Identification and Characterization of Three Calmodulin Binding Sites of the Skeletal Muscle Ryanodine Receptor†

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ABSTRACT: In the present study, we have identified calmodulin binding sequences in the skeletal muscle ryanodine receptor Ca^{2+} release channel. Ligand overlays on RYR fusion proteins indicate that the skeletal muscle RYR contains three calmodulin binding regions defined by residues 2937–3225, 3546–3655, and 4425–4621. The RYR fusion protein PC28 (residues 2937–3225) bound calmodulin in the presence of EGTA and Ca^{2+}, while RYR fusion protein PC26 (residues 3546–3655) exhibited strong calmodulin binding at 10 μM Ca^{2+}. The RYR fusion protein PC15 (residues 4425–4621) did not bind calmodulin in the presence of either EGTA or 10–50 μM Ca^{2+}. In the presence of 100–500 μM Ca^{2+}, the RYR fusion protein PC15 exhibited an affinity for calmodulin of approximately 50 nM. Peptides RYR1 PM2 (residues 3610–3629) and RYR1 PM3 (4534–4552) encompassing putative RYR–calmodulin binding sites were synthesized. The synthetic peptides interacted directly with dansylcalmodulin as demonstrated by their capacity to affect the fluorescence emission of dansylcalmodulin. Missense mutation analysis indicates that the Lys and Arg residues are essential for calmodulin binding to the synthetic peptide RYR1 PM3. The RYR calmodulin binding site defined by peptide PM3 lies in the myoplasmic loop 2, a few residues upstream of the putative transmembrane segment M5; the other two calmodulin binding sites are next to the putative transmembrane segments M′ and M″. Thus, the effect of calmodulin on Ca^{2+} release might involve the regulation of the putative transmembrane segments M5, M′, and M″.

The skeletal muscle sarcoplasmic reticulum (SR) is an intracellular membrane compartment which controls the myoplasmic Ca^{2+} concentration, thereby playing an important role in the excitation–contraction coupling mechanism (Endo, 1985; Franzini-Armstrong, 1980). Skeletal muscle contraction is triggered by release of Ca^{2+} from sarcoplasmic reticulum terminal cisternae (Somlyo et al., 1985) via a Ca^{2+} release channel which is believed to be the ryanodine receptor (RYR)[1] (Rios & Pizzaro, 1991), a large homotetrameric oligomer made up of four 565-kDa subunits (Inui et al., 1987; Smith et al., 1988; Lai et al., 1988; Takeshima et al., 1989; Zorzato et al., 1990; Nakai et al., 1990). *In vitro*, the release of Ca^{2+} from isolated terminal cisternae vesicles via the RYR is modulated by a variety of agents including calmodulin, Ca^{2+}, ATP, and magnesium (Meissner, 1986). The exact molecular mechanism by which the above-mentioned putative physiological RYR modulators interact with the Ca^{2+} channel and affect its function remains elusive. A preliminary step toward the elucidation of this issue is the identification of the binding site of RYR modulators. In this study, we have mapped three calmodulin binding sequences to residues 2937–3225, 3610–3629, and 4534–4552 of the skeletal muscle RYR (Zorzato et al., 1990).

MATERIALS AND METHODS

Materials

Nitrocellulose was from Schleicher & Schuell; restriction enzymes, DNA-modifying enzymes, calmodulin, digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester, anti-digoxigenin peroxidase-conjugated Ab, and BM chemiluminescence were from Boehringer Mannheim; anti-chicken IgG, protein molecular weight markers, and dansylcalmodulin were from Sigma; the bluescript cloning vector was from Stratagene; amino acid derivatives, resin, and reagents were purchased from Novabiochem AG (Laufelfingen, Switzerland); 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).

DNA Manipulations and Production of the trpe Fusion Protein. DNA manipulations were carried out according to standard protocols as described in Maniatis et al. (1989). Fusion proteins covering the entire coding sequence of the rabbit skeletal muscle RYR cDNA were produced as previously detailed by Treves et al. (1993). Briefly, the rabbit skeletal muscle RYR cDNA fragments were fused in-frame with the DNA sequence encoding the *Escherichia coli* trpe protein of pATH plasmid (Koerner et al., 1991). The resulting

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[1] Abbreviations: Ab, antibodies; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; RYR, ryanodine receptor; SDS, sodium dodecyl sulfate; MOPS, 4-morpholinepropanesulfonic acid.

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fusion protein contains, at the NH_2-terminal, 323 amino acids of the trpe protein, while the polypeptide encoded by the RYR cDNA is located at the COOH-terminus. As previously described by Treves et al. (1993), the criteria adopted to identify the RYR fusion proteins were (i) molecular weight and (ii) abundance in the bacterial extracts. To cover the entire RYR coding sequence, we constructed 13 fusion proteins. The details of the construction of PCl, PC3, PC6, PC9, PC10, PC14, and PC15 have been previously described (Treves et al., 1993). The remaining fusion proteins contain RYR cDNA fragments defined by the following restriction sites: PC21 *Bam*HI (4894)–*Xho*I(6424)/*Sal*I vector; PC22 *Sal*I vector/*Xho*I(6424)–*Stu*I(8819)/*Hin*dIII vector; PC26 *Eco*RI vector/*Pst*I(10648)–*Bam*HI(10983); PC27 *Bam*HI-(10983)–*Eco*RI (11973)/*Hin*dIII vector; PC28 *Bam*HI vector/*Stu*I(8819)–*Sma*I(9673)/*Hin*dIII vector; PC31 *Pst*I-(9831)–*Pst*I(10648). The ExoIII/Mung nuclease deletion of the 3′ end of the cDNA encoding the RYR fusion protein PC15 was carried out as previously described (Zorzato et al., 1990).

