Stoichiometric Phosphorylation of Cardiac Ryanodine Receptor on Serine 2809 by Calmodulin-dependent Kinase II and Protein Kinase A*

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Received for publication, February 3, 2002, and in revised form, April 24, 2003
Published, JBC Papers in Press, May 5, 2003, DOI 10.1074/jbc.C301180200

The ryanodine receptor of cardiac muscle plays a central role in the coupling of electrical excitation of the muscle to mechanical contraction. It is a Ca$^{2+}$ channel, which resides primarily in the sarcoplasmic reticulum (SR) at junctions between this organelle and the t-tubular system (a specialized invaginated domain of the plasma membrane). Upon depolarization of the plasma membrane, Ca$^{2+}$ enters the cell across the t-tubule membrane and interacts with the RYR2. Ca$^{2+}$ binding to RYR2 opens the channel, and Ca$^{2+}$ stored in the SR moves through the channel into the cytosol to initiate contraction (1).

A variety of strategies are used by the cell to regulate RYR2 channel activity. It is anticipated that these regulatory strategies facilitate the fine control of E-C coupling, although evidence for this in live cells is rather limited. In vitro studies have shown that the binding of Ca$^{2+}$ (2), Mg$^{2+}$ (3), ATP (4), cADP ribose (5), calmodulin (6), and FKBP12.6 (7) affect channel activity, as does the binding of pharmacological agents such as the plant alkaloid ryanodine (which was originally used to identify the channel protein; Ref. 8). In addition the channel is phosphorylated on at least a single residue, and this phosphorylation alters channel behavior in vitro (7, 9).

In an effort to understand the regulatory role of RYR2 phosphorylation, research has focused on three aspects of the process. First, the identity of sites of phosphorylation in the receptor and kinases capable of using these sites; second, the functional consequence of site-specific phosphorylation in vitro; and third, the incidence and functional consequence of site-specific phosphorylation of the receptor in living cells. To date Ser-2809 (in the rabbit sequence (10) or the corresponding Ser-2808 in man (11)) has been identified as a site of phosphorylation on RYR2, which is used in vitro (7, 9). It has a counterpart in the skeletal muscle ryanodine receptor, Ser-2843, which is also phosphorylated in vitro (12). Other phosphorylation sites may exist in RYR2, but the identity of these await description. CaMKII was first identified as the kinase responsible for Ser-2809 phosphorylation (9), and more recently phosphorylation of this site by PKA has been described (7). It is likely that both of these observations are accurate, although this has not been demonstrated in a single study to date. Resolution of this issue is one of the objectives of the present study.

Phosphorylation of RYR2 by CaMKII or PKA is accompanied by significant changes in channel function in vitro. These changes include an increased open probability ($P_o$) (7, 9), the abrogation of the inhibitory effects of CaM (9) and Mg$^{2+}$ (3), heightened channel activity ($P_o$) in response to step changes in Ca$^{2+}$ (13), dissociation of regulatory factors (e.g. FKBP12.6), expression of subconductance states, and the expression of channel activity at diastolic Ca$^{2+}$ concentrations (7). Furthermore, Ser-2809 phosphorylation appears elevated in clinical situations such as heart failure (14), and may contribute to the abnormal Ca$^{2+}$ handling characteristics of cardiac muscle in these conditions.

Despite good evidence of increased channel activity upon RYR2 phosphorylation in vitro, the anticipated manifestation of this is not observed in single cells (15). Ca$^{2+}$ spark frequency might be expected to increase with increased $P_o$ and with the expression of channel activity at diastolic Ca$^{2+}$ concentrations (which accompany stoichiometric phosphorylation of Ser-2809; Ref. 7). However, Li et al. (15) found that spark frequency did not increase upon cAMP generation in animals lacking the regulatory protein phospholamban. These data suggest that spark frequency was most heavily influenced by the phosphorylation status of phospholamban.

