Calmodulin Binding Sites of the Skeletal, Cardiac, and Brain Ryanodine Receptor Ca^{2+} Channels: Modulation by the Catalytic Subunit of cAMP-Dependent Protein Kinase?†

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ABSTRACT: In this study, we define calmodulin binding sites of skeletal, cardiac, and brain ryanodine receptor (RYR) Ca^{2+} channels. Cardiac and brain RYR peptides corresponding to the calmodulin binding sites present in the skeletal RYR [Menegazzi, P., et al. (1994) *Biochemistry 33*, 9078−9084] were synthesized, and their interaction with calmodulin was monitored by fluorescent techniques. The central portions of the skeletal, cardiac, and brain RYR protomers display one high (CaM1; K_d ranging between 2.7 and 10.2 nM) and one low affinity (CaM2; K_d ranging between 116 and 142 nM) calmodulin binding site. Depending on the RYR model having 4 or 12 transmembrane segments, a third calmodulin binding site (CaM3) was identified a few residues upstream from the putative transmembrane segment M1 or M5. Its affinity for calmodulin varied between the RYR isoforms: the cardiac RYR CaM3 displays a high affinity (9.09 ± 1.0 nM, $n = 5$), while the skeletal and brain RYR CaM3 have low affinity, the lowest affinity being displayed by the brain isoform (234 ± 39 nM, $n = 3$). The RYRs calmodulin binding site CaM1 encompasses the sequence Arg-His-Arg-Val(Ile)-Ser-Leu, which is phosphorylated *in vitro* by the catalytic subunit of the cAMP-dependent protein kinase. Phosphorylation of RYR PM1 peptides occurs on the Ser, corresponding to amino acid number 2919, 3020, and 3055 of the brain, cardiac, and skeletal RYR protomers, respectively. We found that phosphorylation of the RYR PM1 peptides was inhibited by calmodulin binding and that the formation of the PM1 peptide−calmodulin complex was inhibited by peptide phosphorylation. These data indicate that the effect of calmodulin binding to RYR CaM1 may be regulated by the phosphorylation state of the Ser residue localized within the sequence Arg-His-Arg-Val(Ile)-Ser-Leu.

Ca^{2+} release from intracellular stores plays an important role in the regulation of numerous cellular functions ranging from muscle contraction to cell division and neurotransmitter release. The intracellular Ca^{2+} stores of excitable and nonexcitable cells utilize one or two of the intracellular Ca^{2+} channels to release Ca^{2+} upon cell stimulation (Berridge, 1993). The inositol 1,4,5-trisphosphate receptor, $InsP_3R$, which is activated by the second messenger $InsP_3$, mediates Ca^{2+} release mainly from the endoplasmic reticulum of nonexcitable cells (Berridge, 1993; Mikoshiba, 1993). The Ca^{2+} release channel of skeletal and cardiac cells is named ryanodine receptor (Rios & Pizarro, 1991); this channel is a large tetrameric oligomer made up of four subunits, which migrate as a single protein of approximately 560 kDa in a SDS[1]-polyacrylamide gel (Pessah et al., 1985; Inui et al., 1987; Imagawa et al., 1987; Lai et al., 1988; Fleischer & Inui, 1989). In neuronal cells, release of calcium from intracellular stores has been shown to be mediated by both the $InsP_3R$ and the ryanodine receptor.

The primary structures of the skeletal, cardiac, and brain ryanodine receptors have been obtained, and sequence analysis comparison has established an overall homology at the amino acid level of approximately 60% among the three isoforms (Takeshima et al., 1989; Zorzato et al., 1990; Otsu et al., 1990; Nakai et al., 1990; Hakamata et al., 1992). Release of Ca^{2+} via the RYRs is modulated by a variety of putative physiological agents including Ca^{2+}, ATP, Mg^{2+} cADPR, and calmodulin (Smith et al., 1988; Lai et al., 1988; Lee et al., 1994; Meissner, 1994). The latter protein has been shown to inhibit Ca^{2+} release from microsomal vesicles and decrease the activity of RYR reconstituted in planar bilayers (Meissner, 1986; Meissner & Henderson, 1987; Plank et al., 1988; Smith et al., 1989; Fuentes et al., 1994). The effect of calmodulin is most likely the consequence of its direct interaction on the channel protein, since its inhibitory effect occurs in the absence of ATP. The presence

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[1] Abbreviations: Ab, antibodies; DTT, dithiothreitol; EDTA, ethylene(dinitrilo)tetraacetic acid; EGTA, ethylenebis(oxyethylenenitrilo)-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethane sulfonyl fluoride; RYR, ryanodine receptor; SDS, sodium dodecyl sulfate; MOPS, 4-morpholinepropanesulfonic acid; HEPES 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; cADP ribose, cyclic-ADP ribose; cAMP, cyclic-AMP; PKA, protein kinase A.

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of calmodulin binding sites in the RYR has been confirmed by photoaffinity labeling studies on sarcoplasmic reticulum terminal cisternae (Seiler et al., 1984; Yang et al., 1994). The RYR seems to be the only calmodulin binding protein present in terminal cisternae, and it encompasses two classes of calmodulin binding sites: one having high affinity and the other low affinity (Yang et al., 1994). Analysis of the primary structure of the skeletal muscle RYR lead to the identification of putative calmodulin binding sites which are defined by residues 2807−2840, 2909−2930, 3031−3049, 3614−3637, and 4295−4325 (Takeshima et al., 1989; Zorzato et al., 1990). Potential calmodulin binding sites were also identified in cardiac and brain RYR, and their positions in the primary structure appear to be homologous to those observed in their skeletal muscle counterpart (Otsu et al., 1990; Nakai et al., 1990; Hakamata et al., 1992).

In this study we have identified calmodulin binding sites in the cardiac and brain RYRs by following the fluorescence emission of dansyl-calmodulin upon incubation with RYR synthetic peptides. Our results show that the calmodulin binding sequences which we previously identified in the skeletal muscle RYR (Menegazzi et al., 1994) are conserved in both cardiac and brain RYR. The calmodulin binding site having the highest affinity (CaM 1) is located in the central portion of the three RYR isoforms, adjacent to the putative transmembrane segment M′. Interestingly, the binding site CaM1 contains a Ser residue whose phosphorylation by the catalytic subunit of the cAMP-dependent protein kinase is strongly inhibited by calmodulin.

MATERIALS AND METHODS

Materials

Nitrocellulose was from Schleischer and Schuell. Calmodulin, digoxigenin-3-*O*-methylcarbonyl-ϵ-aminocaproic acid *N*-hydroxysuccinimide ester, anti-digoxigenin peroxidase conjugated antibodies, and BM chemiluminescence were from Boehringer Mannheim. Dansyl-calmodulin and heparin agarose resin were from Sigma. Sephacryl S500 HR was from Pharmacia. Amino acid derivatives, resin, and reagents were from Novabiochem A. All other chemicals were reagent grade.

Methods

Preparation of Sarcoplasmic Reticulum Fractions. Terminal cisternae (TC) were obtained from the white skeletal muscle of New Zealand rabbits as described by Saito et al. (1984).

Preparation of Cerebellum RYR. Briefly, 30 mg of bovine cerebellum microsomes were solubilized with 1% Triton X-100, resuspended at a final concentration of 2 mg/mL in 50 mM Tris-HCl, pH 8.5, 1 mM DTT, and 1% Triton X-100, and loaded onto a heparin−agarose column equilibrated with 50 mM Tris-HCl, pH 8.5, 1 mM DTT, 1% Triton-X 100, 1 mM EDTA, and 100 mM NaCl. The column was washed with 80 mL of the same buffer, and the proteins were eluted with 100 mL of a linear gradient of 100−800 mM NaCl in 50 mM Tris-HCl, pH 8.5, 1 mM DTT, and 1% Triton X-100. Fractions of 4 mL each were collected and subjected to SDS−PAGE. Fractions containing the RYR (eluting at approximately 200 mM NaCl) were pooled, dialyzed against 40 mM Tris-HCl, pH 8.0, 20 mM sodium acetate, and 2 mM EDTA, and loaded onto a Sephacryl S500 HR column (40 cm × 2 cm) previously equilibrated with 40 mM Tris-HCl, 20 mM sodium acetate, 2 mM EDTA, and 0.1% SDS, pH 8.0 (solution C). The flow rate was adjusted to 20 mL/h, and fractions of 5 mL each were collected. Fractions enriched in high molecular mass proteins (>450 kDa) were identified and pooled. Protein concentration was determined according to Bradford (1983) using bovine serum albumin as standard.

