Unique Phosphorylation Site on the Cardiac Ryanodine Receptor Regulates Calcium Channel Activity*

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Ryanodine receptors have recently been shown to be the Ca" release channels of sarcoplasmic reticulum in both cardiac muscle and skeletal muscle. Several regulatory sites are postulated to exist on these receptors, but to date, none have been definitively identified. In the work described here, we localize one of these sites by showing that the cardiac isoform of the ryanodine receptor is a preferred substrate for multifunctional Ca"/calmodulin-dependent protein kinase (CaM kinase). Phosphorylation by CaM kinase occurs at a single site encompassing serine 2809. Antibodies generated to this site react only with the cardiac isoform of the ryanodine receptor, and immunoprecipitate only cardiac [18]ryanodine-binding sites. When cardiac functional sarcoplasmic reticulum vesicles or partially purified ryanodine receptors are fused with planar bilayers, phosphorylation at this site activates the Ca" channel. In tissues expressing the cardiac isoform of the ryanodine receptor, such as heart and brain, phosphorylation of the Ca" release channel by CaM kinase may provide a unique mechanism for regulating intracellular Ca" release.

Ca" release from the SR causes an increase in myoplasmic Ca" concentration which leads to muscle contraction (1). Recently, the sites of Ca" release have been identified and purified from both cardiac (2-4) and skeletal muscle SR (5-7) and shown to be the same as the ryanodine receptors or high molecular weight proteins, the structures which attach the transverse tubules to the junctional SR both in intact tissues and isolated membrane fractions (1, 8-10). Although the Ca" release channels from cardiac and skeletal muscle show many similarities such as nearly identical Ca" conductances (2-7), protease sensitivities (11, 12), calmodulin-binding capabilities (11), and modulation by allosteric regulators such as Ca"+, Mg"+, ATP, and calmodulin (13-15), they also exhibit several differences in protein structure and function. Quantitative differences have been noted on the effects of modulators on ryanodine binding to the two proteins (16-18), as well as on Ca"+ channel kinetics (13, 14). In addition, the cardiac ryanodine receptor exhibits a slightly smaller apparent molecular weight than the skeletal muscle receptor on SDS-PAGE (11), and monoclonal antibodies can be made which react with the cardiac receptor but not the skeletal receptor (16).

Recent work on characterization of the two ryanodine receptors has culminated in elucidation of the primary structures of the proteins by sequencing of their cDNAs (19-21). Consistent with the differences between the two protein isoforms noted above, the cardiac and skeletal muscle receptors have been found to be the products of different genes, with overall amino acid identities of 66% (21). Both protein isoforms are very large, containing approximately 5,000 amino acids and exhibiting predicted molecular weights of 564,711 for the cardiac protein (21) and 565,223 (19) or 563,584 (20) for the skeletal muscle protein. In the native state, ryanodine receptors are arranged as tetramers (1-7).

In an earlier study (22), we demonstrated that the cardiac ryanodine high molecular weight protein (or ryanodine receptor; Ref 3) was an excellent substrate for the multifunctional CaM kinase (23, 24) endogenous to junctional SR membranes. In the work described here, we show that phosphorylation of the cardiac receptor by CaM kinase occurs at a single site, which is not substantially phosphorylated in the skeletal muscle receptor, and that phosphorylation of the cardiac ryanodine receptor at this site activates the Ca" channel. Our data are the first to support the theoretical model of Otsu et al. (21), that the modulator-binding sites of the cardiac ryanodine receptor are contained within residues 2619-3016.

EXPERIMENTAL PROCEDURES

Materials—CaM kinase was isolated from rat brain as described previously (25). cAMP kinase (the catalytic subunit from bovine heart) was purchased from Sigma or prepared according to Beavo et al. (26). Identical results were obtained with either cAMP kinase preparation. [γ-32P]ATP and [35S]protein A were obtained from Du Pont-New England Nuclear. Bovine brain calmodulin, protein A-agarose, inomodiacetic acid-agarose, and Sephacyr S-500 were purchased from Sigma.

