Modulation of Inositol 1,4,5-Trisphosphate Receptor Type 2 Channel Activity by Ca\(^{2+}/\)Calmodulin-dependent Protein Kinase II (CaMKII)-mediated Phosphorylation*

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Background: The inositol 1,4,5-trisphosphate receptor (InsP\(_3\)R) is a ubiquitous intracellular calcium release channel. InsP\(_3\)-mediated calcium release through the type 2 inositol 1,4,5-trisphosphate receptor (InsP\(_3\)R2) in cardiac myocytes results in the activation of associated CaMKII, thus enabling the kinase to act on downstream targets, such as histone deacetylases 4 and 5 (HDAC4 and HDAC5). The CaMKII activity also feedback modulates InsP\(_3\)R2 function by direct phosphorylation and results in a dramatic decrease in the receptor-channel open probability (P\(_o\)). We have identified S150 in the InsP\(_3\)R2 core suppressor domain (amino acids 1–225) as the specific residue that is phosphorylated by CaMKII. Site-directed mutagenesis reveals that S150 is the CaMKII phosphorylation site responsible for modulation of channel activity. Nonphosphorylatable (S150A) and phosphomimetic (S150E) mutations were studied in planar lipid bilayers. The InsP\(_3\)R2 S150A channel showed no decrease in activity when treated with CaMKII. Conversely, the phosphomimetic (S150E) channel displayed a very low P\(_o\) under normal recording conditions in the absence of CaMKII (2 \(\mu\)M InsP\(_3\) and 250 nM Ca\(^{2+}\)\(_{\text{free}}\)) and mimicked a WT channel that has been phosphorylated by CaMKII. Phospho-specific antibodies demonstrate that InsP\(_3\)R2 Ser-150 is phosphorylated in vivo by CaMKII. The results of this study show that serine 150 of the InsP\(_3\)R2 is phosphorylated by CaMKII and results in a decrease in the channel open probability.

Intracellular calcium signaling plays an important role in the physiology of the cell, impinging upon a multitude of cellular events ranging from fertilization and cellular proliferation to cellular death (4). One mechanism in which calcium is mobilized from intracellular stores is via the activation of inositol 1,4,5-trisphosphate receptors (InsP\(_3\)Rs) by the second messen-

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3 The abbreviations used are: InsP\(_3\)R, inositol 1,4,5-trisphosphate receptor; CaMKII; Ca\(^{2+}\)/calmodulin-dependent protein kinase II; DN, dominant negative; ET-1, endothelin 1; HDAC, histone deacetylase; P\(_o\), open probability; PP1 and PP2A, protein phosphatases 1 and 2A; RyR2, ryanodine receptor 2; SR, sarcoplasmic reticulum.

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of CaMKII, CaMKIIΔo. Upon stimulation of InsP₃ production, Ca²⁺ released through the InsP₃R₂ activates CaMKIIΔo, allowing it to act on downstream targets, such as histone deacetylase 4 and 5 (HDAC4 and HDAC5) (2, 12). Additionally, CaMKII appears to feedback-modulate InsP₃R₂ function by direct phosphorylation and results in a significant decrease in the channel open probability (Pₒ) (1). The results of this study and others (2, 13, 14) suggest that the activity of InsP₃Rs can be inhibited by CaMKII-mediated phosphorylation. Furthermore, the aminoterminal 1078 amino acids of the InsP₃R₂ have been shown to interact with, as well as be phosphorylated by CaMKII (1). However, it has not yet been established what amino acid(s) or if additional downstream residues of the InsP₃Rs are implicated in channel regulation by CaMKII phosphorylation events.

In this study, we use expressed fragments of the InsP₃R₂ to show that CaMKII can phosphorylate the InsP₃R₂ at Ser-150. Upon phosphorylation, the channel open probability is significantly decreased in planar lipid bilayers. This could be reversed by addition of a specific CaMKII inhibitor (KN-93) and two protein phosphatases (PP1 and PP2A). Single channel studies of the receptor in which the phosphorylation site was mutated to an alanine (S150A) resulted in channels with properties similar to those of WT InsP₃R₂; however, they failed to respond to CaMKII addition and the Pₒ remained unchanged. A phosphomimetic mutation (S150E) displayed a constitutively low open probability and mimicked an InsP₃R₂ phosphorylated by CaMKII. These results strongly suggest that Ser-150 is the site impinged upon by CaMKII and results in negative modulation of the InsP₃R₂.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmid Construction**—The construction of the full-length type 2 InsP₃R protein expression vector (pInsP₃R₂-T2) was described previously (15). Briefly, the expression plasmid was assembled using overlapping cDNA clones originally isolated from a rat brain library (16). The full-length expressed protein includes amino acid residues 1–2701 from the rat type 2 cDNA (accession number X61677).

The pIP3R₂-Stopl078 construct is a mammalian expression vector of the first 1078 amino acids of the type-2 InsP₃ receptor. This sequence is followed by the 12 carboxyl-terminal amino acids of the 116,000 subunit of the proton pump and was described previously (16). The full-length expressed protein includes amino acid residues 1–1078 from the rat type 2 cDNA (accession number X61677).