SDS−Polyacrylamide Gel Electrophoresis, Calmodulin Overlay, and Immunological Techniques. Slab gel electrophoresis was carried out as described by Laemmli (1970). Western blots were performed as described by Gershoni et al. (1985). Calmodulin overlays were performed with digoxigenated hog brain calmodulin as previously described (Menegazzi et al., 1994). Indirect immunoenzymatic staining of Western blots was carried out as described by Menegazzi et al. (1994) using anti-rabbit skeletal muscle RYR polyclonal Ab (Orleans et al., 1961) raised against fusion proteins encompassing amino acid sequences highly conserved among the RYR isoforms.

Peptide Synthesis and Phosphorylation. Peptides were synthesized as described by Temussi et al. (1993). Peptide concentration was determined by weight and by the Bradford protein determination method using bovine serum albumin as a standard. Peptide phosphorylation by the catalytic subunit of the cAMP-dependent protein kinase was carried out as described by Feramisco et al. (1980). Briefly, 1−5 μg of peptide was incubated for 2 min at 30 °C in a solution containing 20 mM MOPS, pH 6.5, 12.5 mM magnesium acetate, 0.3 mg/mL bovine serum albumin, 250 μM ATP (300−1000 cpm/pmol), 100 μM $CaCl_2$, and 10 μg/mL catalytic subunit of PKA (Sigma #P-8289). The reaction was blocked by quick freezing in liquid nitrogen. Phosphorylated peptides were either loaded on a 20% polyacrylamide gel or spotted onto ion exchange Whatmann paper P81. The Whatman paper containing the ^{32}P-labeled peptides was washed three times 5 min each with 50−60 mL of 0.5 M phosphate buffer, and the incorporated radioactivity was measured by liquid scintillation counting. Phosphorylated peptides were purified by reverse-phase liquid chromatography on a Vydac Protein-Peptide C18 column (The Separation Group, Hesperia, CA, catalog no. 218 TP 5415) using a Bruker liquid chromatography LC21-C equipped with Bruker LC313 UV detector. Mobile phases A and B contained 10% acetonitrile/0.1% TFA in water and 60% acetonitrile/0.1% TFA in water, respectively. Elution of peptides from the column was carried out by a linear gradient from 20% B to 60% B at a flow rate of 1 mL/min over 20 min and monitored at 220 nm. Peaks eluting at approximately 15.5 and 16.5 min were collected and lyophilized. Peptides were resuspended in sterile ultrapure water and used immediately for the dansyl-calmodulin binding assays.

Peptide Mass Determinations by Mass Spectroscopy. A triple-stage quadrupole mass spectrometer (TSQ 700, Finnigan MAT, San Jose, CA) was used. A pneumatically assisted electrospray (ion spray) was used for sample introduction to the atmospheric ionization source of the mass spectrometer. The interface capillary sprayer was operated at a positive potential of 4.5 kV. Ionized analyte was then transferred from atmospheric pressure to vacuum (1.5×10^6 Torr) through a heated metal capillary held at 200 °C. Multiply charged molecule ions of the type $M + nH^{n+}$

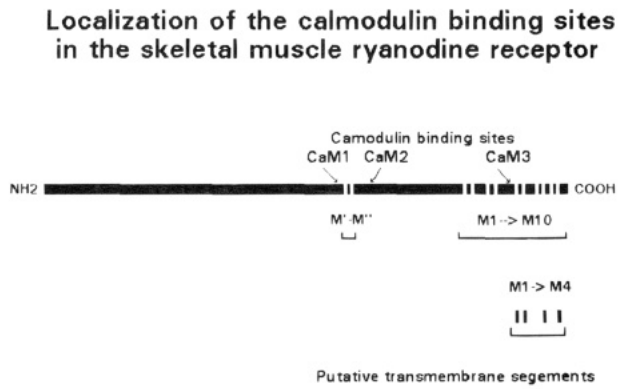

FIGURE 1: Localization of calmodulin binding sites in the skeletal muscle RYR protomer. Putative transmembrane segments are indicate by M′ → M′ and M1 → M10 (Zorzato et al., 1990). The RYR model proposed by Takeshima et al. (1989) includes four transmembrane segments which are indicated by M1 → M4.

exciting the capillary were focused by a tube lens through a skimmer and then focused by an octapole lens into the mass analyzed. Samples were analyzed by direct infusion using a syringe pump (Harvard Apparatus, MA) at a flow rate of 3 μL/min. Peptide concentrations were typically 50 pmol/μL in water/methanol (50/50 v/v) containing 1% acetic acid. Full scan MS experiments were obtained by scanning quadrupole 1 from *m/z* 200 to 1500 in 3 s. Data were acquired onto a DEC station 5000/125 computer (Digital Equipment Corporation, Maynard, MA) and were processed using the deconvolution software package BIOMASS (FinniganMAT) to get molecular weight determinations from the multiply charged ion envelopes. The calculated mass of the peptides matched those determined experimentally (not shown).

Fluorimetric Measurements. spectra were measured in a Perkin Elmer LS50 spectrofluorimeter as described by Kincaid et al. (1982). The excitation wavelength was 340 nm, and emission spectra were recorded between 400 and 600 nm. The dissociation constants were calculated according to Stinson and Hoolbrook (1973).

RESULTS

Identification of Calmodulin Binding Sequences of the Three RYR Isoforms. In a recent report we described the identification of three calmodulin binding sites in the skeletal muscle RYR (Menegazzi et al., 1994). Figure 1 shows a schematic representation of the localization of the calmodulin binding sites within the skeletal muscle RYR protomer: the first two sites (CaM1 and CaM2) are in the central portion of the molecule, while the third (CaM3) is located at the COOH terminus. Calmodulin is known to modulate not only the skeletal muscle RYR but also the RYR contained in heart and brain (Meissner & Henderson, 1987; McPherson & Campbell, 1993).

Earlier studies have shown that cerebella express RYR isoforms to different extents: the cardiac RYR is the predominant isoform, while a low amount of the brain RYR isoform is also present in some cellular types (Furuichi et al., 1994; Lai et al., 1992). Partially purified RYR fractions obtained from bovine cerebella were subjected to calmodulin overlay and western blot analysis with anti-skeletal muscle RYR antibodies. As shown in Figure 2, bovine cerebellum contains a high molecular weight protein having a slightly

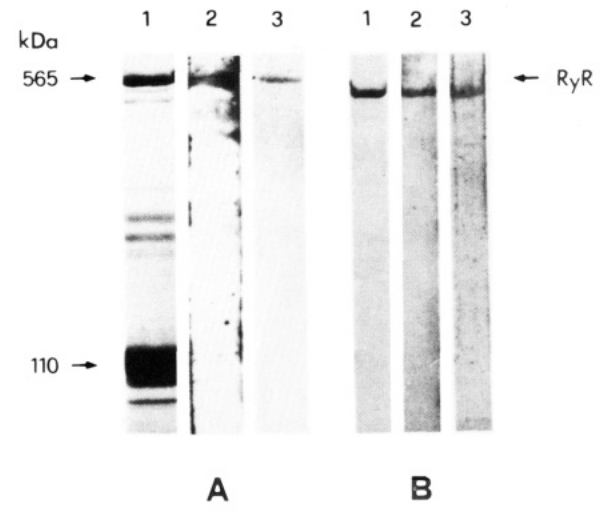

FIGURE 2: Calmodulin binding to RYRs from rabbit skeletal muscle and bovine cerebellum. (Panel A) 5% SDS−polyacrylamide gel of proteins present in rabbit skeletal muscle terminal cisternae (20 μg of protein). (Panel B) 5% SDS-polyacrylamide gel of the high molecular weight proteins present in bovine cerebellum microsomes isolated by gel exclusion chromatography (70 μL of a 5 mL fraction were loaded). Lanes 1: Coomassie Brilliant Blue stained polyacrylamide gel. Lanes 2: autoradiograph of the calmodulin overlay. Lanes 3: indirect immunoenzymatic staining of the western blot with anti skeletal RYR Ab (final concentration 2 μg/mL).

