6674 *Biochemistry* **2009,** *48,* 6674–6683
DOI: 10.1021/bi900322a

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Protein Kinase C Isoforms Differentially Phosphorylate $Ca_v1.2$ α_{1c}[†]

Lin Yang,[‡] Darshan Doshi,[‡] John Morrow,[‡] Alexander Katchman,[‡] Xiang Chen,[‡] and Steven O. Marx*[,‡,§]

[‡]*Division of Cardiology, Department of Medicine and* [§]*Department of Pharmacology, College of Physicians and Surgeons, Columbia University, New York, New York 10032*

Received March 5, 2009; Revised Manuscript Received May 22, 2009

ABSTRACT: The regulation of Ca^{2+} influx through the phosphorylation of the L-type Ca^{2+} channel, $Ca_v1.2$, is important for the modulation of excitation−contraction (E−C) coupling in the heart. $Ca_v1.2$ is thought to be the target of multiple kinases that mediate the signals of both the renin-angiotensin and sympathetic nervous systems. Detailed biochemical information regarding the protein phosphorylation reactions involved in the regulation of $Ca_v1.2$ is limited. The protein kinase C (PKC) family of kinases can modulate cardiac contractility in a complex manner, such that contractility is either enhanced or depressed and relaxation is either accelerated or slowed. We have previously reported that Ser^{1928} in the C-terminus of α_{1c} was a target for PKCα, -ζ, and -ε phosphorylation. Here, we report the identification of seven PKC phosphorylation sites within the α_{1c} subunit. Using phospho-epitope specific antibodies to Ser^{1674} and Ser^{1928}, we demonstrate that both sites within the C-terminus are phosphorylated in HEK cells in response to PMA. Phosphorylation was inhibited with a PKC inhibitor, bisindolylmaleimide. In Langendorff-perfused rat hearts, both Ser^{1674} and Ser^{1928} were phosphorylated in response to PMA. Phosphorylation of Ser^{1674}, but not Ser^{1928}, is PKC isoform specific, as only PKCα, -βI, -βII, -γ, -δ, and -θ, but not PKCε, -ζ, and -η, were able to phosphorylate this site. Our results identify a molecular mechanism by which PKC isoforms can have different effects on channel activity by phosphorylating different residues.

Ca^{2+} homeostasis in the heart is maintained through the actions of channels and pumps, tuned to increase cardiac contractility in response to neurohormonal stimulation. Treatment of several major cardiovascular diseases, including hypertension, heart failure, and cardiac hypertrophy, is dependent, in part, upon the modulation of neurohormonal pathways. $Ca_v1.2$, the L-type, voltage-gated calcium (Ca^{2+}) channel present in the sarcolemma of cardiomyocytes, is required for excitation−contraction (E−C)[1] coupling in the heart (*1*). It is well established that $Ca_v1.2$ plays a key role in modulating cardiac function in response to classic signaling pathways, such as the renin-angiotensin system (RAS) and sympathetic nervous system (SNS) (*2*). Typically, these pathways alter cellular function by regulating kinases. $Ca_v1.2$ is thought to be the target of multiple kinases that mediate the signals of both the RAS and SNS.

The PKC family comprises 12 different isoforms, which are broadly classified according to their activation characteristics (*3*). In heart, PKC isoforms are activated by membrane receptors coupled to phospholipase C via Gq/G11 heterotrimeric G proteins (*4*, *5*). Phospholipases activated via G protein-coupled receptors result in hydrolysis of inositol phospholipids and production of diacylglycerol (DAG). Tumor-promoting phorbol esters act as an analogue of DAG. PKC isoforms are differentially responsive to neurohormones, suggesting that they play distinct and specific roles in cardiac function. Numerous agonists (phenylephrine, norepinephrine, ATP, carbachol, endothelin, angiotensin, and thrombin) accelerate phosphoinositide turnover in cardiac muscle, thereby leading to PKC activation (*6*). Angiotensin II and endothelin-1 have been reported to increase (*7*−*9*), decrease (*10*), or have no effect (*11*) on basal I_{Ca} in the heart. The coupling of α_{1A}-adrenoceptor with the Gq/11-PLC-PKC-CaMKII pathway potentiates I_{Ca}, whereas α_{1B}-adrenoceptor interacts with G_o, of which the $\beta\gamma$ complex might directly inhibit channel activity (*12*). Several direct activators of PKC have variable effects on $Ca_v1.2$, including activation, inhibition, and activation followed by inhibition in cardiomyocytes (*13*−*17*). Techniques that preserve the cytoplasmic environment appear to preserve the upregulation of I_{Ca} in response to agonists.