Regions cloned into the bacterial expression plasmid pET-3a were PCR-amplified using pInsP₃R₂ as template and specific primers engineered to contain an amino-terminal NdeI restriction site and a carboxyl-terminal BglII site in addition to a methionine inserted after the NdeI site and a termination codon directly before the BglII site. PCR products were then digested with NdeI/BglII and ligated into NdeI/BamHI-digested pET-3a plasmid.

Construction of the full-length InsP₃R₂ S150 mutants (pInsP₃R₂-S150A and pInsP₃R₂-S150E) was done as follows. A plasmid coding for amino acids 1–1078 of the type 2 InsP₃R₂ was used as the template along with primers for mutagenesis of Ser-150 to alanine (5'-GAATGCCATGCCTTGCCCTTG-GATGCTGCAGGG-3') or glutamate (5'-GAATGCCATGCCTTGTTGAACCTTGAGCTGCAAGG-3'). Mutagenesis of Ser-150 to either alanine or glutamate was confirmed by DNA sequencing. The region containing S150A or S150E of 1–1078 vector was cut from the plasmid with NdeI and AfeI and ligated into similarly digested pInsP₃R₂, thus creating the full-length InsP₃R₂ expression construct pInsP₃R₂-S150A or pInsP₃R₂-S150E. Mutagenesis was performed using Change-IT Multiple Mutation Site-Directed Mutagenesis Kit (USB Corp.). Constructs were verified by DNA sequence analysis using a commercial facility at University of California Davis (Davis Sequencing) using the Applied Biosystems Big Dye Terminator V3.0 sequencing chemistry.

CaMKIIΔB/C plasmids were a kind gift from Dr. Joan Heller-Brown (University of California San Diego, La Jolla, CA). Adenoviral constructs containing the CaMKIIΔB/C inserts were derived from these plasmids and prepared by the Cell and Molecular Physiology departmental adenosivirus core (Loyola University Medical Center, Maywood, IL) in Adeasy vector backbones.

**COS-1 Cell Transfection**—COS-1 cells were transiently transfected with expression plasmids for pInsP₃R₂ using a DEAE-dextran method as described previously (17). Following the expression period, the COS cells were washed with phosphate-buffered saline (PBS), harvested in IP buffer (50 mM HEPES, pH 7.6, 1.0% bovine serum albumin, 10 mM magnesium acetate, 50 mM NaCl, 0.5 mM CaCl₂, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 100 µg/ml soybean trypsin inhibitor, 10 µM leupeptin, and 10 µM pepstatin) and lysed by 20–40 passages through a 27-gauge needle. The membranes were pelleted by centrifugation at 135,000 × gₖₙₐₓ 4 °C for 10 min, and the supernatant containing any soluble protein fraction was removed from the microsomes. To solubilize any membrane-bound proteins, the membrane fraction was resuspended in IP buffer containing 1.0% Triton X-100 and incubated on ice with stirring for 1–2 h followed by removal of insoluble material by centrifugation at 135,000 × gₖₙₐₓ 4 °C.  

**Bacterial Cell Expression**—Rosetta 2 (DE3) competent cells (Novagen) were transformed with the bacterial expression plasmids and grown at 37 °C with shaking in liquid culture until the A₆₀₀ nm reached 0.6. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside, and the culture was incubated for 3 h at 37 °C. Cells were then pelleted at 3220 × gₖₙₐₓ 40 min and resuspended in lysis buffer (8% sucrose, 0.5% Triton X-100, 10 mM Tris-Cl, pH 8.0, 50 mM EDTA, 1 mM
PMSF) with 10 mg/ml lysozyme (Sigma) and incubated at room temperature with agitation for 20 min. The lysate was then homogenized, and 1 mg/ml DNase and 100 mg/ml RNase were added, and the solution was incubated at room temperature for 25 min with agitation. The sample was then spun at 12,000 × gmax for 10 min, and the pellet was washed with 0.5 M NaCl, 0.5% Triton X-100, 1 mM PMSF. This was repeated twice, with the final wash containing 1 m urea. The resulting inclusion body pellet was then solubilized in 8 m urea, 150 mM NaCl, 50 mM Tris-Cl, pH 7.4, 3 mM 2-mercaptoethanol, 3 mM DTT, and insoluble material was pelleted by centrifugation at 106,000 × gmax for 10 min. Soluble material was dialyzed against a 4000-fold excess of 150 mM NaCl, 50 mM Tris-Cl, pH 7.4, 2 mM 2-mercaptoethanol. Recovered material was cleared by centrifugation at 106,000 × gmax for 10 min and was stored at 4 °C.

**Sf9 Cell Culture and Baculovirus Infection**—Sf9 cells were grown in 75-cm2 tissue culture flasks in culture medium containing TMN-FH medium (Cellgro) and 10% fetal bovine serum. A baculovirus expressing the full-length rat type 2 InsP3R (16, 18) was kindly provided by Dr. Ilya Bezprozvanny (University of Texas Southwestern Medical Center, Dallas, TX). Virus was added to the medium at a multiplicity of infection of 1, and cells were incubated at 27 °C for 72 h to allow for expression of recombinant protein.