faster electrophoretic mobility than the RYR protomer present in skeletal muscle terminal cisternae (Figure 2, lane 1, panels A−C). The identity of the cerebellar high molecular weight band was confirmed by western blotting analysis with a polyclonal anti-skeletal muscle RYR Ab. As expected, these Ab recognized the RYR band contained in the terminal cisternae fraction (Figure 2A, lane 3) and also cross-reacted with the single band present in the cerebellum preparation (Figure 2B, lane 3). Furthermore the immunopositive protein contained in the cerebellum was capable of binding calmodulin when tested by ligand overlay (Figure 2B, lane 2). The present data indicate that the different RYR isoforms are capable of binding calmodulin; however, they convey no information concerning the molecular topology of the calmodulin binding sites within the primary structure of the cardiac and brain RYR protomers. In order to address this issue, we synthesized 16−19 amino acid long peptides encompassing putative calmodulin binding sequences of the cardiac and brain RYR isoforms which are homologous to those that have been identified in the skeletal muscle RYR. Table 1 shows the amino acid sequences of the 10 synthetic peptides: PM1 encompasses amino acids 3042−3057 of the skeletal muscle RYR (RYR1), 3007−3023 of the cardiac RYR (RYR2), and 2906−2922 of the brain RYR (RYR3); PM2 encompasses amino acids 3617−3634 of RYR1, 3583−3601 of RYR2, and 3474−3492 of RYR3; PM3 encompasses amino acids 4540−4557 of RYR1, 4480−4497 of RYR2, and 4393−4410 of RYR3 (Hakamata et al., 1992); RYR PM3 Lys_{11} → Ala is a mutated peptide of RYRPM3 which serves as a control for calmodulin binding. Peptides PM1 and PM2 from skeletal, brain, and cardiac RYR show a high degree of similarity, while RYRs peptides PM3 are quite divergent except for the presence of conserved residues at a position which is crucial to confer calmodulin binding activity (Ikura et al., 1992).

Fluorescence Studies of the Interaction of Calmodulin with the Synthetic RYR Calmodulin Binding Peptides. Calmodu-

Table 1: Calmodulin Binding Sites of Skeletal, Cardiac, and Brain Ryanodine Receptors[a]

RYR1 PM1	L-F-C-K-L-A-A-L-V-R-H-R-V-S-L-F
RYR2 PM1	L-F-C-K-L-G-V-L-V-R-H-R-I-S-L-F-G
RYR3 PM1	L-F-C-K-L-A-A-L-V-R-H-R-I-S-L-F-G
RYR1 PM2	K-A-V-W-H-K-L-L-S-K-Q-R-R-R-A-V-V-A
RYR2 PM2	K-K-A-V-W-H-K-L-L-S-K-Q-R-K-R-A-V-V-A
RYR3 PM2	K-K-A-V-W-H-K-L-L-S-K-Q-R-K-R-A-V-V-A
RYR1 PM3	F-W-G-E-L-E-V-Q-R-V-K-F-L-N-Y-L-S-R
RYR2 PM3	F-W-K-K-I-I-A-Y-Q-Q-K-L-L-N-Y-F-A-R
RYR3 PM3	F-F-K-G-L-E-I-Y-Q-T-K-L-L-H-Y-L-A-R
RYR1 PM3 Lys^{11}->Ala	F-W-G-E-L-E-V-Q-R-V-A-F-L-N-Y-L-S-R

[a] Synthesis was carried out as described in Material and Methods.

lin−RYR peptide interaction was detected by fluorescence measurements by using dansylated bovine brain calmodulin as described by Kincaid et al. (1982). Addition of the RYR peptides to the Ca^{2+}−dansyl-calmodulin complex (closed circles) caused a Ca^{2+}−dependent increase in the fluorescence emission of dansyl-calmodulin. The Ca^{2+} dependency is demonstrated by the fact that the fluorescence emission of dansyl-calmodulin returned to basal levels in the presence of 1 mM EGTA (open squares) (Figure 3). We then titrated dansyl-calmodulin with increasing concentrations of each RYR peptide to determine the affinity of the peptides for calmodulin. At a peptide to calmodulin ratio of 1, the percentage of saturation of the maximal fluorescence emission varied as a function of the three classes of peptides, namely, PM1, PM2, and PM3. The higher percentage saturation of the maximal fluorescence emission of dansyl-calmodulin was achieved with the PM1 peptides, regardless of the RYR isoform, and with cardiac RYR peptides PM3. The end point of titration of dansyl-calmodulin with RYRs peptides was approached at molar ratios ranging between 2 and 4, except for the peptide RYR3 PM3 which reached plateau at a ratio of 8−10 (Figure 4). Table 2 shows the calculated affinities for calmodulin of the RYRs peptides we synthesized. As can be seen, two groups of calmodulin binding sites covered by our RYR peptides exist: the first group defines a high affinity (2−10 nM) binding site, while the second group of peptides contains low affinity binding sites, having K_ds ranging between 100 and 240 nM. Interestingly, the distribution of these sites among the RYR isoforms is different. The cardiac RYR peptides PM1 and PM3 form two high affinity calmodulin binding sites, whereas peptide PM2 makes up a low affinity site. The affinity for calmodulin of the peptides of the skeletal and brain RYR peptides follows a similar pattern, that is, one high affinity and two low affinity binding sites. The high affinity calmodulin binding sites of both skeletal muscle and brain RYR are encompassed by peptide PM1; the low affinity calmodulin binding sites are constituted by peptides PM2 and PM3. The calmodulin binding site displaying the highest (2.7 ± 0.7 nM; $n = 3$) and the lowest (234 ± 39; $n = 3$) affinities for calmodulin are encompassed by brain RYR peptides PM1 and PM3, respectively (Figure 4). The lower affinity for calmodulin of brain RYR peptide PM3 compared to the other PM3 peptides is most likely due to the substitution of the tryptophan residue at position 2 with a phenylalanine.

The formation of a calmodulin−target peptide complex is mainly due to the hydrophobic interaction of aromatic or long hydrophobic amino acids of the peptide with the methionine residues of calmodulin (Ikura et al., 1992). It has been suggested that the stabilization of the calmodulin−peptide complex might be also linked to electrostatic interactions between positively charged amino acids of the target sequence and the glutamate residues of calmodulin. The degree of similarity of RYR PM3 peptides is low except for the presence of conserved residues at positions crucial to confer calmodulin binding activity. In particular, Lys and Arg at positions 11 and 18 are conserved among the RYRs peptides PM3 and appear to be crucial for the interaction between the RYR1 PM3 and dansyl-calmodulin (Menegazzi et al., 1994). We designed experiments to establish whether the Lys at position 11 is important in the interaction of skeletal muscle RYR PM3 peptide with calmodulin. Figure 5A shows the mass spectroscopy spectrum of the RYR1 PM3 $Lys^{11} \rightarrow$ Ala peptide. A single prominent peak corresponding to the expected mass was evident, confirming the identity of the RYR1 PM3$Lys^{11} \rightarrow$ Ala peptide. Addition of the RYR1 PM3 $Lys^{11} \rightarrow$ Ala peptide to Ca^{2+}−dansyl-calmodulin complex caused a negligible shift of the maximum of fluorescent emission of dansyl-calmodulin. Similar results were obtained at a peptide to dansyl-calmodulin ratio of 3 (not shown).