In this study we have sought to develop tools for the study of

* This work was supported by the British Heart Foundation (PG/99166 and PG/99186). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: SR, sarcoplasmic reticulum; CaMKII, calmodulin-dependent kinase II; PKA, protein kinase A; PKI, protein kinase inhibitor; PVDF, polyvinylidene difluoride; ELISA, enzyme-linked immunosorbent assay.
Ryanodine receptor phosphorylation in vitro and in living cells. Phosphorylation site-specific antibodies have proven to be invaluable tools in research to establish the role of phosphorylation of an individual protein in any particular cell biological event. Here we describe the production and characterization of a pair of antisera to the Ser-2809 phosphorylation site of RyR2. One of these antisera is specific for the phosphorylated form of the Ser-2809 epitope, the other is specific for the dephosphorylated form of the same. These antibodies have enabled us to establish that both CaMKII and PKA phosphorylate Ser-2809 to full stoichiometry in vitro. The study has also established that CaMKII phosphorylates RyR2 to a greater extent than PKA, consistent with the use of multiple phosphorylation sites on RyR2 by CaMKII.

EXPERIMENTAL PROCEDURES

Materials—Calmodulin was purified from bovine testes by the method of Gopalakrishna and Anderson (16). CaMKII and PKA (the catalytic subunit from bovine heart) were purchased from Upstate Biotechnology. CaMKII was also obtained from Professor Howard Schulman (Stanford, CA). Phosphorylated and dephosphorylated RyR2 (Ac-S2809KKKKRIS/PO4/QT2812-amide and Ac-2809KKKKRIS-QTS/QT2812-amide, residue numbering according to rabbit sequence) and RyR1 (Ac-S2809KKKKRIS/PO4/QT2812-amide and Ac-2809KKKKRIS-KISS/QT2812-amide) peptides were purchased from NeoSystem (Strasbourg, France), and PKI peptide (TTYADFIASFTGRNRHAMD-amide) was purchased from Alfa Aesar (Wyckoff, NJ). Alkaline phosphatase, type VII-L from bovine intestinal mucosa, was obtained from Sigma, CAMP was from Calbiochem, calycin A and H-89 were purchased from Alexis Corp., KN-93 was from Novagen, and γ[32P]ATP was obtained from ICN.

Preparation of Phospho-specific Antibodies—Phosphorylated and dephosphorylated RyR2 peptides comprising residues 2802–2812 inclusive and 50 mM histidine (pH 7.0), 5 mM MgSO4, 5 mM NaF, and 2 mM EDTA were purified to homogeneity by reverse phase high performance liquid chromatography. It should be noted that Ser-2809 in rabbit RyR2 corresponds to Ser-2808 in the human RyR2 sequence, but that the epitope sequence is identical in these two species. Peptides were conjugated to keyhole limpet hemocyanin using carbodiimide in the case of phosphorylated peptide (17), or glutaraldehyde in the case of dephosphorylated peptide (18), and dialyzed extensively against phosphate-buffered saline. Adult New Zealand White rabbits were immunized with ~150 μg of keyhole limpet hemocyanin and attached peptide at ~6 weekly intervals and immune serum collected 10 days after immunizations 2 and 3. Serum was prepared and stored at ~70°C. Two polyclonal antibodies are described herein: RyR2-PS2809 raised to the phosphorylated peptide and RyR2-2809DP raised to the dephosphorylated peptide.

Dephosphorylation Assay—Canine cardiac sarcoplasmic reticulum vesicles were prepared as described previously (19). SR vesicles were incubated at 37°C in 50 mM histidine (pH 7.0), 5 mM MgSO4, 1 mM EGTA, and 50 (or 100) units/ml alkaline phosphatase for 1 h (20). SR vesicles were recovered by centrifugation at 27,000 × g for 60 min, washed in phosphate-buffered saline, and resuspended in buffer containing 10 mM imidazole HCl (pH 6.9), 0.29 mM sucrose, and 3 mM Na3PO4. Protein concentration was determined by the standard BCA assay.