SDS–Polyacrylamide Gel Electrophoresis and Immunological Techniques. Slab gel electrophoresis was carried out as described by Laemmli (1970). Western blots of bacterial extracts were carried out overnight as described by Gershoni et al. (1985). Indirect immunoenzymatic staining of Western blots was carried out as described by Young et al. (1985) and detailed by Treves et al. (1983) using a sequence-specific polyclonal anti-rabbit skeletal muscle RYR antibody. The sequence-specific anti-rabbit RYR antibodies were raised in chicken using the electrophoretically purified PC15 RYR fusion protein. Immunoglobulins were extracted from serum as described by Orlans et al. (1961) and further purified by using DEAE-cellulose anion-exchange column chromatography (Harlown & Lane, 1988). The immunoglobulin fraction was then batch-extracted for 3 h at room temperature with CNBr–Sepharose coupled to the bacterial protein trpe. The suspension was centrifuged for 10 min at $3000g_{max}$ and the resulting supernatant was incubated with an equal volume of 36% Na_2SO_4. The precipitate was resuspended in PBS and dialyzed overnight against PBS at 4 °C.

Calmodulin Overlay. Hog brain calmodulin (Boehringer Mannheim no. 651222) was labeled with activated digoxigenin according to the manufacturer's instructions (Boehringer Mannheim). Western blots of bacterial extracts were incubated for 60 min in a solution containing 10% low fat milk, 1% bovine serum albumin, 20 mM MOPS, pH 7.0, and 150 mM KCl. After three washes of 5 min each with a solution containing 20 mM MOPS, pH 7.0, 150 mM KCl, and 0.5 mM $CaCl_2$, the blots were incubated for 60 min at room temperature with the latter buffer containing digoxigenin-labeled calmodulin. The blots were then washed 3 times 5 min each with 10 mM Tris, pH 8.0, and 150 mM NaCl (TBS) and incubated with peroxidase-conjugated anti-digoxigenin Ab (1/2000 dilution in TBS). Binding of the anti-digoxigenin Ab to calmodulin was revealed by chemiluminescence.

Synthesis of the Calmodulin Binding Peptides. Peptides were synthesized by the solid phase method with a Milligen Biosearch 9050 peptide synthesizer as described by Temussi et al. (1994). Amino acid analysis was performed using PITC (phenyl isothiocyanate) methodology as the amino acid derivatization reagent. Lyophilized peptide (100 nmol) was placed in heat-treated borosilicate tubes and hydrolyzed in 200 μL containing 6 N HCl/1% phenol in the Pico-Tag work station (Waters-Millipore, Waltham, MA) for 60 min at 150 °C. PITC-amino acid derivatives were separated in a Hypersil ODS column (250 × 4.6 mm, 5-μm size particles). The calculated amino acid compositions of the PM2 and PM3 peptides closely match those determined experimentally (not shown).

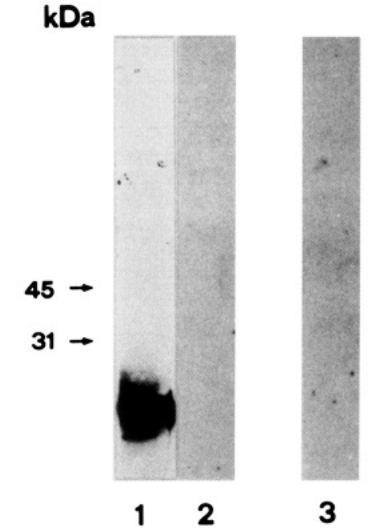

FIGURE 1: Digoxigenin–calmodulin binding to plasmalemma Ca^{2+} pump COOH-terminal peptide. Proteins were separated in a 7.5% SDS–polyacrylamide gel and transferred to nitrocellulose. Calmodulin overlay was carried out in the presence of 500 μM Ca^{2+}/100 nM calmodulin (lanes 1 and 3) and in the presence of 10 mM EGTA (lane 2). Lanes 1 and 2, 3 μg of plasmalemma Ca^{2+} pump COOH-terminal peptide A18–B28; lane 3, 10 μg of uninduced *E. coli* bacterial extracts.

Fluorometric Measurements. Bovine brain dansylcalmodulin (Sigma no. P6654) spectra were measured in a Perkin Elmer LS50 spectrofluorometer as described by Kincaid et al. (1082). Briefly, spectra were performed in 20 mM Tris, pH 8.0, 250 mM NaCl, and 5 mM $MgCl_2$. The excitation wavelength was 340 nm, and emission spectra were recorded between 400 and 600 nm. The dissociation constant of peptide PM3 was calculated according to Stinson and Hoolbrook (1973). Ca^{2+} titration experiments were carried out by measuring the changes in fluorescence emission at 490 nm, after sequential additions of known amounts of $CaCl_2$. The Ca^{2+} concentration of the buffer solutions used for the spectrofluorometric experiments was determined by atomic absorption using a Perkin Elmer 1100B atomic absorption spectrophotometer. Total Ca^{2+} contaminating the solution ranged between 0.4 and 0.6 μM.