cAMP-Dependent Protein Kinase Phophorylation of RYRs PM1 Peptides and Its Modulation by Calmodulin. The RYR PM1 peptides contain a R-H-R-I(V)-S-L motif which satisfies, at least in part, the characteristic sequences of cAMP-dependent kinase substrates (Kemp & Pearson, 1990). Thus, in the following experiments we investigated if the RYR PM1 peptides are indeed a substrate for the catalytic subunit of the cAMP-dependent protein kinase. Phosphorylation assays were first carried out with RYR1 peptides PM1 and PM2. As can be seen in Figure 6, only the RYR1 PM1 peptide incorporated radioactive phosphate, while no phosphorylation was evident with the peptide RYR1 PM2, which does not have a hydroxyl amino acid in the appropriate consensus sequence. The Ser at position 14 of the PM1 peptide is the substrate of the catalytic subunit of protein kinase A (PKA), since peptides in which that residue has been substituted by Ala were not phosphorylated (not shown). Having established that the peptide PM1 is a specific substrate of the catalytic subunit of PKA, we examined if the PM1 peptides of the cardiac and brain RYR isoforms were also phosphorylated. Comparison between the Coomassie Brilliant Blue stained SDS−polyacrylamide gel and the autoradiograph clearly shows that the PM1 peptides (Figure 7), regardless of which RYR isoform they derive from, can be phosphorylated and that the incorporation of phosphate into the peptides occurs only in the presence of the catalytic subunit of the cAMP-dependent protein kinase. After 2 min of incubation with the catalytic subunit of cAMP-dependent kinase, the skeletal, cardiac, and brain RYR PM1 peptides incorporated 174 ($n = 2$), 100 ± 2 ($n = 3$), and 200 ± 7.0 ($n = 3$) pmol of $P_i/\mu g$ of peptide, respectively. We next determined whether phosphorylation was affected by the binding of calmodulin to its target sequence. Figure 8 shows that phosphorylation of the RYR PM1 peptides is inhibited by almost 80% upon incubation with increasing concentrations of calmodulin. Maximal inhibition occurred at a RYR PM1 peptide to calmodulin ratio of 1, a value

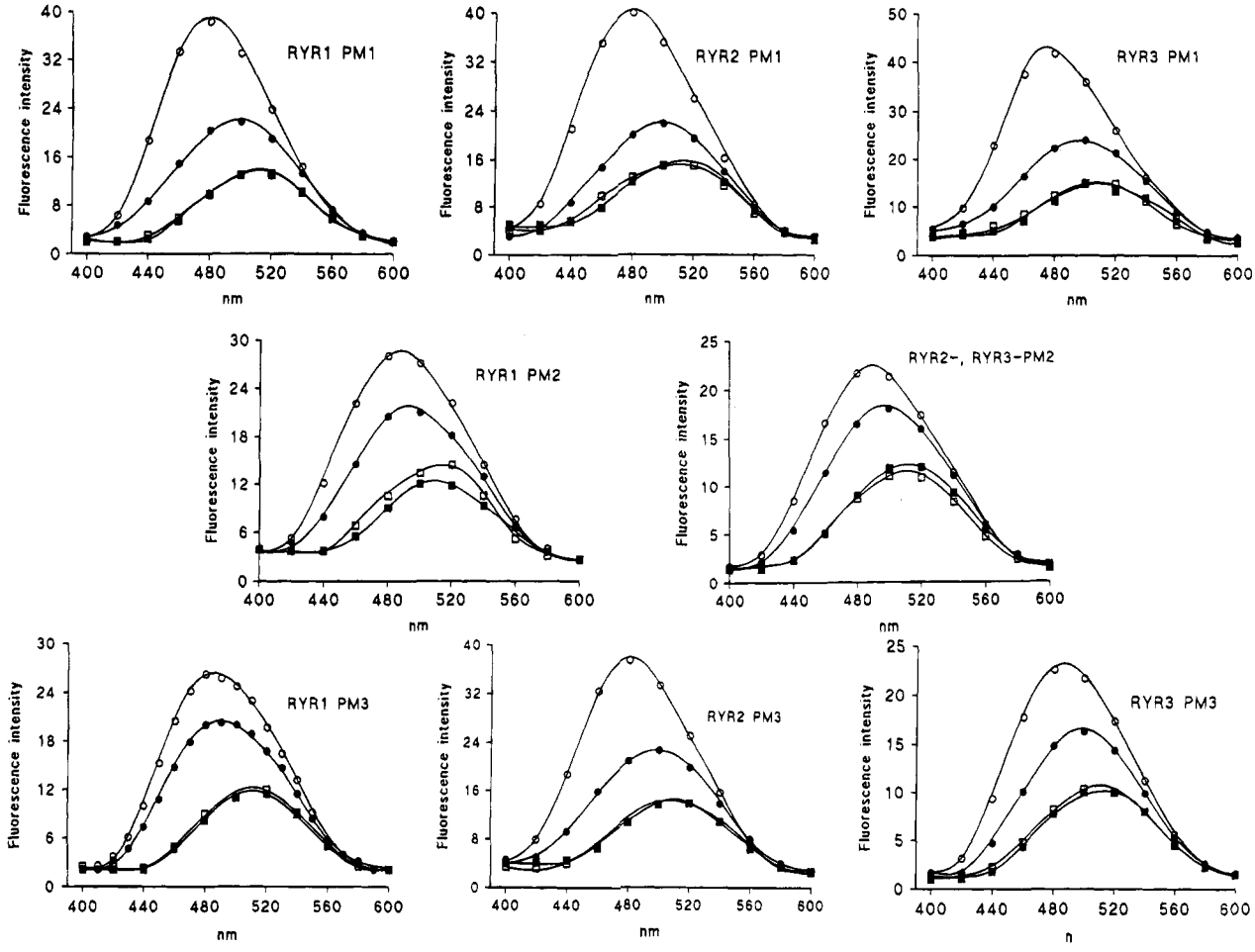

FIGURE 3: Effect of Ca^{2+} and RYR peptides PM1, PM2, and PM3 on the fluorescence spectra of dansyl-calmodulin. The experiments were carried out at equimolar concentrations of dansyl-calmodulin and RYR peptides as described in Material and Methods. Spectra were measured after sequential additions of 100 μM EGTA (filled squares), 500 μM $CaCl_2$ (filled circles), 0.4 μM RYR peptides (open circles), and 600 μM EGTA (open squares).

closely matching those observed during titration experiments with dansyl-calmodulin. The inhibition of phosphorylation is not due to the presence of a high concentration of calmodulin, since in control experiments using the synthetic kinase substrate kemptide (Sigma #K1127), we did not observe any inhibition of phosphorylation (Figure 8, upper left panel).

Effect of Phosphorylation on Calmodulin Binding to RYR1 PM1 Peptide. It has been shown that phosphorylation inhibits the binding of calmodulin to its target sequence (Chapmann et al., 1991). Thus, we examined whether binding of calmodulin was influenced by phosphorylation of the peptide PM1. RYR1 PM1 peptide was phosphorylated for 30 min, and the reaction mixture was subsequently applied to a reverse-phase liquid chromatography column (Vydac Protein-Peptide C18 catalog no. 218TP5415) to separate the phosphorylated peptide from its unphosphorylated conformation. The chromatographic profile of the reaction mixture at time zero shows a peak which elutes at approximately 16.5 min (Figure 9A). The product generated by 30 min of phosphorylation of RYR1 PM1 by the catalytic subunit of PKA eluted approx. 1 min earlier (Figure 9B). Figure 9C shows a chromatographic profile of the reaction mixture incubated in the presence of the catalytic subunit of PKA plus the unphosphorylated RYR1 PM1 peptide as reference. Comparison between panels A, B, and C clearly indicates that the peak eluting at 16.5 min corresponds to the unphosphorylated RYR1 PM1 peptides, while the peak eluting at approximately 15.5 min is the phosphorylated peptide. Such a conclusion is also supported by the autoradiography of the SDS–polyacrylamide gel loaded with the a fraction of the peak eluting at 15.5 min (Figure 10). The ability of the phosphorylated RYR1 PM1 peptide to interact with calmodulin was then tested by a spectrofluorimetric assay with dansyl-calmodulin. Addition of equimolar amount of phosphorylated RYR1 PM1 peptide to the Ca^{2+}–dansyl-calmodulin complex did not cause any fluorescence enhancement (Figure 11). Similar results were obtained even when the phosphorylated peptide to calmodulin ratio was increased up to 3 (not shown).