Phosphorylation Assays—Phosphorylation reactions were conducted at 37°C in 40 μl of buffer containing 10 μg of dephosphorylated SR vesicles and 50 mM histidine (pH 7.0), 5 mM MgSO4, 5 μM NaF, and 2 μM calmodulin A. For phosphorylation by CaMKII (0.2 μM, unless otherwise stated), the buffer contained 0.1 mM CaCl2, 1.5 μg of calmodulin, and 1 μM PKA inhibitor H-89 or PKI (in separate experiments). For the control samples, Ca37+ (1 mM EGTA present), calmodulin and CaMKII were omitted from the assay medium. PKA (0.1 μM) phosphorylation was performed in the presence of 1 mM EGTA, 1 μM cAMP, and 1 μM CaMKII inhibitor, KN-93. cAMP and PKA were omitted in the buffer for the control samples. The phosphorylation reactions were initiated by the addition of 0.2 mM [γ-32P]ATP (0.1 μCi/nmol). After 1 min of incubation, the reactions were stopped by adding 10 μl of SDS sample buffer (5-strength) to the medium.

Immunoblot Analysis—SR proteins were separated by SDS-PAGE using 6% and 15% polyacrylamide gels as described by Laemmli (21). Following separation, proteins were transferred to PVDF membranes (Pall BioSupport, Portsmouth, UK) by semidyblotting, and nonspecific binding sites were blocked for 2–4 h at room temperature using 5% dried milk and Tris-buffered saline (pH 7.4), 0.1% Tween 20. Membranes were probed overnight at 4°C with primary antibodies RyR2-PS2809 or RyR2-2809DP (1:5000) or Ser-2809 (PS-16) and Thr3 (PT-17) (1:5000) for the phosphorylated forms of phospholamban (18). A secondary peroxidase-labeled antibody (Jackson Immunochemicals) was used in combination with an enhanced chemiluminescent detection system (Supersignal West Pico Chemiluminescent, Pierce) to visualize the primary antibodies. Data were captured using a Fuji LAS-1000 Imaging system with a CCD camera (AIDA software for analysis). When both penu-meric and monomeric forms of phospholamban were evident, the optical density of these two bands were summed.

Method of Stripping Membranes—Following immunoblot analysis, membranes were incubated at 55°C in stripping buffer containing 62.5 mM Tris-HCl (pH 6.7), 2% SDS, and 0.1 mM β-mercaptoethanol for 30 min. Membranes were then washed in Tris-buffered saline (pH 7.4) 0.1% Tween 20 and 5% dried milk for 2–4 h at room temperature and probed overnight at 4°C with another primary antibody.

Phosphoimaging and Data Analysis—The amount of 32P incorporation into the cardiac muscle ryanodine receptor was determined by phosphorimaging. Phosphoimaging plates were exposed to PVDF membranes for 24 or 48 h, and densitometric analysis of the data was conducted using Fuji BAS-1000 scanner (AIDA software for analysis).

ELISA—Competitive ELISA experiments were performed by standard protocols (22) using o-phenylene diamine as the substrate for peroxidase-conjugated second antibodies (Jackson Immunochemicals). ELISA plates were coated with RyR2 peptides at 1 μg/well, and RyR2 and PKA peptides were used as competing agents as described in the relevant figure.

Statistics—All the values are expressed as mean ± S.D. for n experiments, and statistical significance was evaluated using the Student’s t test for unpaired data.

RESULTS

Production of Antibodies to Phosphorylated RyR2—In an effort to understand the role of RyR2 phosphorylation at Ser-2809, antibodies specific for the dephosphorylated and the phosphorylated Ser-2809 site were generated. Similar antibodies to other phosphoproteins have proven invaluable in the definition of the incidence and role of site-specific phosphorylation of their respective protein targets (18, 23). A pair of 11-amino acid-long peptides derived from RyR2 sequence 2802–2812 were used as hapten immunogens. One of the peptides was synthesized with a phosphoserine residue at the position, which equates to Ser-2809 (confirmed by mass spectrosopy), whereas the other was a dephosphorylated sequence. The choice and length of sequence was selected to maximize the importance of the phosphorylation status of Ser-2809 (in the molecular recognition event between peptide and antibody), while providing RyR2 flanking sequence to ensure that the antibodies generated were specific for RyR2 protein. Data using raw antisera from the terminal bleed are presented throughout this study.