RESULTS

Calmodulin Binding to RYR Fusion Proteins. To ascertain the specificity of digoxigenin–calmodulin overlays, bindings were carried out on (i) a plasmalemma Ca^{2+} pump COOH-terminal calmodulin binding peptide, A18–B28 (Hofmann et al., 1993) (positive control), and (ii) proteins contained in the total extracts of uninduced *E. coli* cultures (negative control). Figure 1 shows that digoxigenin–calmodulin binds in a Ca^{2+}-dependent manner to the COOH-terminal peptide A18–B28 of the plasmalemma Ca^{2+} pump (lane 1) while no binding is present in the uninduced bacterial extracts (lane 3). Several fusion proteins covering the entire coding sequence of the rabbit skeletal muscle RYR were produced (Treves et al., 1993) and assessed for their capacity to bind calmodulin (Figure 2A,B). As revealed by the calmodulin overlays, RYR fusion proteins PC28, PC26, and PC15 bound calmodulin. Therefore, the skeletal muscle RYR contains three calmodulin binding regions which are defined by residues 2937–3225

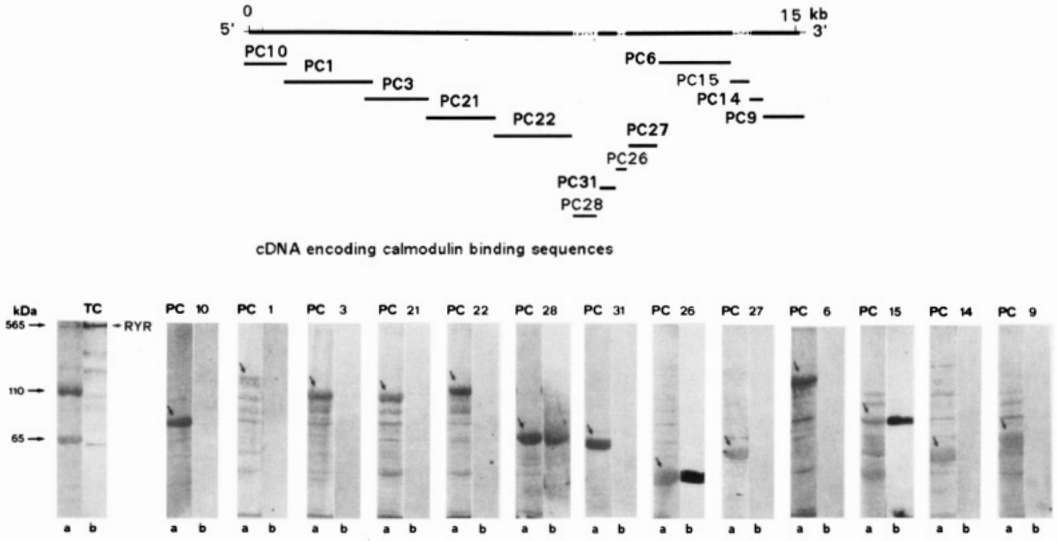

FIGURE 2: Identification of calmodulin binding sequences of the skeletal muscle RYR. (Panel A) Fusion protein production strategy: The top line indicates the size of the full-length rabbit RYR cDNA (Zorzato et al., 1990). The numbering is positive beginning at the first nucleotide of the initiator methionine. The underlying segments indicate the sizes of the cDNA fragments cloned into the pATH vectors. (Panel B) Calmodulin overlays: 10–50 μg of total *E. coli* extracts and 20 μg of terminal cisternae were electrophoretically separated in a 7.5%, 1.5 mm thick SDS–polyacrylamide gel and transferred to nitrocellulose. Lanes a show the Ponceau red staining of the Western blots: lanes b show the autoradiogram of the calmodulin overlay in the presence of 500 nM calmodulin/500 μM $CaCl_2$. Arrows indicate the RYR fusion proteins of different total *E. coli* extracts. TC, terminal cisternae fraction; RYR, ryanodine receptor.

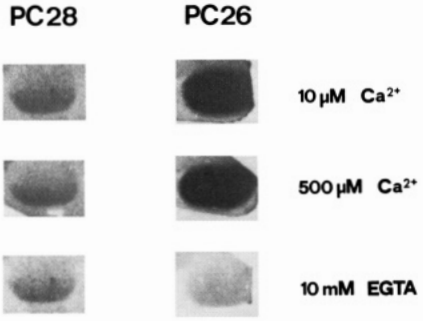

FIGURE 3: Ca^{2+} dependency of calmodulin binding to PC28 and PC26 RYR fusion proteins. Proteins of bacterial cell extracts were separated in a 7.5% SDS–polyacrylamide gel and transferred to nitrocellulose. The bands corresponding to the RYR fusion proteins were cut and ligand overlays were carried out at 50 nM calmodulin as described under Materials and Methods.