DISCUSSION

A number of studies have demonstrated that the RYR Ca^{2+} release channels are modulated by calmodulin (Meissner, 1994), an intracellular Ca^{2+} transducer which regulates the activity of a variety of structurally distinct proteins (O'Neil & DeGrado, 1990). Calmodulin has been shown to interact directly with the channel and reduce the Ca^{2+} release rate from skeletal and cardiac sarcoplasmic reticulum vesicles, without ever causing complete blockade of the Ca^{2+} release activity (Seiler et al., 1984; Meissner, 1986; Meissner & Henderson, 1987; Plank et al., 1988; Smith et al., 1989; Yang et al., 1994; Fuentes et al., 1994). Studies on the brain RYR have suggested that, in neurons as well, Ca^{2+} release from

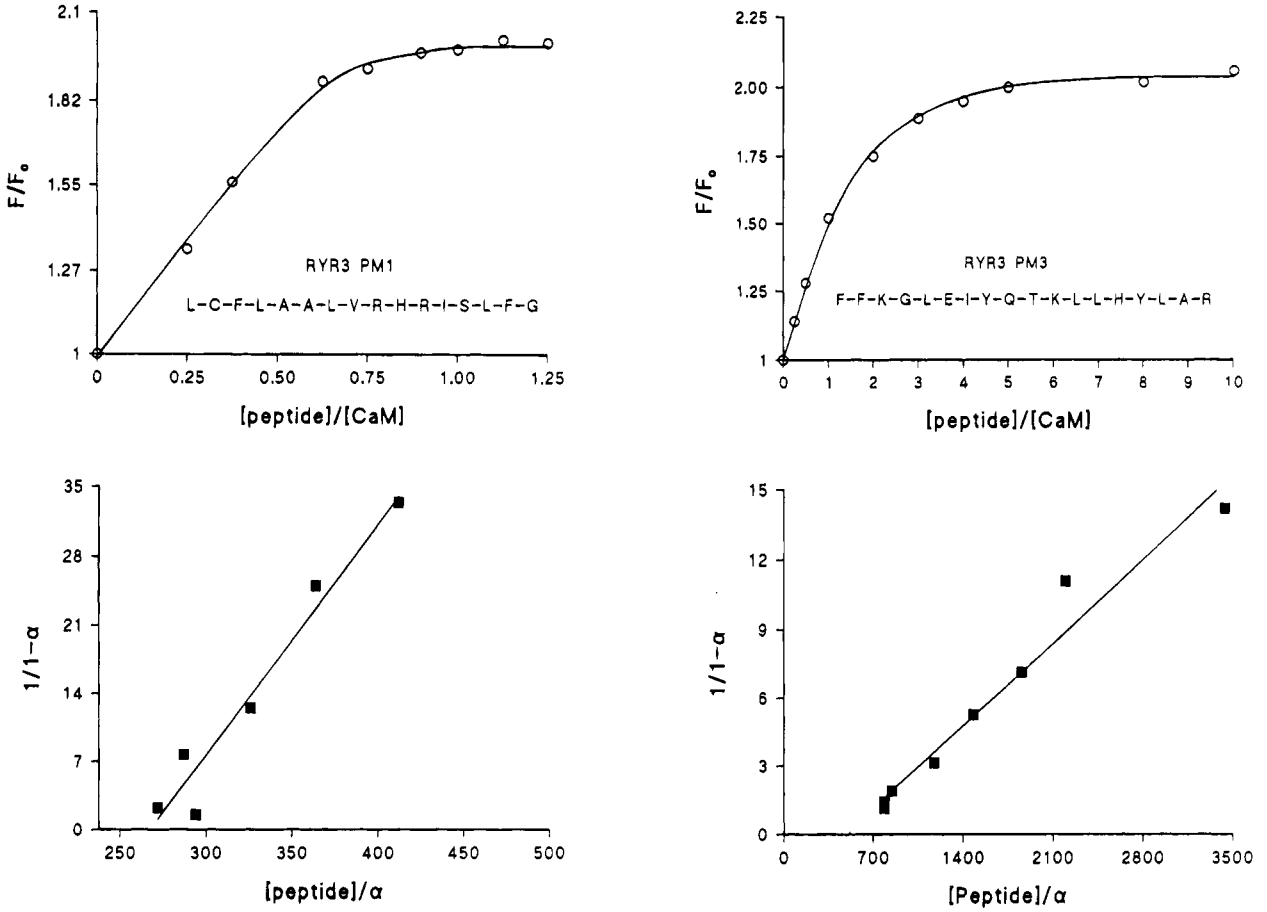

FIGURE 4: Titration of dansyl-calmodulin with RYR3 PM1 and PM3 peptides. (Upper panel) Dansyl-calmodulin (400 nM) was titrated with peptide RYR3 PM1 and RYR3 PM3 (from left to right) in the presence of 500 μM $CaCl_2$. F indicates fluorescence intensity of the peptide/dansyl-calmodulin complex; F_o indicates fluorescence of dansyl-calmodulin. F/F_o was plotted against the ratio between the concentrations of peptide and the concentration of dansyl-calmodulin. (Lower panel) The fractional degree of saturation (α) of dansyl-calmodulin was calculated according to Stinson and Holbrook (1973). A plot of $1/1-\alpha$ against the concentration of peptide divided by α results in a straight line if a 1:1 complex is formed. The K_d is the reciprocal of the slope. Data points of a representative experiment are plotted.

Table 2: Affinity Constants of the Calmodulin Sites of the Skeletal Muscle, Cardiac, and Brain Ryanodine Receptors[a]

	K_d (nM)		
peptide	RYR1	RYR2	RYR3
PM3	92 ± 18 (6)	9.09 ± 1.0 (5)	234 ± 39 (3)
PM2	142 ± 32 (4)	116 ± 14 (3)	116 ± 14 (3)
PM1	10.2 ± 1.6 (5)	3.9 ± 0.8 (4)	2.7 ± 0.7 (3)
PM1 $Lys^{11} \rightarrow$ Ala	undetectable		

[a] Peptides were synthesized as described in Material and Methods. Values are mean ± SD. The number of experiments is indicated in parentheses. The K_ds were calculated as described by Stinson and Brook (1973).

intracellular stores via the RYR might be down-regulated by calmodulin (McPherson & Campbell, 1993). On the other hand, a recent report shows that calmodulin is essential for the activation of the sea urchin egg RYR by cADP ribose (Lee et al., 1994). Taken together, experimental evidence clearly indicate that calmodulin modulates the activity of the members of the RYR Ca^{2+} channel family; however, the exact mechanism of action of calmodulin appears to be quite complicated. In order to help clarify this issue, we thought it would be important to define the calmodulin binding sites present in the three RYR isoforms.