Polyclonal antibodies able to discriminate between phosphorylated and dephosphorylated RyR2 peptides with high fidelity were produced (Fig. 1). Competitive ELISA experiments employing ELISA plates coated with the appropriate peptide and varying concentrations of peptide (phosphorylated or dephosphorylated) in solution were used to analyze the binding characteristics of the two antibodies. A peptide recognized by the antibody is a potent inhibitor of the interaction of antibody with the antigen immobilized on the ELISA plate. Whereas a peptide not recognized by the antibody is unable to affect interaction with immobilized antigen. Antibody RyR2-PS2809 recognized Ser-2809-phosphorylated RyR2 peptide, as the interaction of this antibody with immobilized antigen was inhibited by low concentrations of phospho-RyR2 peptide (IC50 = 10 nM). Low concentrations of dephosphorylated-RyR2 peptide
had no effect on antibody binding to its antigen, but very high concentrations display some inhibitory potential (IC50 12.3 and 41.5 μM, respectively).

The IC50 values for each of these peptides described a 2000-fold preference for phosphorylated RYR2 peptide in the case of antibody RYR2-PS2809. Peptides derived from the equivalent phosphorylation site in RYR1 (Ser-2843) were poorly recognized by the antibody (Fig. 1). Inhibition of antibody binding to antigen immobilized on the ELISA plate was detected using peroxidase-conjugated secondary antibody as described under “Experimental Procedures.” Data represent mean ± S.D. (n = 2). In some cases S.D. is smaller than symbol size.

These experiments confirm that antibodies have been produced which are highly specific for the RYR2 phosphorylation site at Ser-2809 and interact with this site in a phosphorylation-dependent manner. Antibodies of this quality have been described previously with other protein targets (18) and have proven invaluable in the description of the role of protein phosphorylation in cardiac cells.

Antipeptide Antibody Recognition of RYR2 Protein—Having observed peptide and phosphopeptide recognition by these antibodies, we next investigated whether they could interact with RYR2 protein in a phosphorylation dependent manner. Canine cardiac SR vesicles contain appreciable amounts of RYR2 protein; this was phosphorylated by incubation with CaMKII and subjected to Western blot analysis. Fig. 2 shows that RYR2 protein is present in the SR vesicle preparation (stained with monoclonal antibody 34C; Fig. 2, A (panel i) and B (panel i)), and the amount does not differ between control (lanes 1) and CaMKII (lanes 2)-treated samples. RYR2 is separated by electrophoresis into two major species as has been described previously (9). Fig. 2 also shows that antibody RYR2-PS2809 interacts with the RYR2 protein, as it stains a protein doublet of identical molecular weight to that detected by the RYR2 monoclonal antibody. No other proteins or phosphoproteins in cardiac SR were detected by this antibody (Fig. 2). The interaction of antibody RYR2-PS2809 with RYR2 was wholly dependent on the phosphorylation status of Ser-2809, as phosphorylated RYR2 peptide (Fig. 2A (panel iii)), but not dephosphorylated RYR2 peptide (Fig. 2A (panel iv)), inhibited immunorecognition of RYR2 on the blot. Having established that this antibody interacts with RYR2 only when Ser-2809 is phosphorylated, we can assume that the intensity of immunostaining achieved with this antibody reflects the phosphorylation status of Ser-2809 (as is the case for all other phosphorylation-specific antibodies). The detection of RYR2 in control samples by this antibody therefore shows that Ser-2809 phosphorylation is present prior to exposure to kinases, which must be due to the persistence of phosphorylation during the preparation of SR vesicles. This level of phosphorylation is increased upon exposure to CaMKII as seen by the increase in signal intensity with antibody RYR2-PS2809 in the CaMKII (lanes 2)-treated sample compared with the control sample (Fig 2A (panels ii and iv)).