(RYR CaM 1, corresponding to fusion protein PC28), 3546–3655 (RYR CaM 2, corresponding to fusion protein PC26), and 4425–4621 (RYR CaM 3, corresponding to fusion protein PC15). In the presence of 10 μM Ca^{2+}, PC26 exhibited strong calmodulin binding which was almost completely abolished by EGTA (Figure 3). The binding of calmodulin to PC28 RYR fusion protein was weaker than to PC26, and it was unaffected by the presence of EGTA (Figure 3). Analysis of the amino acid sequence contained in the RYR fusion protein PC26 (RYR Cam 2) revealed the existence of a short sequence which displays an invariant structural feature shared by most calmodulin binding sites (Ikura et al., 1992), that is, the presence of 2 aromatic residues spaced by 12 amino acids. On the basis of these observations, we designed a 19 amino acid long peptide, RYR1 PM2 (encompassing residues 3610–3629; Table 1), and studied its interaction with dansylcalmodulin, spectrofluorometrically (Kincaid et al., 1982). In the presence of Ca^{2+}, the formation of the RYR1 PM2–calmodulin complex was demonstrated by the enhancement of fluorescence intensity and by the shift in the peak emission toward 490 nm. Addition of EGTA to the spectrofluorometric cuvette decreased the fluorescence intensity of the complex to a level slightly higher than that observed with calmodulin alone (Figure 4).

Table 1: Alignment of Calmodulin Binding Sequences

SK-MLCK M13[a]	K R R W K K N F I A V S A A N R F K K I S S S G A L M
Ca PUMP C28W[b]	L R R G Q I L W F R G L N R I Q T Q I R V V N A F R S S S
MASTOPARAN X[c]	I N W K G I A A M A K K L L
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 N Y L A R
RYR1 PM2*	K K A V W H K L L S K Q R R R A V V A

[a] Blumenthal et al. (1985). [b] Vorherr et al. (1990). [c] Malencik and Anderson (1983). *Synthesis was carried out as described under Materials and Methods.

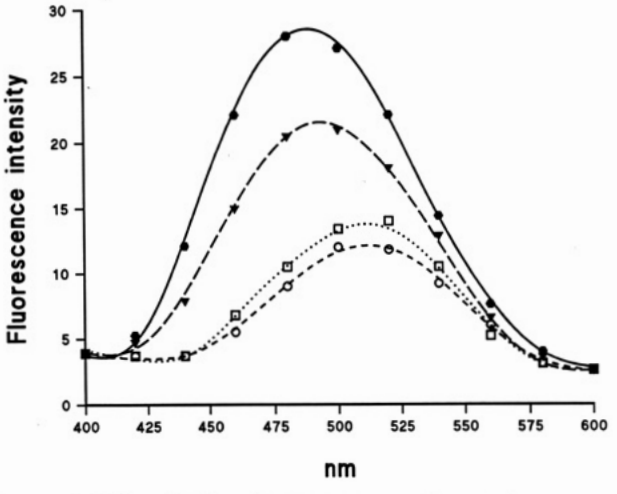

FIGURE 4: Effect of Ca^{2+} and RYR1 PM2 peptide on the fluorescence spectra of dansylcalmodulin. The experiments were carried out at equimolar concentrations of dansylcalmodulin and RYR1 PM2 peptide as described under Materials and Methods. Spectra were measured after sequential additions of 100 μM EGTA (open circles), 500 μM $CaCl_2$ (filled triangles), 0.4 μM RYR1 PM2 peptide (filled hexagons), and 500 μM EGTA (open squares).

Calmodulin Binding Properties of RYR1 CaM 3. We wished to study in more detail the characteristics of calmodulin binding to the RYR fusion protein PC15 (residues 4425–4621), because this region has been implicated in the Ca^{2+}-

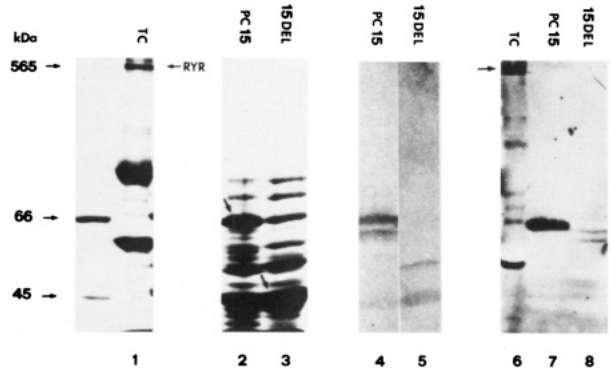

FIGURE 5: Calmodulin binding to the terminal cisternae fraction and the wild-type and COOH-terminal-deleted PC15 RYR fusion protein. Proteins in terminal cisternae vesicles and bacterial extracts were separated in a 7.5% SDS–polyacrylamide gel and stained with Coomassie BB: 15 μg of terminal cisternae (lane 1); 40 μg of bacterial extracts containing wild-type (lane 2) and COOH-terminal-deleted (lane 3) RYR fusion protein PC15. Lanes 4 and 5, indirect immunoenzymatic staining of the Western blot with sequence-specific anti-RYR Ab (final concentration 2 μg/mL) of wild-type (lane 4) and COOH-terminal-deleted (lane 5) RYR fusion protein. Lanes 6, 7, and 8, autoradiogram of a calmodulin overlay of terminal cisternae (lane 6) and wild-type (lane 7) and COOH-terminal-deleted (lane 8) RYR fusion protein. The overlay was carried out in the presence of 0.5 mM $CaCl_2$/0.75 μM calmodulin. Arrows indicate RYR fusion proteins. Abbrevations: (1) PC15, wild-type RYR fusion protein PC15; (2) 15Del, COOH-terminal-deleted RYR fusion protein PC15; (3) TC, terminal cisternae.