Membrane Preparation—Canine cardiac junctional SR vesicles (vesicles sedimenting at the 1.0/1.5 M sucrose interface) were isolated as described previously (3) and extracted with 50 mM Tris, 300 mM KCl, and 25 mM sodium pyrophosphate (pH 7.0). Salt-extracted skeletal muscle SR membranes were isolated (27) from predominately fast (tensor fasciae latae) and slow (vastus intermedius) muscles.
Fig. 1. Phosphorylation of canine cardiac and canine fast and slow skeletal muscle ryanodine receptors in SR membranes by CaM kinase. Phosphorylation reactions were conducted for 5 min and stopped with SDS. 46 µg of membrane protein were electrophoresed per gel lane. The left panel shows the Coomassie Blue-stained gel, and the right panel is the corresponding autoradiogram. The arrowheads and small arrows indicate the cardiac and skeletal muscle ryanodine receptors, respectively. The asterisk designates a phosphorylated protein residue in the slow skeletal fractions not related to the ryanodine receptor. Purified CaM kinase (PK) and calmodulin (CaM) were added as indicated. Mobility standards (M, x 10^-3) are displayed between the two panels.

Fig. 2. CaM kinase and cAMP kinase phosphorylation of the cardiac ryanodine receptor. 0.06 and 1.4 µg of added CaM kinase (Δ) and CaM kinase (O), respectively, were used to phosphorylate 40 µg of cardiac junctional SR vesicles for the times indicated. Filled circles (●) show phosphorylation by the endogenous CaM kinase. 32P incorporation into ryanodine receptors was quantitated by scintillation counting after excision of radioactive bands from SDS gels (22). Higher concentrations of added kinases had no further effect on ryanodine receptor phosphorylation.

Vesicles were stored frozen at -40°C in 0.25 M sucrose, 30 mM histidine (pH 7.4).

Membrane Protein Phosphorylation—Phosphorylation reactions were conducted at 30°C in 50 µl of buffer containing 40-50 µg of membrane protein and 25 mM MOPS (pH 7.4), 7.5 mM MgCl₂, 0.5 mM EGTA, 0.75 mM CaCl₂, 0.1 mM NaF, and 50 µM [γ-32P]ATP with or without 0.06 µg of brain CaM kinase and 2 µg of calmodulin. Phosphorylation by cAMP kinase (1.4 µg of the catalytic subunit) was conducted under identical conditions with the omission of Ca²⁺ and calmodulin from the incubation buffer. Reactions were terminated by adding 20 µl of SDS dissociation medium, and samples were subjected to SDS-PAGE in 6% 1.5-mm thickness gels (28), followed by autoradiography (11). To achieve the greatest resolution of ryanodine receptors, gels were electrophoresed at 35 mA, and electrophoresis was continued for 20-30 min after the dye front exited the bottom of the gels.

Purification of Phosphorylated Cardiac Ryanodine Receptor—50 µg of cardiac junctional SR vesicles were phosphorylated for 5 min at 30°C in 50 ml of buffer containing 50 mM MOPS (pH 7.4), 10 mM MgCl₂, 4.8 mM EGTA, 5 mM CaCl₂, 0.5 mM NaF, 0.4 mg of calmodulin, 25 µg of CaM kinase, and 40 µM [γ-32P]ATP. The membranes were then rapidly pelleted at 4°C and resuspended to 4 ml in 0.25 M sucrose, 30 mM histidine (pH 7.4). SDS and NaF were added to final concentrations of 4% and 0.5 M, respectively, and the sample was heated to 50°C and sedimented. The supernatant, containing solubilized ryanodine receptors, was loaded onto a 90 × 2.9-cm Sephacryl S-500 column equilibrated with 20 mM Tris, 0.5 mM NaCl, 0.2% SDS, and 1 mM dithiothreitol (pH 8). The column was run at room temperature at a flow rate of 12 ml/h. Three fractions were collected while monitoring absorbance at 280 nm. 300-400 µg of ryanodine receptor protein were routinely recovered from 50 µg of membrane protein. Protein concentrations were determined by the method of Schaffner and Weissman (29).