**Immunoprecipitation of Recombinant Proteins**—Recombinant InsP3R2 proteins from either mammalian or bacterial expression were immunoprecipitated as follows. Proteins were divided into equal volume aliquots, and 10 μg of the appropriate primary antibodies was added and the samples incubated overnight at 4 °C with agitation. Then 30 μl of a 10% slurry of protein A-Sepharose CL-4B beads (Amersham Biosciences) was added and incubated for 2 h at 4 °C with agitation. Immune complexes were then washed three times with IP buffer before addition of kinase. Following kination the samples were treated with SDS-PAGE sample buffer, boiled 5 min, and resolved on SDS-PAGE.

**CaMKII Phosphorylation of Recombinant Proteins**—IP complexes were used as substrates for in vitro CaMKII phosphorylation 32P incorporation assays. Immune complexes were incubated at 30 °C for 20 min with 500 units of exogenous pre-activated CaMKII (New England Biolabs) or with 10 μM concentrations of the specific CaMKII inhibitor, KN-93 (Seikagaku Corp.). CaMKII enzyme was activated by incubation in reaction buffer (50 mM Tris-Cl, pH 7.5, 10 mM MgCl2, 2 mM DTT, 0.1 mM Na2EDTA, 2 mM CaCl2, 1.2 μM calmodulin, 200 μM ATP) for 10 min at 37 °C. Following CaMKII activation, the reaction buffer was supplemented with [γ-32P]ATP to a final specific activity of 200 μCi/μmol for visualization of phosphorylated proteins via autoradiography.

The in vivo phosphorylation of InsP3R2 experiments used COS-1 cells that were singly or co-transfected with InsP3R2 and the CaMKII plasmids. Phosphorylation of endogenous InsP3R2 was achieved by adenoviral expression of CaMKIIβC and its dominant negative derivative (DN) in neonatal rat ventricular myocytes. The cells were harvested 24 h after infection, and protein extracts were subjected to Western immunoblotting with the phospho- and non-phospho-specific antibodies. Neonatal rat ventricular myocytes were prepared in the Cell and Molecular Physiology myocyte core (Loyola University Medical Center, Maywood, IL).

**Antibodies**—The affinity-pure InsP3R2-specific amino-terminal (T2NH) and carboxyl-terminal (T2C) antibodies were directed against the sequences CPDYRDQNEGKTVRD-GERL (residues 320–338) and CNKQRGFLGSNTPH ENHH-MPPH (residues 2679–2701) of the rat InsP3R2, respectively (19). The Ser-150-T2 and phospho-specific Ser(P)-150-T2 antibodies were directed against CKNAMRVLDAAG (residues 144–155). For Ser(P)-150-T2 rabbits were immunized with phosphopeptide where Ser-150 was phosphorylated. Both antibodies were affinity-purified using immunogen peptide. Anti-FLAG antibody was purchased from Affinity BioReagents and is a rabbit polyclonal against the peptide DYK-DDDDDKC.

**SDS-PAGE and Western Blotting**—SDS-PAGE and Western blotting were performed as described previously (17) using 5, 7.5, or 10% SDS-polyacrylamide gels. Visualization was accomplished using ECL reagents (GE Healthcare).

**Preparation of Microsomes for Lipid Bilayers**—Microsomal fractions from Sf9 and COS-1 cells were prepared for use in planar lipid bilayers as described previously (20). Briefly, COS-1 or Sf9 cells were harvested in homogenization buffer (50 mM Tris-HCl, pH 8.3, 1 mM EDTA, 5 mM sodium azide, 0.25 mM PMSF, 10 μM leupeptin, 10 μM pepstatin, 100 μg/ml trypsin inhibitor) and lysed with a glass/Teflon Potter-Elvehjem tissue grinder. An equal volume of buffer containing 0.5 M sucrose was added, and the material was rehomogenized and centrifuged for 5 min at 1,200 × gmax. The supernatant containing the microsomes was recovered, and KCl and disodium pyrophosphate were added to a final concentration of 0.6 M and 20 mM, respectively. The material was homogenized and mixed for 30 min at 4 °C. Following a final homogenization, the microsomes were centrifuged for 5 min at 200 × gmax and the supernatant was recovered. Finally, the microsomes were pelleted at 100,000 × gmax for 10 min, resuspended in storage buffer containing 10% sucrose, and snap-frozen in liquid nitrogen.

**Planar Lipid Bilayers**—Single channel recordings of recombinant InsP3R2 activities were performed by fusing microsomes into planar lipid bilayers. Bilayers were formed around a 150-μm-diameter hole in the wall of a Delrin cup using a 7:3 lipid mixture of phosphatidylethanolamine and phosphatidylcholine (50 mg/ml in decane; Avanti Polar Lipids, Alabaster, AL). The bilayer separated two pools (cis and trans). The microsomes were added to the cis-side of the bilayer. Standard solution contained 20 mM HEPES-Tris, pH 7.4, 1 mM EGTA, [Ca2+]free = 250 nm, and 220 mM CsCH3SO3 in the cis-chamber (20 mM, trans). Free calcium concentration was calculated using MaxChelator software. 2 μM InsP3 and 10 μM ryanodine were used. The trans pool was held at virtual ground. The channels were positively identified by their sensitivity to InsP3 and heparin. Open probability was determined by using the half-threshold crossing from 3-min records at 0 mM. Unitary currents were recorded using a conventional patch clamp amplifier (Axopatch 200B, Axon Instruments, Union City, CA). The current signal was digitized at 10 kHz with a 32-bit AD/DA converter (Digidata 1322A; Axon Instruments) and filtered at 1 kHz with a low pass eight-pole Bessel filter. Data acquisition, unitary current

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measurement, statistical analysis, and data processing were performed using commercially available software packages (pClamp V10.2, Axon Instruments and Origin, Microcal).