Here we demonstrate that the cardiac and brain RYRs have three calmodulin binding sites: two of these sites are located in the central portion of the molecule (RYR CaM1 and RYR CaM2), while the third is located at the COOH terminus (RYR CaM3). According to the 12 transmembrane segments model of the RYR (Zorzato et al., 1990), the binding sites CaM1 and CaM2 are next to the putative transmembrane segments M′ and M″, whereas CaM3 is adjacent to the putative transmembrane segment M5. This implies that the effect of calmodulin on the activity of the cardiac and brain RYRs could involve a direct action on the putative transmembrane segments M′, M″, and M5. The number of reported calmodulin binding regions of the skeletal muscle RYR varies between three (Figure 1) and six (Chen & MacLennan, 1994). Such a discrepancy may be due to the different conditions used to quench unspecific binding during the calmodulin ligand overlay (Menegazzi et al., 1994; Chen & MacLennan, 1994). Three calmodulin binding regions were detected in the RYR1 (Menegazzi et al., 1994) when nonspecific binding was abolished using a protocol slightly different from that of Kessler et al. (1992), that is, the inclusion (i) of BSA and (ii) of low fat milk proteins in the blocking step. Omission of the incubation with low fat milk during the calmodulin overlay may account for the existence of additional nonspecific calmodulin binding sites in the RYR1 (Chen & MacLennan, 1994). Although we cannot exclude that the cardiac and brain RYR isoforms contain additional calmodulin binding sites, we think that this

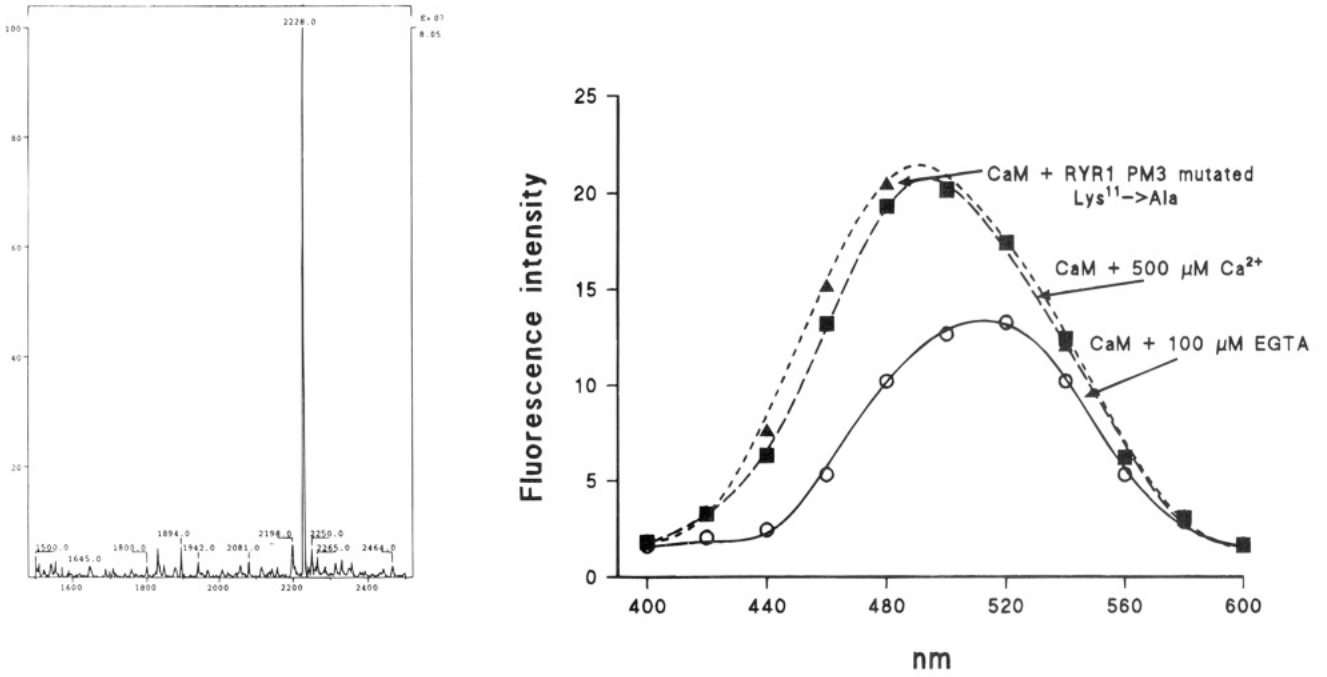

FIGURE 5: Effect of RYR1 PM3 Lys11 → Ala peptide on the fluorescence properties of dansyl-calmodulin. (Left panel) Mass spectroscopy spectrum of RYR1 Lys_{11} → Ala peptide. (Right panel) Fluorescent spectra of 0.4 μM dansyl-calmodulin were measured after sequential additions of 100 μM EGTA (open circles), 500 μM $CaCl_2$ (filled squares), and 0.4 μM RYR1 PM3 Lys_{11} → Ala peptide (filled triangles).

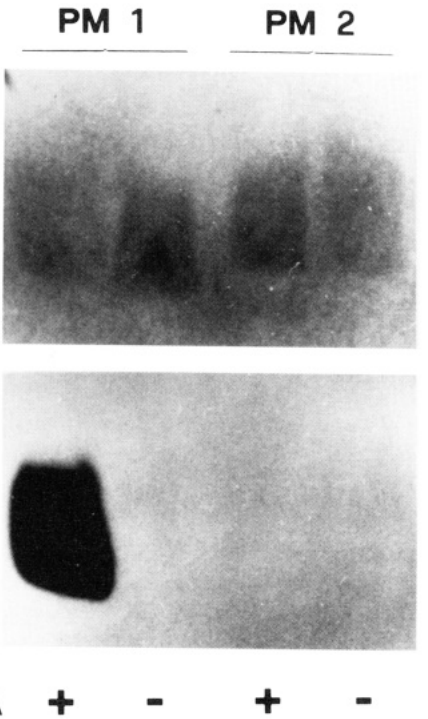

FIGURE 6: Phosphorylation of RYR1 PM1 and PM2 peptides by the catalytic subunit of cAMP-dependent protein kinase. Peptides (1−2 μg) were incubated with radioactive ATP either in the presence (+) or in the absence (−) of the catalytic subunit of PKA. At the end of the reaction, the assay medium was loaded on a 20% SDS−polyacrylamide gel. (Upper panel) Coomassie Brilliant Blue stained gel. (Lower panel) Autoradiograph of the phosphorylated peptides.

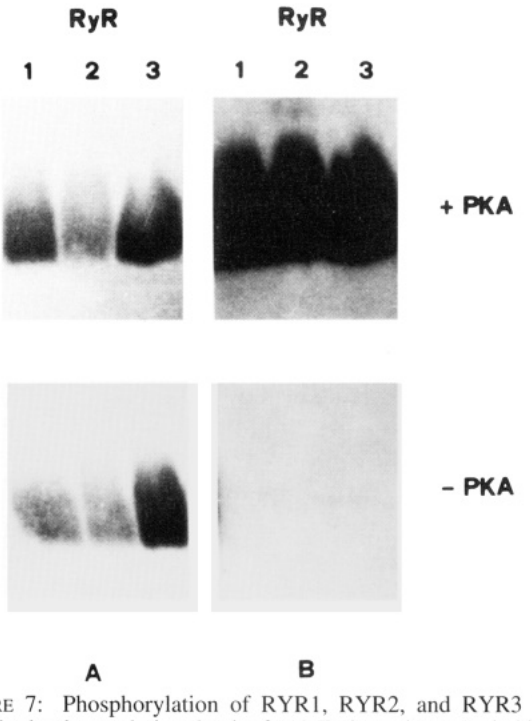

FIGURE 7: Phosphorylation of RYR1, RYR2, and RYR3 PM1 peptides by the catalytic subunit of cAMP-dependent protein kinase. Peptides were phosphorylated as described in Material and Methods. (Panel A) Coomassie Brilliant Blue stained 20% SDS−polyacrylamide gel. (Panel B) Autoradiograph of the phosphorylated peptides.

possibility is unlikely since the presence of many binding sites is a rather unusual feature of calmodulin-modulated proteins. The physiological relevance of the calmodulin binding sites identified here is not known. However, with the only possible exception of the RYR3 PM3 peptide, they are deemed to play an important role in the regulation of the Ca^{2+}-release channel since their interaction with calmodulin occurs at concentrations of ligand which are well below those that have been estimated in striated muscles and neuronal cells (Grand et al., 1979; Watterson et al., 1980).