Tryptic Proteolysis and Isolation of Phosphopeptide—600-800 µg of purified phosphorylated ryanodine receptors from two column runs were dialyzed against 4 liters of 50 mM NH₄HCO₃, 1 mM dithiothreitol for 16 h at room temperature. Tryptsin was added at a 1:50 weight ratio, and proteolysis conducted for 24 h at 37°C. The digest was then lyophilized, solubilized in 0.1 M acetic acid, and applied to a 0.4-mL column of iminodiacetic acid–agarose saturated with Fe²⁺ as described (30). The column was washed with two column volumes of 0.1 M acetic acid (load), then three column volumes of the following buffers: 0.1 M sodium acetate (pH 5.2); 0.1 M ammonium acetate (pH 5.9); 0.1 M ammonium acetate (pH 8.6); 0.1 M ammonium acetate (pH 10); and 0.2 M EDTA (pH 8.0). Essentially all of the radioactivity applied to the column was recovered. The pH 8.6 and 10 column eluates were pooled, lyophilized, and solubilized in 10% trifluoroacetic acid in H₂O. Peptides were injected into a Beckman reverse-phase C18 column equilibrated with 0.1% trifluoroacetic acid in H₂O and the column developed with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Absorbance was measured at 214 nm and radioactivity of recovered fractions was monitored by Cerenkov counting. Only a single major peak of radioactivity was detected, which eluted at 24% acetonitrile and accounted for 77% of the total radioactivity applied to the column. The sequence of the recovered radioactive peptide was determined by automated Edman degradation using an Applied Biosystems Model 470A gas-phase sequenator (31). Phosphoamino acid analysis was performed as described (31).

Antiserum Production—Peptide 2805-2819 of the cardiac ryanodine receptor (Fig. 5) was synthesized with an added cysteine at the carboxyl terminus and coupled to thyroglobulin (32). A rabbit was immunized subcutaneously with 300 µg of peptide conjugate in complete Freund's adjuvant, and booster injections of 200 µg of peptide conjugate in incomplete adjuvant were given at monthly intervals. Antiserum was obtained on day 7 after booster injections.

Immunoprecipitation of [3H]Ryanodine-binding sites—3 µg of canine cardiac junctional SR, fast skeletal SR, and slow skeletal SR vesicles were solubilized in 300 µl of buffer containing 2% Triton X-100 and 10-µl aliquots from supernatant fractions added to 190 µl of binding buffer (20 mM MOPS, pH 7.2, 0.25 M sucrose, 0.5 M KCl, 0.06 mM CaCl₂, 1 mg/ml bovine serum albumin, and 20 mM [3H]ryanodine or with or without 10 M cold ryanodine). After a 1-h incubation at 37°C, [3H]ryanodine binding was solubilized using polyethylene glycol precipitation followed by filtration over glass fiber filters (3). For immunoprecipitation, replicate 200-µl aliquots of receptors plus binding buffer incubated as above were added to Eppendorf tubes containing 20 µl of cardiac ryanodine receptor antiserum. Samples were incubated at 4°C for 2 h, then 100 µl of protein A–agarose beads equilibrated with binding buffer were added, and the incubation continued for 2 h at 4°C. Disintegrations/min of [3H]ryanodine binding recovered in the sedimented washed beads and in supernatant fractions was then measured. Values are the averages of triplicate determinations.
FIG. 3. **Purification of the phosphorylated cardiac ryanodine receptor by SDS gel filtration chromatography.** The purified phosphorylated ryanodine receptor (RR) eluted early from the column, as shown by SDS-PAGE and Coomassie Blue staining of 120-μl aliquots of column fractions (lower panel). UV absorbances of column fractions are shown in the upper panel. Fraction numbers are listed between the two panels.

FIG. 4. **Purification of cardiac ryanodine receptor phosphopeptide.** The main graph shows elution of "P-labeled tryptic peptides from the Fe" column, and the inset shows reverse-phase chromatography of the combined pH 8.6 and 10 column eluates. Numbers in parentheses above the bars in the main graph indicate the percent radioactivities recovered in the different fractions. In the inset only, the portion of the reverse-phase profile containing the radioactive peak is shown. The continuous tracing denotes absorbance and the filled bars indicate counts/min.
that employed for the tryptic peptide. Except that CNBr followed by abbreviations are used.

The canine cardiac phosphorylation site. The latter peptide was purified with a strategy similar to that employed for the tryptic peptide, except that CNBr followed by Lys-C (Boehringer Mannheim) cleavages were used.2 Corresponding sequences reported for rabbit cardiac (21), rabbit skeletal (19), and human skeletal (20) ryanodine receptors are listed. Vertical lines and asterisks designate identical residues and mismatches, respectively. X indicates indeterminate residues sequenced. One-letter amino acid abbreviations are used.