Data Presentation—Statistical comparisons were made using Student’s t test for paired and unpaired data, with statistical significance from wild-type control InsP3R2 channels set at \( p / 0.05 \). Bar graphs are presented as average open probabilities \( / / \) S.E. of \( n \) measurements, where \( n \) refers to the number of channels. All Western blot data and channel traces shown are representative of three or more identical experiments.

RESULTS

CaMKII Modulation of InsP3R2 Channel Activity Is Reversible with Phosphatase Treatment—Previous data from our laboratory have shown that the InsP3R2 and CaMKII form a macromolecular complex in cardiac ventricular myocytes and that CaMKII can modulate InsP3R2 channel activity (1). To investigate further the role of CaMKII in mediating InsP3R2 activity, we fused microsomes from Sf9 insect cells infected with a baculovirus coding for the rat InsP3R2 into planar lipid bilayers for channel analysis. In Fig. 1A, a single InsP3R2 channel is recorded under normal conditions \( / / \) \( 20 \text{ min} \). This treatment resulted in a decrease in the \( P_o \) to 0.09. The addition of protein phosphatases (10 units of PP1 and 50 ng of PP2A) and KN-93 \( (30 \mu \text{m}) \) was able to relieve CaMKII-mediated regulation of the channel activity, and the \( P_o \) returned to approximately starting level \( (0.33) \). The control experiment was performed by adding...
InsP$_3$R2 Channel Modulation by CaMKII Phosphorylation

CaMKII Phosphorylates the Full-length InsP$_3$R2 on Ser-150—To determine the specific residue(s) of the InsP$_3$R2 phosphorylated by CaMKII, we used expressed fragments of the InsP$_3$R2 in combination with in vitro CaMKII-dependent phosphorylation assays. Receptor subfragments spanning residues 1–1078, 1074–1640, 1635–2118, and 2114–2701 (Fig. 2) were expressed in COS-1 cells and immunoprecipitated with an amino-terminal antibody (T2NH, for 1–1078) or anti-FLAG antibodies for the 1–1078 construct showed significant incorporation of $^{32}$P, whereas no signal could be detected with the 1074–1640, 1635–2118, or 2114–2701 constructs. Lower panel, Western blotting of the membrane with T2NH (1–1078) or anti-FLAG (1074–1640, 1635–2118, and 2114–2701) antibody confirmed the success and uniformity of immunoprecipitations used in the phosphorylation reactions. Diagrams of the fragments used in this experiment are shown at the bottom with amino-terminal FLAG tags on fragments 1074–1640, 1635–2118, and 2114–2701.

A summary of the planar lipid bilayer results is shown in Fig. 1C. These data, together with the previous data from Bare et al. (1), support the hypothesis that CaMKII-mediated phosphorylation of the channel is responsible for the decrease in $P_0$ seen upon CaMKII addition. Additionally, these results indicate that the inhibitory site on the receptor is likely localized to the large cytoplasmic region of the protein oriented on the cis side of the bilayer membrane, as that was the side the kinase was added. Of note, addition of active CaMKII to the trans side of the bilayer had no effect on the channel properties (data not shown). This is consistent with our previous in vitro phosphorylation results indicating that a fragment encompassing the amino-terminal 1078 residues of the receptor is phosphorylated by CaMKII.

**Figure 2. CaMKII-dependent phosphorylation of InsP$_3$R2 (1–1078) fragment.** CaMKII phosphorylation assays performed using immunoprecipitations of InsP$_3$R2 subfragments expressed in COS-1 cells as substrates. Upper panel, with the addition of CaMKII, the 1–1078 construct showed significant incorporation of $^{32}$P, whereas no signal could be detected with the 1074–1640, 1635–2118, or 2114–2701 constructs. Lower panel, Western blotting of the membrane with T2NH (1–1078) or anti-FLAG (1074–1640, 1635–2118, and 2114–2701) antibody confirmed the success and uniformity of immunoprecipitations used in the phosphorylation reactions. Diagrams of the fragments used in this experiment are shown at the bottom with amino-terminal FLAG tags on fragments 1074–1640, 1635–2118, and 2114–2701.

all components present in the CaMKII activation mixture except the kinase to the bath solution during recording of an InsP$_3$R2 single channel (Fig. 1B). These had no effect on InsP$_3$R2 activity and suggest that the diminution of InsP$_3$R2 channel activity was a consequence of phosphorylation events mediated by CaMKII.