In a recent report it was demonstrated that the native skeletal muscle RYR tetrameric complex displays two classes of calmodulin binding sites, one with low and the other with high affinity. The results presented here confirm, at least in part, the observations on the skeletal muscle RYR and also indicate that cardiac and brain RYR might have two

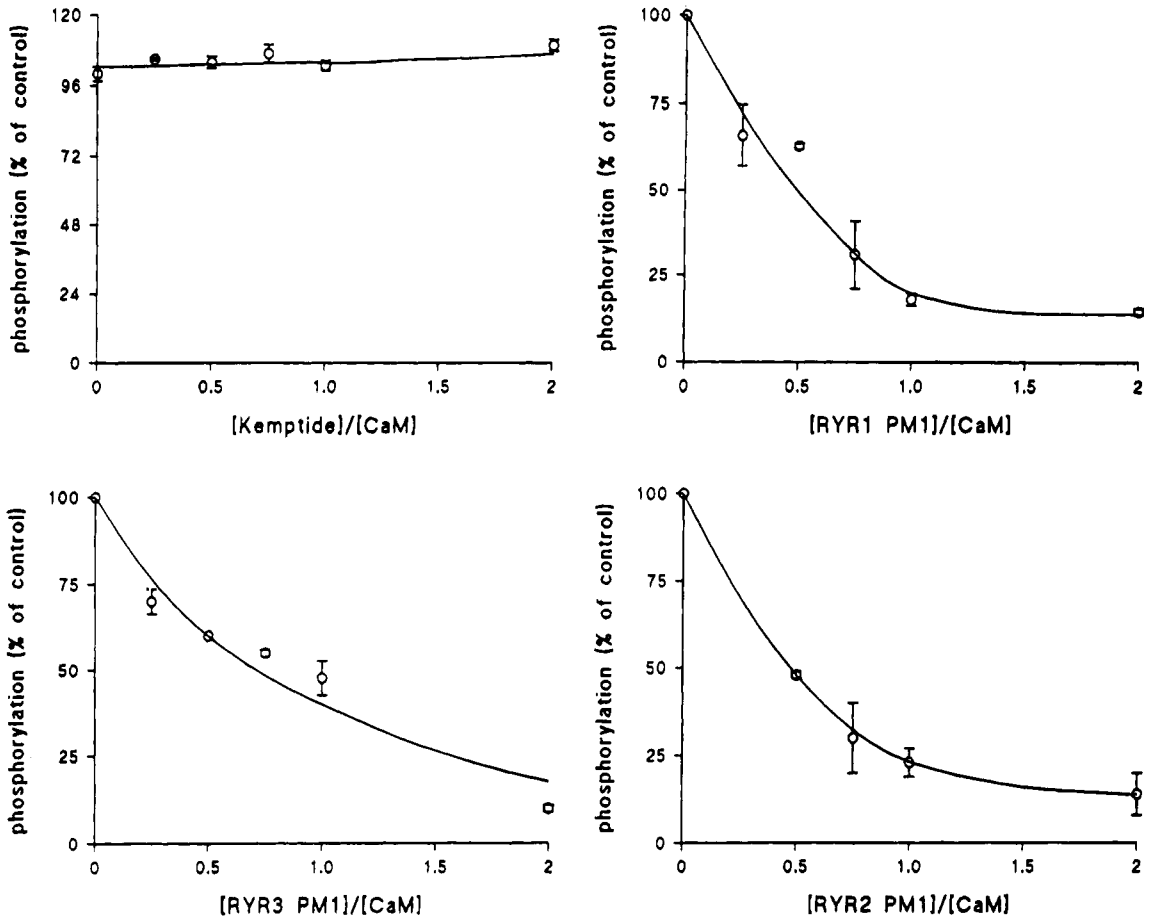

FIGURE 8: Effect of calmodulin on the phosphorylation of RYR1, RYR2, and RYR3 PM1 peptides by the catalytic subunit of cAMP-dependent protein kinase. 400 μM RYRs peptides were phosphorylated as described in Materials and Methods in the presence of increasing concentrations of calmodulin. The data are mean ± SD (n = 3). Control values are 174 (n = 2), 100 ± 2 (n = 3), and 200 ± 7 (n = 3) pmol of $P_i/\mu g$ of peptide for RYR1, RYR2, and RYR3 PM1 peptides, respectively.

classes of calmodulin binding sites with low and high affinities. As for the affinity constants of the two classes of calmodulin binding sites defined by the RYR1 peptides, they are higher than those reported by Yang et al. (1994) for the native RYR tetrameric complex under similar ionic condition (0.1 vs 10 and 17 vs 100−150 nM, respectively). This discrepancy may result from the different experimental systems exploited to determine the affinity constants. In our assay we used the minimal RYR1 sequence required to form the calmodulin binding site, while Yang et al. (1994) determined the affinity of the binding sites by using SR vesicles and found that the affinity constants varied as a function of the ionic composition of the medium assay. That is, in the presence of millimolar Mg^{2+} and 0.1 mM Ca^{2+}, the affinity for calmodulin is greater than in the presence of Ca^{2+} alone. This observation implies either that Mg^{2+} binds to RYR sequences distinct from those forming the calmodulin binding sites or an interaction of junctional Mg^{2+}-binding proteins with the RYR. In this latter case, the Mg^{2+}-binding proteins would modulate the affinity of the calmodulin binding sites.

The cardiac RYR calmodulin binding peptides that have been analyzed in this study encompass two high affinity and one low affinity binding site, while the skeletal and the brain RYR peptides form one high affinity and two low affinity calmodulin binding sites. The amino acid sequences of peptides PM1 and PM2 are highly homologous in the three isoforms, form high and low affinity binding sites, and display similar affinity constants. The major differences both at the amino acid level and concerning the affinity constants for calmodulin are attributable to the peptides PM3. In the case of the cardiac RYR, PM3 forms a high affinity site, whereas the lowest affinity was observed for the brain RYR peptide. Skeletal RYR PM3 peptide also binds calmodulin with low affinity, and the RYR1 PM3−calmodulin complex seems to involve electrostatic interactions since the substitution of Lys at position 11 abolishes the peptide's ability to bind to calmodulin. Because of its low affinity (250 nM), the functional relevance of the brain RYR calmodulin binding site defined by peptide PM3 remains to be established. However, a physiological role for this low affinity calmodulin binding site cannot be totally excluded since the ligand required to saturate it could be provided by proteins that sequester calmodulin in proximity to the membrane compartment containing the RYR. A possible candidate is neuromodulin (Slemmon & Martzen, 1994), a calmodulin-sequestering protein present in neuronal cells, whose affinity for calmodulin decreases upon phophorylation by protein kinase C or by exposure to micromolar free Ca^{2+} (Liu & Storm, 1990; Chapmann et al., 1991; Coggins & Zwiers, 1991). Neuromodulin is distributed in several regions of the brain including the hippocampus (Liu & Storm, 1990), an area particularly enriched in RYR (Lai et al., 1992; Sharp et al., 1993). Thus, if neuromodulin colocalizes with the RYR, it may be possible that the Ca^{2+} released from intracellular stores causes a negative feedback on the RYR by dissociating