The upper row gives the sequence of the canine cardiac phosphorylation site. The horizontal lines demarcate the tryptic and CNBr/endoproteinase Lys-C peptides sequenced. The latter peptide was purified with a strategy similar to that employed for the tryptic peptide, except that CNBr followed by Lys-C (Boehringer Mannheim) cleavages were used.2 Corresponding sequences reported for rabbit cardiac (21), rabbit skeletal (19), and human skeletal (20) ryanodine receptors are listed. Vertical lines and asterisks designate identical residues and mismatches, respectively. X indicates indeterminate residues sequenced. One-letter amino acid abbreviations are used.

RESULTS AND DISCUSSION

Preferential Phosphorylation of the Cardiac Ryanodine Receptor—Fig. 1 shows that the canine cardiac ryanodine receptor (arrowheads) is phosphorylated in junctional SR vesicles by an endogenous calmodulin-requiring protein kinase and that this phosphorylation is stimulated severalfold when exogenous CaM kinase is added. In contrast, the ryanodine receptor in canine fast and slow skeletal muscle SR vesicles, which migrates with a slightly higher molecular weight on SDS-PAGE (2, 11, 16), is not significantly phosphorylated by either endogenous or exogenous protein kinase (Fig. 1, small arrows). Similar results were obtained with rabbit skeletal muscle SR vesicles. The identity of the skeletal muscle ryanodine receptor in these studies (Fig. 1, small arrow) was confirmed by immunoblotting with a skeletal muscle isoform-specific antibody (supplied by K. Campbell, University of Iowa). We did detect a low level of phosphorylation of a protein in slow skeletal muscle samples migrating slightly faster than the cardiac receptor (Fig. 1, asterisk), but this protein did not cross-react with skeletal muscle (or cardiac, see below) antibodies, suggesting that it is unrelated to the ryanodine receptor. Although we reported earlier that a rabbit skeletal muscle high molecular weight protein could be phosphorylated by an endogenous CaM kinase (11), it is probable that much of the 32P incorporation detected in this earlier study was due to phosphorylation of this unrelated protein. Pretreatment of SR membranes with acid phosphatase to remove endogenous phosphate did not affect the results described presently; CaM kinase-catalyzed phosphorylation of the cardiac ryanodine receptor was always at least 10-fold greater than skeletal receptor phosphorylation. Chu et al. (35) recently reported that the skeletal muscle ryanodine receptor is phosphorylated by CaM kinase, but the present results demonstrate that this phosphorylation is insignificant compared to cardiac protein phosphorylation. Consistent with our results, Otsu et al. (21) have recently shown that the cardiac isoform of the ryanodine receptor is absent from fast and slow skeletal muscle.

As reported earlier (11), and later confirmed by Takasago et al. (36), CAMP kinase also phosphorylates the cardiac ryanodine receptor. However, maximal phosphorylation by added CAMP kinase is no greater than that achieved with endogenous CaM kinase (Fig. 2). In contrast, 1/20th the amount of exogenous CaM kinase increases receptor phosphorylation 4-fold, to a maximal level of 26 pmol of P/mg of SR protein (Fig. 2). Thus, efficient phosphorylation of the cardiac ryanodine receptor occurs only with CaM kinase. In agreement with recent results (35, 36), we observed no significant phosphorylation of canine fast and slow or rabbit skeletal muscle ryanodine receptors by CAMP kinase, although the adventitious protein mentioned above was phosphorylated.