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CaMKII Phosphorylates the Full-length InsP$_3$R2 on Ser-150—To determine the specific residue(s) of the InsP$_3$R2 phosphorylated by CaMKII, we used expressed fragments of the InsP$_3$R2 in combination with in vitro CaMKII-dependent phosphorylation assays. Receptor subfragments spanning residues 1–1078, 1074–1640, 1635–2118, and 2114–2701 (Fig. 2) were expressed in COS-1 cells and immunoprecipitated with an amino-terminal antibody (T2NH, for 1–1078) or anti-FLAG antibody (for 1074–1640, 1635–2118, and 2114–2701). The immune complexes were then incubated with [$\gamma$-$^{32}$P]ATP and either preactivated CaMKII (500 units) or the CaMKII inhibitor KN-93 (10 $\mu$m), resolved on 7.5% SDS-PAGE, transferred to nitrocellulose membrane, and autoradiographed. Following autoradiography the membranes were Western blotted with either a InsP$_3$R2 amino-terminal (T2NH) or anti-FLAG antibodies to visualize the protein bands. As shown in Fig. 2, the fragment spanning amino acids 1–1078 was the only fragment that showed consistent $^{32}$P incorporation upon treatment with CaMKII. The incorporation of the $^{32}$P label in this fragment was blocked with KN-93 and is in agreement with our previous results demonstrating CaMKII-mediated phosphorylation of this span of amino acids (1). These results suggest that the only detectable CaMKII phosphorylation site on the InsP$_3$R2 is localized to the amino-terminal 1078 amino acids.

To elucidate further the site of CaMKII-dependent phosphorylation on the InsP$_3$R2, we used eight bacterially expressed subfragments spanning the amino-terminal 1078 residues. Fragments spanning residues 1–338, 1–546, 234–1078, and 320–1078 were subjected to in vitro kinase assays. Expression products from constructs spanning residues 1–338 and 1–546 showed significant incorporation of $^{32}$P after CaMKII treatment, whereas no signal could be detected with the 234–1078 and 320–1078 constructs (Fig. 3A). The incorporation of label for the 1–338 and 1–546 constructs was inhibited with 10 $\mu$m KN-93, demonstrating CaMKII-specific $^{32}$P incorporation. These results suggest that the site of CaMKII phosphorylation on the InsP$_3$R2 lies within the amino-terminal 234 residues. Some very weak label incorporation could be observed for the two fragments encompassed by residues 234–1078, and this was interpreted as nonspecific trapping of label although it cannot be ruled out that the fragments were misfolded and possible sites were inaccessible to CaMKII.

The localization of the phosphorylation site was further delineated by preparing amino-terminal deletions that preserve the NH$_2$-terminal antibody epitope (T2NH, amino acids 320–338) used for protein immunoprecipitation. These include residues 106–338, 134–338, 151–338, and 172–338. As shown in Fig. 3B, the 106–338 and 134–338 constructs showed robust incorporation of $^{32}$P after treatment with CaMKII, which was sensitive to inhibition with 10 $\mu$m KN-93. No significant signal could be detected with the CaMKII-treated 151–338 or 172–338 expression products. These results imply that the CaMKII phosphorylation site lies within amino acids 134–150 of the InsP$_3$R2.

Within this span of amino acids, there is one potential CaMKII site (either serine or threonine residue) located at Ser-150. This serine is part of an S-X-D amino acid motif (RVSLSDAAGN) in the InsP$_3$R2. This motif was shown to be the CaMKII-dependent phosphorylation site on the RyR2 (21). Mutagenesis of the serine 150 to alanine in the two fragments (1–338 and 134–338) resulted in no appreciable CaMKII-dependent $^{32}$P incorporation for either fragment (Fig. 3C). Site-directed mutagenesis was also performed to mutate the serine at residue 150 to alanine (S150A) in the full-length InsP$_3$R2 protein. Upon treatment with CaMKII, the InsP$_3$R2-S150A showed no $^{32}$P incorporation compared with the InsP$_3$R2-WT protein that could be phosphorylated with exogenously supplied CaMKII (Fig. 4). These results clearly identify Ser-150 as the CaMKII phosphorylation site on the InsP$_3$R2. Furthermore,
it can be concluded that at the levels of sensitivity of this assay, this is likely the only CaMKII phosphorylation site present on the InsP₃R₂ because mutagenesis of this site abolished CaMKII-mediated phosphorylation of the full-length protein.

**Homology of CaMKII Phosphorylation Sites**—Serine 150 is part of an S-X-D motif that was also found to be a target for CaMKII phosphorylation in the RyR₂ (21). The conservation of this region in the other isoforms of the InsP₃R and other CaMKII substrates (3, 21, 22) is shown in Table 1. Homology of the CaMKII phosphorylation site in the InsP₃R₂ with the RyR₂ and InsP₃R₁ and InsP₃R₃. Interestingly, Ser-150 is conserved as a threonine in the type 1 and type 3 InsP₃R homologues. We have observed that CaMKII can phosphorylate the InsP₃R₁ on the first 1081 amino acids (data not shown). This supports the hypothesis that Thr-150 of the InsP₃R₁ is a potential CaMKII phosphorylation site. This provides a potential mechanism of differential regulation between isoforms given that one is a serine and the other is a threonine. CaMKII has not been observed to show any preferential phosphorylation of either residue.