As would be expected the staining intensity pattern with the dephosphorylated antibody, RYR2-2809deP, was exactly the opposite of that seen with the phosphorylation antibody. It too recognized the RYR2 protein doublet exclusively, and the immunorecognition was lost upon addition of dephosphorylated RYR2 peptide (Fig. 2B (panel iv)), but not upon addition of phosphorylated-RYR2 peptide (Fig. 2B (panel iii)). Finally the signal intensity was decreased somewhat (25–40%) upon CaMKII treatment of SR samples. These data confirm that both of these antibodies recognize RYR2 protein in a manner which is wholly dependent upon the phosphorylation status of antibody RYR2-2809deP, where the inhibition of binding of antibody to peptide immobilized on the plate displayed a shallow slope with respect to dephosphorylated RYR2 peptide concentration, but a steep slope with respect to phosphorylated RYR2 peptide concentration. This, we believe, is a function of the polyclonal nature of the antiserum generated. A number of different antibodies exist within the polyclonal serum, which possess a range of binding affinities for the peptide. In the case of RYR2-2809deP, the affinity of the antibodies for dephosphorylated RYR2 peptide has a broad range that centers around a half-maximal inhibitory concentration of 10 nM, whereas the affinity displayed by this antibody for phosphorylated RYR2 peptide is more uniform, centered around a half-maximal inhibitory concentration of 44 μM.

Fig. 1. Antibodies discriminate between peptide and phosphopeptide. ELISA plates were coated with phosphorylated RYR2 peptide (1 μg/well) and probed with RYR2-PS2809 (1:500) in the presence of phosphorylated peptide (RYR1P (■), RYR2P (○)) and dephosphorylated peptide (RYR1deP (□), RYR2deP (○)) at a range of concentrations 0.8 nM to 50 μM (A) or dephosphorylated RYR2 peptide (1 μg/well) and probed with RYR2-2809deP (1:500) in the presence of phosphorylated peptide (RYR1P (■), RYR2P (○)) and dephosphorylated peptide (RYR1deP (□), RYR2deP (○)) at the same range of concentrations 0.8 nM to 50 μM (B). Antibody binding to antigen immobilized on the ELISA plate was detected using peroxidase-conjugated secondary antibody as described under “Experimental Procedures.” Data represent mean ± S.D. (n = 2). In some cases S.D. is smaller than symbol size.
Ser-2809; antibody RYR2-PS2809 is specific for Ser-2809 phosphorylated RYR2, and antibody RYR2-2809deP is specific for Ser-2809 dephosphorylated RYR2.

**Which Kinases Phosphorylate Ser-2809?**—Separate reports suggest that different kinases phosphorylate RYR2 on Ser-2809 (7, 9). It is likely that both reports are correct and that both kinases, CaMKII and PKA, phosphorylate this site in vitro. A degree of uncertainty surrounds this point, which could be dispelled by demonstration of the action of both kinases within a single study. This was addressed in the following experimental series. First, the basal phosphorylation status of RYR2 was minimized by phosphatase treatment in an effort to make subsequent kinase action clear. A reduction in immunorecognition by RYR2-PS2809 and an increase in staining by RYR2-2809deP provide evidence of near complete dephosphorylation of Ser-2809 of RYR2 by treatment of canine cardiac SR with alkaline phosphatase (50 or 100 units/ml) for 60 min (Fig. 3).

A batch of dephosphorylated SR was produced by treatment with alkaline phosphatase (50 units/ml) for 60 min followed by two centrifugation steps at 27,000 × g for 60 min and an intervening wash step (in phosphate-buffered saline) to harvest SR vesicles and remove the soluble phosphatase. The SR was then exposed to a purified protein kinase (CaMKII, PKA) in the presence of selective inhibitors of the other kinase. The site-specific phosphorylation of phospholamban (CaMKII, Thr-17; PKA, Ser-16; Ref. 18) was used to confirm that the exclusive activity of each kinase in turn was observed (Fig. 4). The dephosphorylation treatment removed most, but not all, phosphate from Ser-16 and Thr-17 (Fig. 4, B and C, samples C1 and C2). Subsequent Ser-16 phosphorylation of phospholamban in a radioassay was not observed upon CaMKII addition (remained at this base-line level, Fig. 4B, lanes 2–5) but in separate assays increased dramatically upon PKA addition (Fig. 4B, lanes 7–10). Conversely, Thr-17 phosphorylation was unresponsive to PKA addition (remained at base-line level, Fig. 4C, lanes 7–10) but increased substantially in the presence of CaMKII (Fig. 4C, lanes 2–5). These data confirm that the conditions used permit observation of the activity of individual kinases (CaMKII, PKA) exclusively.