Maximal [3H]ryanodine binding (3) in these preparations ranged between 5 and 6 pmol/mg of protein, a value nearly identical to the level of receptor phosphorylation achieved with exogenous CAMP kinase (see also, Ref. 36) or endogenous CaM kinase, but one-fourth the value achieved with added CaM kinase. Since the functional unit of the cardiac Ca2⁺ release channel contains only one high affinity ryanodine-binding site/tetramer (4), our results suggest that the endogenous CaM kinase is capable of phosphorylating only one-fourth of the available sites, whereas the exogenous kinase can fully phosphorylate the receptor, i.e. all four subunits (see below).
Cardiac Ryanodine Receptor Phosphorylation Site

**Table 1**

*Immunoprecipitation of [H]ryanodine receptors from CHAPS-solubilized canine SR membranes*

Values are expressed for aliquots of the following fractions: S, solubilized receptors after treatment of membranes with 2% CHAPS; B, bound fraction, containing ryanodine receptors immunoprecipitated from CHAPS supernatant; F, free fraction, containing ryanodine receptors not immunoprecipitated. Total binding was measured using 20 nM [H]ryanodine. For nonspecific binding, 10 μM cold ryanodine was added.

| Binding condition | [H]Ryanodine bound |
|-------------------|--------------------|
|                   | Cardiac SR         | Slow skeletal SR | Fast skeletal SR |
|                   | dpm × 10^4/10-μl fraction | dpm × 10^4/10-μl fraction | dpm × 10^4/10-μl fraction |
| Total             |                     |                 |                 |
| Nonspecific       | 887.0               | 1154.8          | 1718.6          |
| Difference        | 39.8                | 65.0            | 50.0            |

**Fig. 7.** Effect of ATP and calmodulin on the cardiac Ca^2+ release channel. The cis chamber contained 1 mM Ca(OH)_2, 1 mM EGTA, 5 mM MgCl_2, 250 mM HEPES, and 125 mM Tris (pH 7.2). The trans chamber contained 50 mM Ba(OH)_2, 250 mM HEPES, and 125 mM Tris (pH 7.2). Holding potential was 0 mV, with upward current deflections representing movement of Ba^2+ from the trans to the cis chamber. Arrows to the left of each trace denote the fully closed state of the channel. Representative continuous sweeps for each condition are shown at left, and amplitude histograms of 2 min of continuous activity are displayed to the right. Gaussian distributions were fit to the peaks of activity in the histograms. Signals were filtered at 300 Hz (low pass Bessel) and digitized at 1 KHz (Axotape, Axon Instruments) for off-line analysis. In the control (A), p(open) was 0.26. Addition of 1 mM ATP (B) produced prolonged openings of the channel, increasing p(open) to 0.81. Subsequent addition of 3 μM calmodulin (C) decreased p(open) to 0.12, producing long closures and brief aborted openings.

Sequencing of the Cardiac Phosphorylation Site—In order to sequence the phosphorylation site of the cardiac ryanodine receptor, we phosphorylated junctional SR membranes on large scale with added CaM kinase and purified the phosphorylated denatured ryanodine receptor to homogeneity in one step using SDS-gel filtration chromatography (Fig. 3). The purified cardiac ryanodine receptor was digested with trypsin, and the radioactive peptides recovered using Fe^3+ affinity chromatography (30,37). 90% of the loaded radioactivity was recovered in the pH 8.6 and 250 mM Ca(OH)_2, 1 mM EGTA, and 125 mM Tris (pH 7.2). Holding potential was 0 mV, with upward current deflections representing movement of Ba^2+ from the trans to the cis chamber. Arrows to the left of each trace denote the fully closed state of the channel. Representative continuous sweeps for each condition are shown at left, and amplitude histograms of 2 min of continuous activity are displayed to the right. Gaussian distributions were fit to the peaks of activity in the histograms. Signals were filtered at 300 Hz (low pass Bessel) and digitized at 1 KHz (Axotape, Axon Instruments) for off-line analysis. In the control (A), p(open) was 0.26. Addition of 1 mM ATP (B) produced prolonged openings of the channel, increasing p(open) to 0.81. Subsequent addition of 3 μM calmodulin (C) decreased p(open) to 0.12, producing long closures and brief aborted openings.

The fact that R-R-X-S and R-X-X-S/T are minimal consensus phosphorylation sequences (38,39) for CaM kinase and CaM kinase, respectively, makes this residue the likely phosphorylation site. Consistent with this, the ratio of dithiothreitol-serine to phenylthiohydantoin-serine recovered during cycle 3 of sequencing of this peptide was 10 times greater than that recovered during cycles 6 and 9. It is known that dithiothreitol-serine is the predominant breakdown product of phosphoserine (40, 41). Phosphoamino acid analysis revealed that this peptide contained only phosphoserine; moreover, >90% of the 32P, was released from the peptide by cycle 10 (40, 42), demonstrating that no serine residue downstream of this region was significantly labeled. Based on these results, we conclude that serine 2809 is the amino acid phosphorylated by CaM kinase.