dependent regulation of the Ca^{2+} release channel (Zorzato et al., 1989; Fill et al., 1990; Treves et al., 1993). Lanes 1, 2, and 3 of Figure 5 show a Coomassie-stained 7.5% SDS–PAGE of rabbit skeletal muscle terminal cisternae (lane 1), and of bacterial cell extracts containing the intact RYR fusion protein PC15 and the COOH-terminal-deleted RYR fusion protein PC15 (arrows in lanes 2 and 3, respectively). Lanes 4 and 5 show that both the wild-type and COOH-terminal-deleted PC15 fusion proteins were recognized by a sequence-specific polyclonal anti-rabbit RYR Ab (Treves et al., 1993). The lower band in lane 4 probably represents a degradation product of PC15. The immunological reactivity in correpondence of a band with slower mobility than that of the COOH-terminal-deleted PC15 fusion protein could be due to different conformational states of the deleted fusion protein due to its high density of negatively charged amino acids. Lanes 6, 7, and 8 show autoradiograms of calmodulin overlays to the terminal cisternae fraction of rabbit skeletal muscle and to the RYR fusion proteins. Terminal cisternae contain few calmodulin binding proteins, including a component with an apparent molecular weight identical to that of the RYR (arrow, lane 6). We did not investigate in any further detail the identity of the calmodulin binding proteins present in the terminal cisternae fraction. The intact RYR fusion protein PC15 (lane 7) was positive on a calmodulin overlay carried out in the presence of 0.75 μM calmodulin and 500 μM Ca^{2+}. On the contrary, the RYR fusion protein PC15 carrying a deletion at its COOH terminus did not bind calmodulin (lane 8). This result demonstrates that calmodulin binding is specific, and that its binding to the RYR fusion protein requires the presence of the COOH-terminal portion.

Figure 6 shows the Ca^{2+} and dose dependency of calmodulin binding to the full-length PC15 RYR fusion protein. Binding of calmodulin occurred at a high [Ca^{2+}] (100–500 μM), and reached saturation at 100 nM calmodulin. At 500 μM Ca^{2+}, the half-maximal concentration for calmodulin binding was approximately 50 nM.

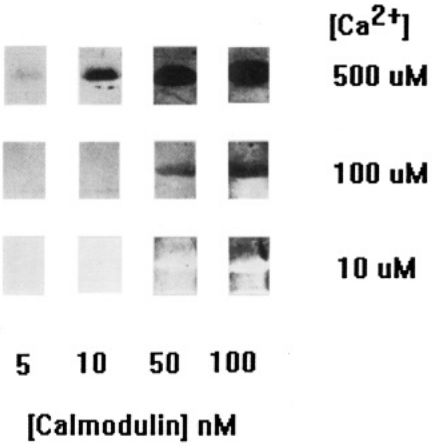

FIGURE 6: Dose and calcium dependency of calmodulin binding to PC15 RYR fusion protein. Proteins in the bacterial extracts were electrophoretically separated on a 7.5% SDS–PAGE and blotted onto nitrocellulose. The band corresponding to the RYR fusion protein was cut, and the calmodulin overlay was carried out as described under Materials and Methods. Autoradiograms after approximately 60 min exposure are shown.

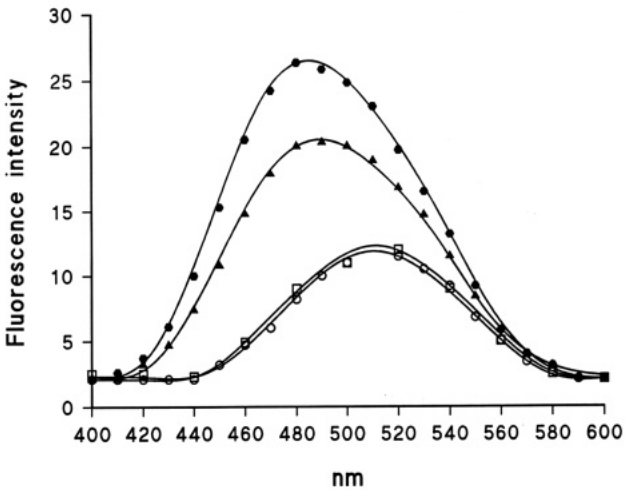

FIGURE 7: Effect of Ca^{2+} and RYR1 PM3 peptide on fluorescent spectra of dansylcalmodulin. Spectra were carried out at a peptide:dansylcalmodulin ratio of 1. Experimental conditions as in Figure 4.