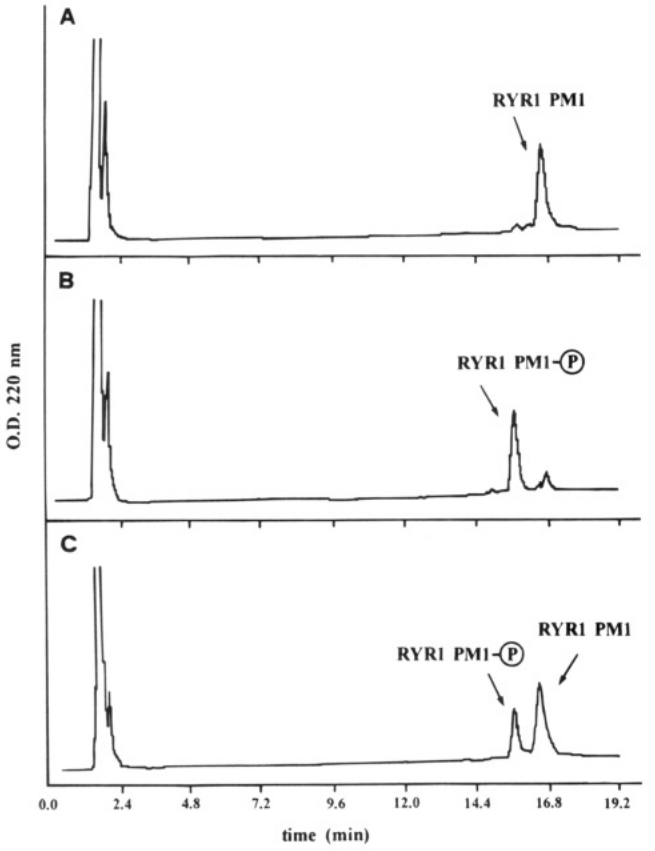

FIGURE 9: Separation of phosphorylated RYR PM1 from unphosphorylated RYR1 PM1 by reverse-phase liquid chromatography. Twenty micrograms of RYR1 PM1 peptides was phosphorylated by catalytic subunit of PKA for 30 min at 37 °C. The reaction was terminated by quick freezing in liquid nitrogen. The reaction mixture (20 μL) was injected in a reverse-phase liquid chromatography Vidac protein-peptide C18 HPLC column. Panel A and B show the chromatographic profile of reaction mixture at time zero and after 30 min, respectively. Panel C shows the chromatographic profile of the 10 μL of reaction mixture plus unphosphorylated RYR1PM1 (20 μg) as reference. The phosphorylated peptide eluted at 15.5 min, and the unphosphorylated eluted 1 min later.

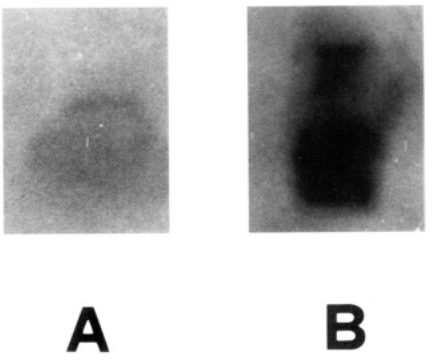

FIGURE 10: SDS−polyacrylamide gel electrophoresis and autoradiograph of the phosphorylated RYR1 PM1 peptide. (Lane A) Four aliquots of 20 μg of RYR1 PM1 were phosphorylated as described in the legend to Figure 9 and eluted separately from the reverse-phase chromatography column. The peaks eluting at 15.5 min were pooled, lyophilized, and resuspended in 200 μL of sterile water. Twenty microliters was loaded on SDS−polyacrylamide gel and stained with Coomassie BB. (Lane B) Twenty micrograms of RYR1 PM1 was phosphorylated in the presence of [^{32}P]ATP. The radioactive peak eluting at 15.5 min was collected, lyophilized, and resuspended in 100 μL of sterile water. Fifty microliters of the phosphorylated peptide was loaded in a 20% SDS−polyacrylamide gel. The autoradiogram of the polyacrylamide gel is shown.

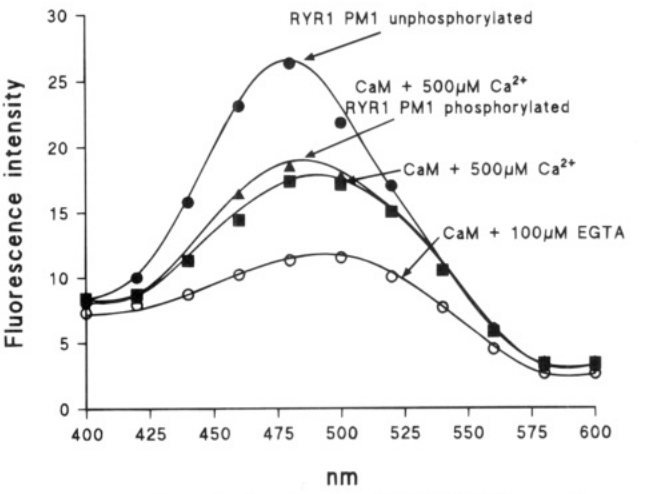

FIGURE 11: Effect of phosphorylated RYR1 PM1 peptide on fluorescence emission of dansyl-calmodulin. Fluorescent spectra of 200 nM dansyl-calmodulin were measured after sequential addition of 100 μM EGTA (open circles), 500 μM $CaCl_2$ (filled squares), 200 nM phosphorylated RYR1 PM1 peptide, (filled triangles), and 200 nM unphosphorylated RYR1 PM1 peptide (filled circles). Excitation and emission slits, 8.

calmodulin from neuromodulin; calmodulin in turn would bind Ca^{2+} to form the Ca^{2+}−calmodulin complex that decreases the Ca^{2+} release rate from the RYR.

Several reports show that the RYR isoforms are substrates for a variety of protein kinases, including the cAMP-dependent kinase (Seiler et al., 1984; Chu et al., 1990; Takasago et al., 1991; Witcher et al., 1991, 1992; Damiani & Margreth, 1991; Yoshida et al., 1992; Suko et al., 1993; Hohenegger & Suko, 1993; Herrmann-Frank & Varsàny, 1993). These results are in agreement with the identification of several putative consensus sequences for protein kinases in the primary structure of the RYRs. The consensus sequence for cAMP-dependent protein kinase is generally made up by Arg-Arg-X-Ser and Lys-Arg-X-X-Ser(Thr) motifs; however, in the case of β casein B, the cAMP-dependent kinase substrate is made up by an Arg-X-Ser motif (Kemp & Pearson, 1990). The presence of a hydrophobic residue immediately adjacent to the phosphate acceptor serine is often found in sequences surrounding cAMP-dependent kinase substrates. As demonstrated in Figure 8, the RYR peptides PM1 can be phosphorylated by the catalytic subunit of protein kinase A. In particular, the Arg-His-Arg-Ile(Val)-Ser-Leu motif of peptide PM1 is the sequence recognized by the catalytic subunit of protein kinase A since no phosphorylation of peptides containing inappropriately positioned hydroxylated amino acids was observed. The phosphate acceptor site of peptide PM1 is the Ser residue which corresponds to the Ser at position 2919, 3020, and 3055 of the brain, cardiac, and skeletal RYR, respectively. Analysis of phosphopeptides derived from phosphorylation of native RYRs revealed that the major substrate of cAMP-, cGMP-, and CaM-dependent protein kinases is the Ser residue at position 2809 of the cardiac RYR and at position 2843 of the skeletal RYR (Witcher et al., 1991; Suko et al., 1993; Hoenneger & Suko, 1993). Similar results have also been obtained with the brain RYR (Witcher et al., 1992). The lack of experimental evidence supporting the existence of the phosphorylated Ser within the RYR calmodulin binding site defined by peptides PM1 may be due to the presence of endogenous calmodulin that protects the acceptor site from phosphorylation by protein kinases, a conclusion

supported by the data showing that the binding of calmodulin to the PM1 peptides inhibits phosphorylation. On the other hand, the introduction of a negatively charged phosphate group into the RYR calmodulin binding site defined by peptide PM1 in turn compromise its ability to bind calmodulin. We do not know whether in the native RYRs the Ser residues corresponding to those localized in the PM1 peptide are phosphorylated. If this proves to be so, however, it may be the activity of protein phosphatases and kinases which ultimately modulate calmodulin binding to RYRs sites CaM1.

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