**CaMKII-dependent phosphorylation of bacterially expressed InsP₃R₂ fragments.** CaMKII phosphorylation assays were performed using immunoprecipitations of bacterially expressed InsP₃R₂ subfragments as substrates. A, with the addition of CaMKII, the 1–546 and 1–338 constructs showed significant incorporation of ³²P, whereas no signal could be detected with the 234–1078 and 320–1078 constructs. B, the 106–338 and 134–338 constructs showed significant incorporation of ³²P with the addition of CaMKII. No significant signal could be detected with the 151–338 or the 172–338 constructs (A and B, upper panels). Western blotting of the membrane with T2NH antibody confirmed the success and uniformity of immunoprecipitations used in the phosphorylation reactions (A and B, lower panels). Diagrams of the fragments used in this experiment are shown at the bottom. C, mutation of Ser-150 → Ala could abolish CaMKII-mediated phosphorylation of fragments 1–338-S150A and 134–338-S150A. Upper panel, fragments 1–338 and 134–338 not containing the mutation showed significant incorporation of ³²P when treated with CaMKII. Lower panel, Western blotting of the membrane with T2NH antibody confirmed the presence of similar protein amounts within samples used in the phosphorylation reactions.

**CaMKII can phosphorylate the full-length InsP₃R₂ on Ser-150.** Upper panel, CaMKII phosphorylation assays performed using full-length InsP₃R₂ expressed in COS-1 cells as substrates show that mutation of Ser-150 → Ala could abolish CaMKII-mediated phosphorylation of full-length InsP₃R₂-S150A. Full-length InsP₃R₂ not containing the mutation showed significant incorporation of ³²P when treated with CaMKII. Lower panel, Western blotting of the membrane with T2NH antibody confirmed the presence of similar protein amounts within samples used in the phosphorylation reactions.

**FIGURE 3.** CaMKII-dependent phosphorylation of bacterially expressed InsP₃R₂ fragments. CaMKII phosphorylation assays were performed using immunoprecipitations of bacterially expressed InsP₃R₂ subfragments as substrates. A, with the addition of CaMKII, the 1–546 and 1–338 constructs showed significant incorporation of ³²P, whereas no signal could be detected with the 234–1078 and 320–1078 constructs. B, the 106–338 and 134–338 constructs showed significant incorporation of ³²P with the addition of CaMKII. No significant signal could be detected with the 151–338 or the 172–338 constructs (A and B, upper panels). Western blotting of the membrane with T2NH antibody confirmed the success and uniformity of immunoprecipitations used in the phosphorylation reactions (A and B, lower panels). Diagrams of the fragments used in this experiment are shown at the bottom. C, mutation of Ser-150 → Ala could abolish CaMKII-mediated phosphorylation of fragments 1–338-S150A and 134–338-S150A. Upper panel, fragments 1–338 and 134–338 not containing the mutation showed significant incorporation of ³²P when treated with CaMKII. Lower panel, Western blotting of the membrane with T2NH antibody confirmed the presence of similar protein amounts within samples used in the phosphorylation reactions.

**FIGURE 4.** CaMKII can phosphorylate the full-length InsP₃R₂ on Ser-150. Upper panel, CaMKII phosphorylation assays performed using full-length InsP₃R₂ expressed in COS-1 cells as substrates show that mutation of Ser-150 → Ala could abolish CaMKII-mediated phosphorylation of full-length InsP₃R₂-S150A. Full-length InsP₃R₂ not containing the mutation showed significant incorporation of ³²P when treated with CaMKII. Lower panel, Western blotting of the membrane with T2NH antibody confirmed the presence of similar protein amounts within samples used in the phosphorylation reactions.

**it can be concluded that at the levels of sensitivity of this assay, this is likely the only CaMKII phosphorylation site present on the InsP₃R₂ because mutagenesis of this site abolished CaMKII-mediated phosphorylation of the full-length protein.**

**Homology of CaMKII Phosphorylation Sites**—Serine 150 is part of an S-X-D motif that was also found to be a target for CaMKII phosphorylation in the RyR₂ (21). The conservation of this region in the other isoforms of the InsP₃R and other CaMKII substrates (3, 21, 22) is shown in Table 1. Homology of the CaMKII phosphorylation site in the InsP₃R₂ with the RyR₂ and InsP₃R₁ and InsP₃R₃. Interestingly, Ser-150 is conserved as a threonine in the type 1 and type 3 InsP₃R homologues. We have observed that CaMKII can phosphorylate the InsP₃R₁ on the first 1081 amino acids (data not shown). This supports the hypothesis that Thr-150 of the InsP₃R₁ is a potential CaMKII phosphorylation site. This provides a potential mechanism of differential regulation between isoforms given that one is a serine and the other is a threonine. CaMKII has not been observed to show any preferential phosphorylation of either residue.