On the basis of the extent of deletion at the 3′ end of the cDNA encoding the wild-type RYR fusion protein, we defined a 90-residue-long sequence containing the putative calmodulin binding site. Analysis of this sequence, using the method devised by Ikura et al. (1992), allowed us to design an 18 amino acid long peptide (Table 1; RYR1 PM3). The interaction between the RYR1 PM3 peptide and calmodulin was investigated by measuring changes in the fluorescence properties of dansylcalmodulin, according to the protocol of Kincaid et al. (1982). As expected, the addition of 500 μM Ca^{2+} to the spectrofluorometric cuvette elicited a blue shift in the emission maximum (from 520 to 495 nm), and an increase in the fluorescence emission of dansylcalmodulin (final concentration 400 nM). Addition of equimolar amounts of the RYR1 PM3 peptide (400 nM) caused a further 5–10 nm blue shift of the maximum, and an increase in fluorescence emission (Figure 7), which were reversed upon addition of EGTA. Qualitatively similar results were obtained by lowering the dansylcalmodulin concentration to 50 nM (not shown). To determine the K_d of the RYR1 PM3 peptide for dansylcalmodulin, we carried out fluorescence titration experiments. At a peptide:dansylcalmodulin ratio of 1, we observed 63 ±

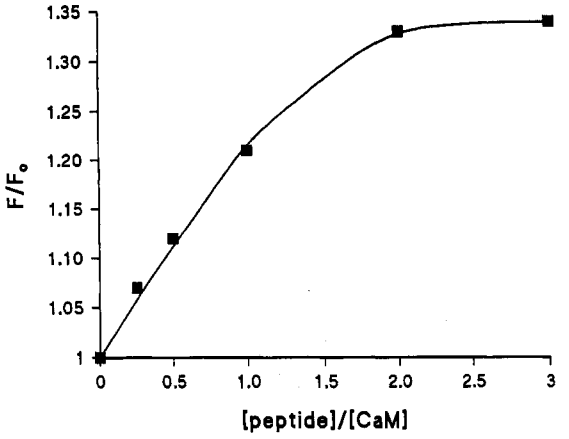

FIGURE 8: Titration of dansylcalmodulin with peptide RYR1 PM3. Dansylcalmodulin (400 nM) was titrated with peptide RYR1 PM3 in the presence of 500 μM $CaCl_2$. F indicates the fluorescence intensity of the peptide/dansylcalmodulin complex; F_0 indicates the fluorescence of dansylcalmodulin. F/F_0 was plotted against the ratio between the concentrations of peptide and the concentration of dansylcalmodulin. Data points of a representative experiment are plotted.

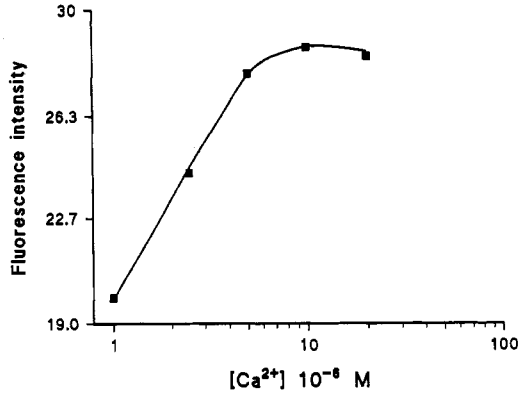

FIGURE 9: Fluorescence intensity emission of the RYR1 PM3 peptide/dansylcalmodulin complex as a function of the total Ca^{2+} concentration. The experiment was carried out in a solution containing 20 mM Tris, pH 8.0, 250 mM NaCl, and 5 mM $MgCl_2$. Known amounts of $CaCl_2$ were sequentially added to the spectrofluorometric cuvette containing 400 nM RYR1 PM3 and dansylcalmodulin. Points of a representative experiment are plotted. The concentration of Ca^{2+} contaminating the buffer solution was determined by atomic absorption spectroscopy.

3.4% saturation (mean ± SD, n = 5) of the maximal fluorescence emission. Figure 8 shows a representative titration experiment which was used to calculate the K_d according to Stinson and Holbrook (1973). The K_d of peptide RYR1 PM3 for dansylcalmodulin was calculated to be 88 nM.

We next examined the Ca^{2+} sensitivity of calmodulin binding to the site contained within the RYR1 PM3 peptide. As shown in Figure 9, the binding of the RYR1 PM3 peptide to dansylcalmodulin was strictly Ca^{2+} dependent. The fluorescence enhancement, which is indicative of the formation of the peptide–calmodulin complex, occurred within a Ca^{2+} concentration which ranged between 1 and 10 μM; maximal fluorescence enhancement was obtained at a Ca^{2+} concentration of 10 μM (n = 5).