The phosphorylation status of RYR2 on Ser-2809 was then examined by Western blotting in these same samples (Fig. 5). Low levels of Ser-2809 phosphorylation were detected in control samples (no kinase added), but substantially greater Ser-2809 phosphorylation was detected following treatment with either purified CaMKII or purified PKA (Fig. 5A). Densitometric analysis confirmed that a statistically significant increase in Ser-2809 phosphorylation occurred upon addition of either CaMKII or PKA (Fig. 5C).

We next sought to establish whether RYR2 was phosphorylated to the same extent by each of these kinases. This was most clearly investigated by measurement of the amount of unphosphorylated RYR2 following treatment with each kinase. As is evident in Fig. 5B PKA phosphorylated RYR2 to apparent stoichiometry on Ser-2809, as there was no detectable unphosphorylated protein remaining in the sample. CaMKII did not achieve this high level of phosphorylation in this experiment. These data suggest that Ser-2809 is predominantly a phosphorylation site for PKA, as this enzyme is able to catalyze the stoichiometric phosphorylation of this site. Marx et al. (7) suggested the same using a back phosphorylation strategy. However, in the present study, the amount of CaMKII and PKA employed in the experiment differed, which might contribute to the dissimilar magnitude of phosphorylation observed in each case. To explore this issue, canine cardiac SR was phosphorylated by comparable amounts of CaMKII (2 nmol/min of activity) and PKA (1 nmol/min of activity). This represents a substantial increase in CaMKII activity (but not total protein), as
the specific activity of CaMKII used in the subsequent experiments was substantially greater than that used in the former. Under these conditions, Ser-2809 was phosphorylated to apparent stoichiometry by each kinase (Fig. 6), since the dephosphorylation-specific antibody was unable to detect any protein phosphorylation of a further four sites or stoichiometric phosphorylation of Ser-2809 plus stoichiometric phosphorylation of more than four additional sites. Examination of the sequence of human RYR2 has identified 21 potential CaMKII phosphorylation sites (Fig. 8) and only a single PKA site (Ser-2808). A similar number of sites are observed in the rabbit RYR2 sequence.

**DISCUSSION**

In this study we have produced and characterized a pair of antibodies that interact with the cardiac ryanodine receptor in a manner dependent upon the phosphorylation status of Ser-2809. One of the antibodies, RYR2-2809deP, interacts with RYR2, while Ser-2809 is dephosphorylated, and the binding affinity of this antibody is reduced 4410-fold when the epitope is phosphorylated. The other antibody, RYR2-PS2809, interacts with RYR2 when Ser-2809 is phosphorylated, and the apparent binding affinity is reduced 2000-fold upon dephosphorylation of this site. These antibodies are essentially wholly specific for the dephosphorylated and phosphorylated RYR2-2809 epitope, respectively. The antibodies were used to interrogate RYR2 phosphorylation at Ser-2809 in isolated cardiac SR vesicle preparations. These studies provided data that reconcile an uncertainty in the literature, whether CaMKII and/or PKA phosphorylate Ser-2809. Both CaMKII and PKA were able to phosphorylate Ser-2809 to high stoichiometry. CaMKII appears to phosphorylate at least four additional sites on RYR2.

**Quality of Antibodies—**Phosphorylation site-specific antibodies are proving to be a valuable resource in life science research, with in excess of 500 different antibodies in use today. The data of this study confirm our previous description (18) that highly specific anti-phospho-antibodies can be produced by the immunization of rabbits with short peptide sequences (−10−12 residues), purified to homogeneity and conjugated to a carrier protein. The method of conjugation does not appear to be critical, as two different methods were used in this study (glutaraldehyde and carbodiimide), and both delivered high quality antisera. These antibodies report on the phosphorylation status of Ser-2809 of RYR2 faithfully and are unaffected by the phosphorylation status of other sites in the receptor protein. This fidelity arises from the molecular recognition between antibody and epitope in which the phosphorylation status of Ser-2809 is critical but not the only determinant of binding. The importance of other residues within the epitope