**Ser-150 Is the Site of CaMKII-dependent Modulation of the InsP₃R₂ Channel**—In addition to the InsP₃R₂-S150A nonphosphorylatable mutation, a InsP₃R₂-S150E phosphomimetic mutation changing Ser-150 to a glutamate (S150E) was made for use in planar lipid bilayer studies. Wild type and the two mutant (S150A and S150E) constructs were transiently transfected into COS-1 cells for expression of the proteins. Membrane fractions from the three samples were prepared for use in...
channel recordings from planar lipid bilayers. In Fig. 5, a sample recording from WT microsomes shows that upon CaMKII treatment, the open probability ($P_o$) of the channel is significantly decreased from 0.57 to 0.04. Conversely, the channel trace shown in Fig. 5B from InsP$_3$R2-S150A microsomes did not show any decrease in open probability when active CaMKII enzyme was added to the bath solution ($P_o = 0.58$ and 0.57, respectively), indicating that the channel was insensitive to the effect of CaMKII-dependent phosphorylation. Finally, Fig. 5C is a representative channel trace from InsP$_3$R2-S150E microsomes showing that this channel protein exhibits a constitutively low open probability ($P_o = 0.02$), thus mimicking an InsP$_3$R2 that has been phosphorylated by CaMKII. Furthermore, it confirms that replacing Ser-150 with a glutamate did not alter the ability of the receptor to form a functional tetrameric channel. Fig. 5D is a summary bar graph showing the open probability of the three InsP$_3$R2 channels with or without CaMKII treatment. There may be other CaMKII sites on the receptor that we were unable to detect; however, here we show that Ser-150 is a site that is phosphorylated by CaMKII and the site that is responsible for the channel modulation by CaMKII.

InsP$_3$R2 Is Phosphorylated in Vivo by CaMKII at Ser-150 in COS Cells and Neonatal Rat Ventricular Myocytes—In support of the planar lipid bilayer studies demonstrating the functional consequences of Ser-150 phosphorylation to channel activity, we examined whether the InsP$_3$R2 was phosphorylated in vivo as a consequence of CaMKII activity. COS 1 cells were transfected with either the WT or S150A or S150E mutants, with and without CaMKII and HDAC6C were also shown to illustrate the homology the InsP$_3$R2 Ser-150 site shares with these known CaMKII phosphorylation sites on other cardiac proteins.

TABLE 1

| Homology of CaMKII phosphorylation sites |
|-----------------------------------------|
| CaMKII phosphorylation site alignment with the three isoforms of the InsP$_3$R and from various species (mouse, rat, human, bovine) shows that Ser-150 of the type 2 receptor is conserved as a threonine in the type 1 and type 3 receptors. The region surrounding Ser-150/Thr-150 is also highly homologous in the three isoforms and among species. The CaMKII phosphorylation sites for human RyR2 and HDAC6 are also shown to illustrate the homology. |
| **RVTLDATGN: mlnS$_3$P$_3$R3** | **QVSVDAAH: hRyR2** |
| **RVTLDATGN: rlnS$_3$P$_3$R3** | **QHSLDQSSP: HDAC4** |
| **RVTLDATGN: hlnS$_3$P$_3$R3** | **RVTLDATGN: blnS$_3$P$_3$R3** |

Additionally, we examined the endogenous InsP$_3$R2 in acutely isolated neonatal rat ventricular myocytes infected with adenovirus expressing CaMKII to a and a dominant negative mutation (CaMKII$<$C:DN). Western blots (Fig. 6B) using Ser(P)-150-T2 reveal a strong phospho-InsP$_3$R2 signal in CaMKII$<$C infected cells and little if any signal in the cells expressing CaMKII$<$C:DN. Together these results demonstrate that phosphorylation of InsP$_3$R2 at S150 by CaMKII occurs in vivo.

DISCUSSION

The InsP$_3$R2 is localized predominantly to the nuclear envelope and to a lesser extent in the SR of cardiac myocytes (1, 23). InsP$_3$R2s are intracellular Ca$^{2+}$ release channels that play a role in the regulation of Ca$^{2+}$ signaling in the cardiac myocyte. Their role in the regulation of ET-1-induced positive inotropy in both atrial and ventricular myocytes has been recently well characterized, along with their part in a nuclear signal transduction cascade involving CaMKII-mediated activation of gene expression (2, 7, 23). Thus, CaMKII-dependent phosphorylation of the InsP$_3$R2 is an important method of physiological regulation of the channel in a cellular environment. This is the first report of the specific site of CaMKII-mediated phosphorylation of the InsP$_3$R2 and the functional affects of this post-translational modification on the channel activity. The results demonstrate that serine-150 of the rat InsP$_3$R2 is the site of modulation of the channel activity by CaMKII-dependent phosphorylation. Phosphorylation leads to inhibition of the InsP$_3$R2, and this functional effect can be abolished by mutation of Ser-150 to alanine. Furthermore, a phosphomimetic InsP$_3$R2 channel (S150E) exhibits a constitutively low open probability very similar to that of a WT channel phosphorylated by CaMKII.

Functional Consequences of CaMKII Phosphorylation of the InsP$_3$R2—Type 2 InsP$_3$R-mediated signals have a sigmoidal Ca$^{2+}$ dependence, meaning termination of this signal is not intrinsically controlled by a Ca$^{2+}$-dependent inactivation mechanism (15). Of significance in cardiac tissues where type 2 receptors are present, this sigmoidal Ca$^{2+}$ dependence makes the InsP$_3$R2 resistant to the RyR-mediated Ca$^{2+}$ signals driving the cardiac contractile cycle and allows InsP$_3$-dependent intracellular signaling cascades to operate independent of the global Ca$^{2+}$ fluxes associated with contraction. We show a potential mechanism of termination of InsP$_3$R2 calcium release by which CaMKII directly phosphorylates the InsP$_3$R2 at Ser-150 and inhibits channel activity, thus terminating InsP$_3$-mediated SR and nuclear Ca$^{2+}$ release.