The formation of the calmodulin–target peptide complex is mainly due to hydrophobic interactions of aromatic or long hydrophobic amino acids of the peptide with the methionine residues of calmodulin (Ikura et al., 1992). In addition, electrostatic interactions between positively charged amino acids of the target sequence and glutamate residues of

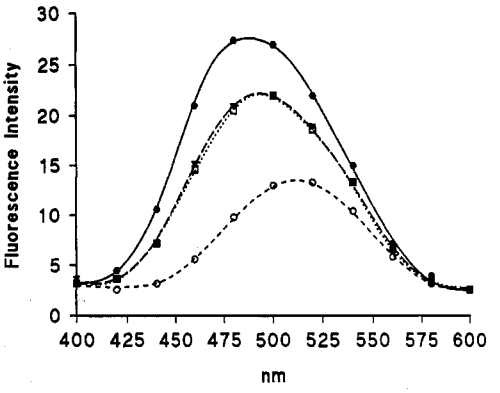

FIGURE 10: Effect of Ca^{2+} and mutated RYR1 PM3 peptide on fluorescent spectra of dansylcalmodulin. The experiment was carried out as described in Figure 4. Spectra were measured after sequential additions of 100 μM EGTA (open circles), 500 μM $CaCl_2$ (filled triangles), 400 nM mutated RYR1 PM3 peptide (open squares), and 400 nM wild-type RYR1 PM3 peptide (filled hexagons).

calmodulin may also be involved in the stabilization of the calmodulin–target peptide complex (Ikura et al., 1992). In the next set of experiments, we examined whether the Lys and Arg residues (positions 11 and 18, Table 1) are important to confer calmodulin binding activity to the RYR1 PM3 peptide. We changed these two residues since comparison of the corresponding sequence of both cardiac and brain RYRs indicates that the position of these two residues is conserved among the different isoforms (Table 1). The lysine and arginine at positions 11 and 18 of the RYR1 PM3 peptide were substituted by a helix-forming amino acid (Chou & Fassman, 1978) such as alanine. Addition of the mutated RYR1 PM3 to Ca^{2+}–dansylcalmodulin in the presence of Ca^{2+} did not cause any fluorescence enhancement (Figure 10). Identical results were obtained even when the mutated peptide:dansylcalmodulin ratio was increased up to 3 (not shown).

DISCUSSION

Calmodulin is an intracellular Ca^{2+} transducer which up-regulates the activity of a variety of structurally distinct proteins. The RYR Ca^{2+} channel has recently entered the growing list of calmodulin-modulated proteins (Seiler et al., 1984; Meissner, 1986; Meissner & Henderson, 1987; Plank et al., 1988; Yang et al., 1994). However, as opposed to these, the activity of the striated muscle Ca^{2+} channel appears to be down-regulated by a direct interaction of calmodulin with the channel protein itself (Meissner, 1986; Plank et al., 1988). In fact, calmodulin has been shown to reduce the Ca^{2+} release rate from skeletal and cardiac sarcoplasmic reticulum vesicles, without ever causing complete blockade of the Ca^{2+} channel. The exact mechanism by which calmodulin regulates the Ca^{2+} release mechanism remains elusive. As a preliminary step toward the understanding of this issue, we have defined the calmodulin binding sites on the Ca^{2+} release channel.

In this study, we have identified three calmodulin binding sequences in the skeletal muscle RYR by digoxigenin–calmodulin overlay. Although the exact physiological relevance of the calmodulin binding sites identified by such a technique is not known, they are deemed to play an important role in the regulation of the Ca^{2+} release channel since their interaction with calmodulin occurs at physiological concentrations of ligand (5–100 nM) (Grand et al., 1979). Two of these calmodulin binding sites are located in the central portion of the molecule (RYR CaM 1 and RYR CaM 2) near the putative transmembrane segments M′ and M″, while the third is located at the COOH-terminus of the molecule (RYR CaM

3). The total number of calmodulin binding sites which we have identified per protomer is lower than that reported by Yang et al. (1994). They suggested that the native RYR in SR vesicles contains four to five calmodulin binding sites per protomer. Such a discrepancy may result from the different experimental approaches exploited to characterize the calmodulin binding properties of the RYR. The number of calmodulin binding sites calculated by Yang et al. (1994) relies on affinity-labeling experiments, the underlying assumption being that the RYR is the predominant calmodulin receptor of the terminal cisternae fraction (Seiler et al., 1984). These studies cannot exclude that the total rhodamine–calmodulin binding activity displayed by the terminal cisternae fraction may also be due to the contribution of minor protein components capable of binding calmodulin. Thus, the differences in the number of binding sites may reflect binding to such minor protein components. Nevertheless, we believe that the RYR protomer contains at least three calmodulin binding sites, a rather unusual feature for calmodulin-modulated proteins. Sequence analysis of a variety of calmodulin binding proteins has indicated that a high-affinity binding site is made up of repeated basic–hydrophobic residues which are arranged in a positively charged amphiphilic helix (O'Neil & DeGrado, 1991). The use of such a pattern has been inadequate to predict not only the RYR calmodulin binding site (Takeshima et al., 1989; Zorzato et al., 1990) but also one of the two calmodulin binding sites located at the COOH-terminus of the γ subunit of phosphorylase kinase (Dasgupta et al., 1989). On the other hand, in the case of adenylyl cyclase, a predicted calmodulin binding site was not demonstrated to be the calmodulin binding domain of the adenylyl cyclase (Vorrher et al., 1993). The above-mentioned method has now been superseded by a more reliable one, based on sophisticated structural analysis of the atomic interaction within the calmodulin–target sequence complex (Ikura et al., 1992). In this study, we have adopted this new model. On the basis of the results obtained using calmodulin overlays on RYR fusion proteins, we designed peptides encompassing putative calmodulin binding sites. The demonstration that the synthetic peptides RYR1 PM2 (residues 3610–3629) and RYR1 PM3 (residues 4534–4552) were indeed able to complex with dansylcalmodulin indicates that ligand overlays are useful as an initial approach to screen for calmodulin binding sequences in large proteins such as the RYR protomer. Various pieces of evidence are consistent with the specificity of the PM3 peptide–calmodulin interaction. Substitution of the Lys and Arg residues at positions 11 and 18 of the RYR1 PM3 peptide abolished its capacity to affect the fluorescence emission of dansylcalmodulin. In addition, peptides corresponding to the cardiac and brain isoforms gave similar Ca^{2+}-dependent fluorescence enhancements when incubated with dansylcalmodulin (unpublished observations), suggesting that such a calmodulin binding site is conserved among the known RYR isoforms.