When only endogenous CaM kinase was used to phosphorylate the cardiac ryanodine receptor, the same labeled tryptic peptide was recovered and sequenced in four separate runs. Thus, although exogenously added kinase gives a 4-fold stimulation of receptor phosphorylation (Fig. 2), no new sites are phosphorylated. The reason for the low level of phosphorylation obtained with endogenous CaM kinase remains undefined, but could be due to membrane-limited structural
Cardiac Ryanodine Receptor Phosphorylation Site

Fig. 8. Time course of the effects of CaM kinase on cardiac Ca^{2+} channel activity. The experiment is a continuation of that depicted in Fig. 7. 5 min after addition of calmodulin to the cis chamber (Fig. 7C), 1 μg of CaM kinase was added (downward vertical arrow). The analog record is a continuous tracing of channel activity, revealing an increase in prolonged openings of the channel in each subsequent sweep. The histogram labeled A represents cumulative data from the first minute after addition of CaM kinase, when p(open) was 0.15. Panel B demonstrates the cumulative data from the final minute of exposure to kinase, when p(open) was increased to 0.86. Open and shut times for the channel were determined from analysis of idealized traces using a 50% crossing threshold for determination of the open state. In the presence of calmodulin (Fig. 7C), prolonged closures were present, giving a mean closed time of 280 ms. CaM kinase produced long openings, eliminating the long closures produced by calmodulin (15), decreasing the mean closed time to 27 ms.

constraints, or to endogenous phosphatase activity in the membranes (43) overwhelming a low level of kinase. It should be pointed out that the heart contains both soluble (44) and particulate (22) forms of CaM kinase.

The sequence surrounding residue 2809 of the rabbit and human skeletal muscle receptors is quite different from that of the cardiac receptor (Fig. 5). Out of 25 residues, 14 or 15 residues, respectively, are altered. These differences may account for the insigificant phosphorylation of the skeletal muscle isoforms (see last section).

Antibody to the Cardiac Phosphorylation Site—In order to confirm that the phosphorylation site sequence originated from the cardiac isoform of the ryanodine receptor, we raised a rabbit antiserum to a synthetic peptide (residues 2905–2919) encompassing serine 2809.

Immunoblot analysis revealed that this antiserum reacted only with the cardiac ryanodine receptor (Fig. 6). Ryanodine receptors in canine fast and slow and rabbit skeletal muscle membranes were unreactive. Moreover, the antiserum immunoprecipitated [3H]ryanodine-binding sites solubilized from cardiac membranes but did not sediment binding sites solubilized from either fast or slow skeletal muscle membranes (Table 1). The antiserum also recognized the human cardiac ryanodine receptor, but not the human skeletal muscle ryanodine receptor.2

In preliminary experiments, we have used this same antiserum to immunoprecipitate >90% of the cardiac receptors solubilized from neuronal membranes, which like cardiac receptors, are substrates for CaM kinase (45). Thus our results confirm that the cardiac isoform of the ryanodine receptor is expressed in brain (21), and demonstrate that the unique phosphorylation site is conserved.

Phosphorylation Effect on Ca^{2+} Channel Activity—Previous studies have shown that ATP activates, whereas calmodulin inhibits the ryanodine receptor/Ca^{2+} release channel (13–15). Consistent with previous results (3, 13–15), we observed that cardiac junctional SR vesicles fused with the planar bilayer exhibited a characteristic 75 picosiemens divalent cation conductance, which required micromolar Ca^{2+} on the cis side of the bilayer and was regulated by ATP and calmodulin. With 11 μM Ca^{2+} and 3 mM Mg^{2+} on the cis side of the bilayer (Fig. 7A), addition of 1 mM ATP to the cis side produced prolonged openings of the channel and increased the open state probability p(open) from 0.26 to 0.81 (Fig. 7B). Cis addition of 3 μM calmodulin then inhibited channel activity, reducing the p(open), shortening the mean open time, and producing prolonged closures (Fig. 7C).

