer, S., and Schindler, H. (1997) *Cell Calcium 21*, 283−290.
55. Mayrleitner, M., Timerman, A. P., Wiederrecht, G., and Fleischer, S. (1994) *Cell Calcium 15*, 99−108.
56. Cameron, A. M., Steiner, J. P., Roskams, A. J., Ali, S. M., Ronett, G. V., and Snyder, S. H. (1995) *Cell 83*, 463−472.
57. Timerman, A. P., Onoue, H., Xin, H. B., Barg, S., Copello, J., Wiederrecht, G., and Fleischer, S. (1996) *J. Biol. Chem. 271*, 20385−20391.
58. Menegazzi, P., Larini, F., Treves, S., Guerrini, R., Quadroni, M., and Zorzato, F. (1994) *Biochemistry 33*, 9078−9084.
59. Yang, H., Reedy, M. M., Burke, C. L., and Strasburg, G. M. (1994) *Biochemistry 33*, 518−525.
60. Wagenknecht, T., and Radermacher, M. (1995) *FEBS Lett. 369*, 43−46.
61. Lester, L. B., and Scott, J. D. (1997) *Recent Prog. Horm. Res. 52*, 409−429.
62. Silberman, S. R., Speth, M., Nemani, R., Dombradi, V., Paris, H., and Lee, E. Y. C. (1984) *J. Biol. Chem. 259*, 2913−2922.

BI982144K