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**Fig. 4. Site-specific phosphorylation of phospholamban identifies conditions for exclusive activation of CaMKII and PKA.** Canine cardiac sarcoplasmic reticulum vesicles were treated with alkaline phosphatase as described under “Experimental Procedures.” SR proteins were then phosphorylated by either exogenous CaMKII (3 μg/ml, lanes 2–5) in the presence of 1 μM PKA or exogenous PKA (2.5 μg/ml, lanes 7–10) in the presence of 1 μM KN-93 using 0.2 mM [γ-32P]ATP (0.1 μCi/nmol). Control SR samples that lack exogenous CaMKII, Ca2+, and calmodulin (1 mM EGTA present), but contain all other components of the assay, were prepared (C1). Control SR samples that lack exogenous PKA and cAMP but contain all other components of the assay were prepared (C2). SR proteins (3 μg) were separated on 15% SDS-PAGE gels and transferred to PVDF membranes. A, radiolabeled phosphoproteins were visualized by phosphorimage analysis. B, serine 16-phosphorylated phospholamban was detected using antibody PS-16 (1:5000). C, threonine 17-phosphorylated phospholamban was detected using antibody PT-17 (1:5000). An arrow indicates the location of phospholamban protein (monomer and oligomer).
was established by examining antibody recognition of the equivalent phosphorylation site in RYR1 (Ser-2843). The amino acid sequence of this RYR1 site (12) is very similar to the RYR2-2809 site (Fig. 9); however, the antibodies described in this study show clear discrimination between these sequences (Fig. 1). The binding of either antibody to the corresponding RYR1 sequence (phosphorylated or not, as appropriate) is ~1000-fold weaker than binding to RYR2 sequence (Fig. 1). From this we can conclude that both antibodies interact with multiple residues in the epitope sequence and that the differences in sequence (between RYR2 and RYR1) reduce the affinity of interaction enormously. RYR2 does not contain any other potential phosphorylation site which resembles the Ser-2809 epitope more closely than does the RYR1–2843 sequence (see Fig. 9). As such, we can anticipate that the antibodies used in this study would fail to interact with other (potential) RYR2 phosphorylation sites and that staining observed with these antibodies is dependent upon Ser-2809 phosphorylation status exclusively.

The pair of antibodies, one to the dephosphorylated (Ser-2809) receptor and the other to the phosphorylated (Ser-2809) receptor, were created in this study, as it was unclear from the literature which of the extremes of phosphorylation was going to be important in physiology and pathophysiology. At a technical level the complementary pair of antibodies has proven advantageous. They provide an internal check of experimental data, as the staining intensity observed with one antibody is the inverse of staining intensity of the other antibody. This approach was used previously in the first description of phosphorylation state-specific antibodies, which were raised to neuronal protein, G-substrate (26). Furthermore, the antibodies allow ready access to the stoichiometry of phosphorylation, as the amount of dephosphorylated protein was reduced to undetectable levels by PKA or CaMKII treatment of SR, suggesting that stoichiometric phosphorylation of RYR2 on Ser-2809 is achieved in vitro by either PKA or CaMKII. These new data

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**Fig. 5. Phosphorylation of RYR2 on Ser-2809 by PKA and CaMKII.** Dephosphorylated SR proteins were phosphorylated by exogenous CaMKII (5 μg/ml) in the presence of 1 μM H-89 or 1 μM PKI or by exogenous PKA (2.5 μg/ml) in the presence of 1 μM KN-93 for 60 s at 37 °C. SR proteins (10 μg) were separated by SDS-PAGE (6% acrylamide) and transferred to PVDF membranes. Ser-2809 phosphorylated RYR2 was detected with RYR2-PS2809 (1:5000) (A) and dephosphorylated RYR2 was detected by RYR2-2809deP (1:5000) (B). In A and B, lane 1 contains dephosphorylated SR, lanes 2–5 contain control SR (lacking exogenous kinase), and lanes 6–9 contain SR treated with kinase. Quantification of Ser-2809-phosphorylated RYR2 (C) and Ser-2809-dephosphorylated RYR2 (D) was performed by densitometry. Solid bar represents control data, mean ± S.D. (n = 4); open bar represents kinase-treated samples, mean ± S.D. (n = 4). **, p < 0.01; ***, p < 0.001.