Subsequent addition of CaM kinase to the cis side of the bilayer, under conditions known to give phosphorylation of the cardiac ryanodine receptor, reversed the inhibitory effect of calmodulin on p(open) (Fig. 8, A and B), and restored the prolonged openings of the channel (Fig. 8, analog tracing) observed in the presence of ATP (Fig. 7B). No effect on the unitary current of the channel was seen. In multiple experiments, we observed that the net effect of CaM kinase was to reverse the inhibitory effect of calmodulin. No effect of the kinase on channel activity was observed when it was added to the trans side of the bilayer, nor was an effect seen in the absence of ATP or calmodulin. We also observed no effect of added CaM kinase when skeletal muscle SR vesicles were used.

To rule out the possibility that phosphorylation of an accessory protein by CaM kinase was activating the Ca^{2+} channel in cardiac SR vesicles, we prepared a partially purified preparation of the ryanodine receptor by solubilization of cardiac membranes in CHAPS followed by sucrose density gradient centrifugation (Fig. 9). The ryanodine receptor in this partially purified preparation was visualized by Coomassie Blue staining (left panel) and, importantly, was the only protein phosphorylated by exogenously added CaM kinase (right panel). Phosphorylation by CaM kinase was blocked by the affinity purified antibody (data not shown).
Cardiac Ryanodine Receptor Phosphorylation Site

FIG. 9. Phosphorylation of the partially purified cardiac ryanodine receptor by CaM kinase. 3 mg of cardiac junctional SR vesicles in 20 mM MOPS (pH 7.2), 1 mM NaCl, 0.1 mM EGTA, 0.15 mM CaCl₂, and protease inhibitors (3) were solubilized by addition of 10% CHAPS containing phosphatidylcholine to give final protein, phospholipid, and detergent concentrations of 1.5 mg/ml, 2.5 mg/ml, and 1.5%, respectively. The supernatant was subjected to density gradient centrifugation and the fractions containing the ryanodine receptor identified by [³H]ryanodine-binding assay (3). The fractions constituting the [³H]ryanodine-binding peak were pooled and concentrated with a Centricon-30 (Amicon), and an aliquot of the concentrate phosphorylated by added CaM kinase as described under "Experimental Procedures." Shown is the Coomassie Blue-stained gel (C.B.) and corresponding autoradiogram (Auto) after SDS-PAGE of phosphorylated samples. During SDS-PAGE the dye front (DF) was not run off the gel in order to retain all potentially phosphorylated proteins. T indicates the top of the running gel. Purified ryanodine receptor (RR) and CaM kinase (PK) were added as indicated. The minor phosphoprotein band migrating between the 66- and 43-kDa protein standards is the autoprophosphorylated CaM kinase. No phosphorylation of the ryanodine receptor was observed when the CaM kinase was omitted from the incubation buffer (not shown).

demonstrating that the same region of the receptor was phosphorylated in the partially purified preparation as in native SR vesicles.

The partially purified receptor was fused with the planar bilayer, and single channel activity was measured. As shown in Fig. 10, addition of CaM kinase to the bilayer bath (B) increased channel activity substantially over that observed in the presence of ATP and calmodulin (A), similar to results obtained with native SR vesicles. Thus, phosphorylation of the ryanodine receptor itself was sufficient to activate the channel.

Functional Considerations—As our work was being completed, Otsu et al. (21) reported the deduced primary structure of the rabbit cardiac ryanodine receptor, which contains the phosphorylation site we have presently sequenced. Our results suggest that phosphorylation at a single site is sufficient to change channel function. Since serine 2809 is remote in sequence from the putative pore-forming carboxyl-terminal transmembrane segments (19-21), it is apparent that some well-orchestrated mechanism must exist to transduce the phosphorylation signal into a change in Ca²⁺ conductance. Although our results do not address this mechanism, they do show that at least one regulatory site of the molecule is located within the region recently postulated to contain the modulator-binding sites (residues 2619-3016) (21). As a corollary, our results are the first to definitively localize this region of the molecule to the cytoplasmic side of the SR membrane.

At present, it is not possible to conclude whether phosphorylation at one subunit or all four subunits is required for activation of the channel; in this regard it would be helpful to know whether the endogenous CaM kinase of native SR vesicles survives fusion with the planar bilayer, and remains capable of phosphorylating the ryanodine receptor to a low stoichiometry. In multiple experiments, we observed significant activation of the channel only when exogenous CaM kinase was added.