CaMKII-mediated phosphorylation of InsP$_3$R2 most likely has different physiological effects based on which population of receptors is being phosphorylated. Data show that knock-out of InsP$_3$R2 in mouse myocytes abolished the positive inotropic and arrhythmogenic effects of ET-1 in cardiac myocytes (24) and the ET-1 induced HDAC5 nuclear translocation in ventric-
ular myocytes (2). This HDAC5 pathway seems to be isolated from the beat-to-beat global fluctuations in $[\text{Ca}^{2+}]_{\text{i}}$, creating an elegant system that is dependent on very local control of $[\text{Ca}^{2+}]_{\text{i}}$. InsP$_3$R$_2$s localized to the nuclear envelope release $\text{Ca}^{2+}$ into the nucleoplasm, activating CaMKII which can phosphorylate HDAC5 and cause its translocation out of the nucleus (2). Furthermore, it has been proposed that the activated CaMKII can feedback-inhibit the InsP$_3$R to close the channel and terminate the InsP$_3$-mediated release of $\text{Ca}^{2+}$ from the nuclear envelope into the nucleoplasm (1, 2). The results presented here strengthen this hypothesis and show that CaMKII feedback phosphorylates the InsP$_3$R$_2$ at Ser-150, and this phosphorylation results in a decrease of the channel open probability which is an important part of signaling pathway leading to hypertrophy and heart failure (2).

In heart failure, the InsP$_3$R$_2$ protein is up-regulated (25). Functionally, the majority of experimental evidence indicates that in cardiac myocytes the modest inotropic effect of the InsP$_3$R$_2$s also comes with the considerable ability to disrupt ECC at normal SR $\text{Ca}^{2+}$ load (23). Despite this, the up-regulation of the InsP$_3$R$_2$ seen in heart failure may be an attempt to preserve and enhance the inotropic response of ET-1 in this condition. In addition to the increased InsP$_3$R$_2$ protein levels, the levels of the RyR are decreased in heart failure (25). The increased InsP$_3$R$_2$ protein level may contribute to arrhythmogenesis and the mishandling of $\text{Ca}^{2+}$ regulation also seen in heart failure, or the InsP$_3$R$_2$s may create an environment where this increased expression in the SR can help potentiate $\text{Ca}^{2+}$-induced calcium release from the decreased population of RyRs.

FIGURE 5. Representative single channel recordings of WT, S150A, and S150E InsP$_3$R$_2$. A, for WT-InsP$_3$R$_2$ single channels, a channel $P_o$ of 0.57 was recorded. Active CaMKII enzyme was added to the bath solution and recording continued. This treatment resulted in a decrease in the $P_o$ of 0.04 after 20 min. B, for S150A channels, a channel $P_o$ of 0.58 was recorded. Active CaMKII enzyme was added to the bath solution and recording continued. This treatment resulted in no change in the recorded $P_o$ of the channel after 20 min. C, for S150E channels, a channel $P_o$ of 0.02 was recorded. A, B, and C were taken as representative windows having $P_o$ values reflecting the 10-min recordings used for the actual determination of $P_o$. Channel openings are shown as upward deflections from the zero current level. D, bar graphs summarize the planar lipid bilayer studies ($n = 5$ for all conditions; *, $p < 0.001$; error bars, S.E.)
InsP$_3$R2 Channel Modulation by CaMKII Phosphorylation

InsP$_3$R2s co-localize with RyRs in both atrial and ventricular cells and can modulate the activity of the RyR (7, 23, 26). InsP$_3$-induced calcium release can potentiate Ca$^{2+}$-induced calcium release from RyRs leading to enhanced systolic SR Ca$^{2+}$ release and positive inotropy. Due to the relatively low expression levels of InsP$_3$R2s compared with RyRs (27), SR Ca$^{2+}$ release from InsP$_3$R2s alone does not affect the beat-to-beat global Ca$^{2+}$ transient during action potential stimulation in the cell. However, it can influence RyR-mediated SR Ca$^{2+}$ release which does affect the global SR Ca$^{2+}$ release (7, 23, 26). Thus, it seems the effect of CaMKII-mediated phosphorylation of the SR-localized InsP$_3$R2s would have an indirect effect on global SR Ca$^{2+}$ release through the RyR.

Possible Mechanism of Inhibition—InsP$_3$ binds to a distinct region of the InsP$_3$R located at the amino terminus of each subunit, known as the ligand binding domain. This region is composed of an amino-terminal suppressor domain and an adjacent InsP$_3$-binding core (28–30). The suppressor domain reduces the affinity of InsP$_3$ for the InsP$_3$-binding core and is required for channel gating, as it was shown that removal of suppressor domain increased affinity for InsP$_3$ but did not form functional channels (31). It is possible that phosphorylation of Ser-150 localized within the suppressor domain could perturb InsP$_3$ binding to the InsP$_3$-binding core or that phosphorylation impedes the interactions necessary for channel gating, thus preventing ligand-induced activation.

In summary, we have identified Ser-150 of the InsP$_3$R2 as the target of CaMKII-mediated phosphorylation. This event results in the negative modulation of the InsP$_3$R2 receptors intrinsic calcium channel activity.

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