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**Fig. 6. Phosphorylation of Ser-2809 to apparent stoichiometry by CaMKII or PKA.** Dephosphorylated canine cardiac SR vesicles were phosphorylated by CaMKII (5 μg/ml, 2 nmol/min of activity in each assay) in the presence of a PKA inhibitor (1 μM PKI) for 60 s or by PKA (2.5 μg/ml, 1 nmol/min of activity in each assay) in the presence of a CaMKII inhibitor (1 μM KN-93) for 60 s. Proteins were separated by SDS-PAGE (6% acrylamide) and transferred to PVDF membrane. Dephosphorylated RYR2 was detected using antibody RYR2-2809deP (1:50000). Dephosphorylated SR vesicles are shown in lanes 1 and 5 and kinase-treated SR in lanes 2–4 (CaMKII-treated) and 6–8 (PKA-treated).
PKA data. The radioactivity associated with RYR2 was quantified by densitometry, arrow phosphorylation by PKA. Dephosphorylated SR vesicles were phosphorylated by exogenous CaMKII (5°C endogenous kinases, were prepared in parallel. SR proteins (10°/H9262 lacking the exogenous kinase and the activating agents for relevant subsequent dissociation) with RYR2 needs further investiga-
tion of Ser-2809 by PKA (7) and extend this significantly suggests that the molecular event of FKBP association (and the binding and dissociation of FKBP12.6. The present work of potential phosphorylation sites in human RYR2. The sequence used to generate antibodies is shown aligned with the comparable site in RYR1 (2843) and the most closely related potential phosphorylation sites in human RYR2. Shading highlights identical residues, and the phosphoamino acid is highlighted in a box. It should be noted that Ser-2809 in rabbit corre-
sponds with Ser-2808 in the human sequence.

tion, since CaMKII would be expected to achieve stoichiometric phosphorylation of Ser-2809 and should therefore be able to affect FKBP12.6 binding. This was not observed in Marx et al. (7), which might be explained in one of the following ways: first, CaMKII phosphorylation of RYR2 might have been incomplete in the study of Marx et al. (7), thereby obscuring the subse-
quent effect on FKBP binding. Fig. 5 (of the present study) shows that substoichiometric phosphorylation of RYR2 is observed when suboptimal amounts of CaMKII are employed. Alternatively, since CaMKII phosphorylates a number of sites on RYR2 (details below), the binding of FKBP12.6 might be regulated by the phosphorylation status of multiple sites, such that dissociation occurs when Ser-2809 is phosphorylated ex-
cursively, but not if Ser-2809 and another (to be identified) site are phosphorylated.

Phosphorylation of Ser-2809 (or the corresponding residue, Ser-2808, in human RYR2) appears elevated in heart failure (14, 27, 28). To date this has been attributed to PKA activity exclusively; however, the results of this study suggest that CaMKII would be capable of contributing to this event. Both kinases (PKA and CaMKII) are likely to be active in situations CaMKII would be capable of contributing to this event. Both CaMKII and PKA are known to be active in this region of the heart, and their activation might be a consequence of the increased demand for calcium release from the SR in heart failure. The presence of both kinases would allow for a greater degree of regulation of RYR2 phosphorylation, which could be important for the proper functioning of the heart in conditions of increased workload.

**Fig. 9.** Sequence divergence between known and potential RYR phosphorylation sites. The sequence used to generate antibodies is shown aligned with the comparable site in RYR1 (2843) and the most closely related potential phosphorylation sites in human RYR2. Shading highlights identical residues, and the phosphoamino acid is highlighted in a box. It should be noted that Ser-2809 in rabbit corre-
sponds with Ser-2808 in the human sequence.

**Acknowledgments**—We are grateful to Prof. Howard Schulman (Stanford) for providing CaMKII, to Prof. Clive Orchard (Leeds) for critical discussion, to Ralph Hyde for assistance with bioinformatics, and to Peter Jones for providing canine SR vesicles.

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