The interaction of calmodulin with target proteins occurs in the presence of 3–5 μM Ca^{2+}, i.e., at a concentration of Ca^{2+} in which the high-affinity Ca^{2+} binding sites are saturated (Keller et al., 1982; Kincaid et al., 1982). However, while the calmodulin binding site defined by the RYR1 PM3 peptide complexed with its ligand at the expected [Ca^{2+}], binding of calmodulin to the blotted PC15 RYR fusion protein occurred at a 5–10-fold higher [Ca^{2+}]. A number of possibilities may account for this discrepancy. (i) The overlay technique relies on the fact that functional features of short amino acid sequences can be preserved or regained during electrophoresis and blotting procedures. Thus, the altered Ca^{2+} sensitivity of calmodulin binding to the blotted PC15 RYR fusion protein might reflect partial renaturation after gel electrophoresis. (ii) The Ca^{2+} sensitivity of calmodulin binding to the PC15 fusion protein and/or to the RYR might be influenced by sequences surrounding the calmodulin binding site(s). RYR CaM 3 is located 40 residues downstream of a sequence characterized by a high density of negative charges (D-G-E-E-E-E-L-V-P-E-P-E-P-E-P-E-P-E-P-E) which may perturb the interaction of calmodulin with its site. One can hypothesize that after blotting, the above-mentioned glutamate-proline domain forms a negatively charged screen that needs to be saturated with Ca^{2+} before calmodulin can gain access to its binding site. In this context, it should be mentioned that in the presence of 1 mM Mg^{2+} the optimal [Ca^{2+}] for calmodulin binding to native RYR is 50 μM (Yang et al., 1994), a value close to that observed with the blotted PC15 RYR fusion protein. These results imply that at least one of the calmodulin binding sites present in the RYR is occupied by the ligand when the surrounding [Ca^{2+}] reaches values of at least 50 μM. It has been estimated that the maximal Ca^{2+} released during a depolarization of sufficient magnitude is 36 mmol (L of muscle)$^{-1}$ s^{-1} (Baylor et al., 1983). Assuming that all Ca^{2+} releasing sites are localized at the junctional SR and that the space corresponding to the transverse tubules–terminal cisternae gap is approximately equal to 1/200 myoplasmic volume, very high [Ca^{2+}] would be obtained in the junctional gap after a few milliseconds of Ca^{2+} release. Thus, it is likely that during excitation–contraction coupling the [Ca^{2+}] surrounding the RYR CaM 3 reaches a value which would allow the interaction between the Ca^{2+} release channel and calmodulin. The conformational modification generated by the binding of calmodulin to RYR CaM 3, the peptide which lies a few residues upstream from the putative transmembrane segment M5, might be directly transmitted to the gate within the pore-forming unit of the channel. Such an event would in turn decrease the mean open probability of the channel, thereby decreasing the Ca^{2+} release rate.

The definition of a functional role for calmodulin binding to the RYR fusion proteins PC28 and PC26 awaits more data. The ability of RYR fusion protein PC28 to bind calmodulin in the presence of EGTA confirms data by Yang et al. (1994), who demonstrated that calmodulin is able to bind to the native RYR at very low Ca^{2+} concentrations. These results are certainly provocative because they indicate that not only the Ca^{2+}–calmodulin complex but also calmodulin alone can bind to the RYR channel. A small number of other proteins have been shown to bind calmodulin in the presence of very low [Ca^{2+}] (Cohen et al., 1978; Ladant, 1989; Geiser et al., 1991; Espreafico et al., 1992). As to the functional relevance of the interaction between the RYR channel and calmodulin in the presence of Ca^{2+} concentrations similar to those of resting muscle, we can advance a hypothesis. Analysis of the primary structure of the RYR failed to identify a clear consensus sequence for high-affinity Ca^{2+} binding site(s). On the other hand, there is a large body of evidence which shows that the activity of the RYR Ca^{2+} channel is stimulated by micromolar concentrations of Ca^{2+}. We propose that the calmodulin molecules which interact with the RYR at [Ca^{2+}] similar to those found in resting muscle operate as Ca^{2+} sensors of the Ca^{2+}-induced Ca^{2+} release mechanism. Thus, the model predicts that conformational changes caused by the binding of Ca^{2+} to the high-affinity binding site of calmodulin are mechanically transmitted to the channel complex to elicit its opening. Further characterization of this calmodulin binding

site will help to clarify its exact role in the structure to function relationship of the RYR.

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