Distributed throughout the cardiac ryanodine receptor (21) are 16 candidate phosphorylation sites for multifunctional CaM kinase, even when the consensus site of the kinase is narrowly defined as R-X-X-S/T (38, 39). There are also five candidate phosphorylation sites for cAMP kinase based on a consensus of B-B-X-S/T, where B represents a basic residue, either Arg or Lys (38). Remarkably, only serine 2809 of the cardiac ryanodine receptor is phosphorylated by CaM kinase.

In particular, although CaM kinase phosphorylates pyruvate kinase as well as synthetic peptides at a sequence containing R-R-A-S, with a motif similar to R-R-I-S in the cardiac isoform of the ryanodine receptor (39, 46, 47), it does less well when a lysine replaces an arginine residue (19), as occurs in the rabbit skeletal muscle receptor (Fig. 5). For example, a synthetic peptide containing the sequence K-R-I-S is not phosphorylated at all (39). This same region of the skeletal muscle ryanodine receptor (Fig. 5) has been postulated to bind calmodulin (20), and it is also possible that phosphorylation of this site could be prevented by the presence of bound calmodulin. Such protection of a phosphorylation site by calmodulin is seen when myosin light chain kinase in its calmodulin-bound state is used as a substrate for cAMP kinase (48). The cardiac ryanodine receptor would be expected to bind calmodulin less well at this site since it differs markedly in the number of basic residues on the amino-terminal side of serine 2809 (Fig. 5).

Interestingly, the serine 2809 phosphorylation site is at the carboxyl-terminal end of the third of four large repeated sequences found in the cardiac ryanodine receptor (21). While the function of these repeats is unknown, it is possible that phosphorylation may modulate the receptor by altering the function of these repeated sequences.

Phosphorylation of the ryanodine receptor/Ca²⁺ release channel offers an attractive mechanism for increasing myoplasmic Ca²⁺ concentration during β-adrenergic stimulation of the heart. Work with another cardiac SR protein, phospholamban, already demonstrates that during β-adrenergic stimulation both CaM kinase and cAMP kinase are activated and phosphorylate SR proteins (42). In the case of phospholamban phosphorylation, Ca²⁺ uptake into the SR is stimulated, resulting in an increased rate of myocardial relaxation (49, 50). Simultaneous phosphorylation of the ryanodine receptor could increase the amount of Ca²⁺ released from the SR, contributing to the increased force of contraction. In neurons, phosphorylation of the cardiac isoform of the ryanodine receptor could contribute to the increase in cytoplasmic Ca²⁺ con-
centration occurring during membrane depolarization and/or neurotransmitter release. In either case, the phosphorylation effect on Ca²⁺ channel activity seems to be modulatory, relieving an inhibitory effect already caused by calmodulin.

Acknowledgments—We thank David Mendel for performing the planar bilayer experiments, P. Hanson for generously providing the CaM kinase, Steve Austin for single channel analysis software development, and Noel Davies for the computer program to calculate calcium levels. Thanks is also given to Joyce Dwulet for performing the sequence analyses. The excellent secretarial assistance of Lisa Lewis is greatly appreciated. Finally, we thank Kevin Campbell for supplying us with antisera specific for the skeletal muscle isoform of the ryanodine receptor.

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Fig. 10. CaM kinase effect on partially purified cardiac ryanodine receptor. The partially purified receptor was incorporated into the planar bilayer in buffer solutions identical to that used in Figs. 7 and 8. The top trace (A) shows single channel activity recorded in the presence of 1 mM ATP and 3 μM calmodulin, cis. The bottom three sweeps (B) were taken 1, 3, and 5 min following the addition of 1 μg of CaM kinase to the cis chamber. A second channel becomes apparent in the second sweep from the bottom. In the bottom sweep, one channel is open nearly the entire time, with interspersed brief openings of a second channel. Side arrows denote the fully closed state. Amplitude histograms are shown at the right for activity in the presence of ATP and calmodulin (A) and for 5 min of continuous activity following the addition of CaM kinase (B). p(open) increased from 0.15 to 0.60 after the addition of CaM kinase.
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