Although all PKC isoforms preferentially phosphorylate peptides with hydrophobic amino acids at position 1 C-terminal of the phosphorylated serine and basic residues at position −3, individual PKC isoforms have distinct optimal substrates (*18*). PKC, purified from avian brain, has been shown to phosphorylate the α_{1c} and β_{2a}

[†]This work was supported in part by National Institutes of Health Grant R01 HL68093 and the Arlene and Arnold Goldstein Family Foundation. D.D. is supported by a Glorney-Raisbeck Medical Student Fellowship from the New York Academy of Medicine and a Heritage Affiliate-American Heart Association Medical Student Fellowship. S.O.M. is an Established Investigator of the American Heart Association.

*To whom correspondence should be addressed: 630 W. 168th St., New York, NY 10032. E-mail: sm460@columbia.edu. Phone: (212) 305-0271. Fax: (212) 342-0475.

[1]Abbreviations: E−C, excitation−contraction; RAS, renin-angiotensin system; SNS, sympathetic nervous system; PKC, protein kinase C; PS, phosphatidylserine; PMA, phorbol 12-myristate 13-acetate; IP, immunoprecipitation; PKD, protein kinase D; GST, glutathione *S*-transferase; Bis, bisindolylmaleimide; Cal, calyculin; WT, wild type; SDS, sodium dodecyl sulfate.

 Published on Web 06/15/2009

subunits in vitro (*19*). A systematic study of the phosphorylation of α_{1c} by different PKC isoforms has not been completed. Several studies have suggested that the N-terminus of α_{1c} is important for PKC upregulation of channel function (*20*, *21*). Phosphorylation of α_{1c} Thr^{27} and Thr^{31} was proposed, on the basis of electrophysiological studies utilizing heterologous expression of mutant channels, to mediate PKC-induced inhibition of channel activity (*22*). No biochemical evidence exists for the phosphorylation of these residues in cells or in the heart. Recently, we reported that α_{1c} Ser^{1928} was phosphorylated by PKCα, PKCε, and PKCζ (*23*). Here, we demonstrate the PKC phosphorylation of several targets within the α_{1c} protein, in an isoform specific manner. We demonstrate that the phosphorylation occurs in response to a PKC activator in a heterologous expression system and in cardiac myocytes. The results suggest that the α_{1c} subunit can be differentially regulated by the different PKC isoforms, based upon phosphorylation of specific residues.

EXPERIMENTAL PROCEDURES

cDNA Clones and Site-Directed Mutagenesis. The rabbit α_{1c} subunit (NCBI accession number X15539) and the β_{2a} subunit (NCBI accession number X64297) in pcDNA3 (Invitrogen) were used for HEK cell expression. The preparation of the rabbit α_{1c}−GST fusion proteins was described previously (*23*). Site-directed mutagenesis was performed using the QuikChange XL kit (Stratagene). All clones were sequenced on both strands prior to use. Transfections into HEK293 cells were performed with Lipofectamine 2000 (Invitrogen).

Preparation of Phospho-Epitope Specific Antibodies. The general α_{1c}, α_{1c} phospho-Ser^{1928}, phospho-Ser^{528}, and phospho-Ser^{533} antibodies have been previously described (*23*, *24*). The phospho-Ser^{1674} (pS1674) antibody was prepared at Zymed utilizing the peptide NH_2-CEQGLVGKPpSQRN-COOH. The phospho-PKC substrate antibody was purchased from Cell Signaling Technology (catalog number 2261).

PKC Kinase Assay. For PKC kinase reactions, samples were washed twice with PKC washing buffer [for conventional PKCs, 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, and 100 μM $CaCl_2$; for novel and atypical PKCs, 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, and 100 μM EGTA]. Conventional PKC kinase assays were performed in 15 μL of phosphorylation buffer containing 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 100 μM $CaCl_2$, 1 mg/mL phosphatidylserine (PS), 200 μg/mL DAG, and 100 μM ATP. Novel and atypical PKC isoform phosphorylation assays were performed with an identical buffer except the Ca^{2+} was replaced with 100 μM EGTA. [γ-^{32}P]ATP (5 μCi) was added to the assay buffer to radiolabel the substrates of the kinase assay as indicated. Phosphorylation reactions, which were optimized for individual PKC isoforms (Panvera, Invitrogen), were performed for 10−30 min at 30 °C. Samples were size-fractionated via SDS−PAGE, extensively washed, stained with Coomassie, fixed, and dried. [γ-^{32}P]ATP was detected using autoradiography. All in vitro kinase assays were repeated at least three times.

PKD Kinase Assay. Samples were washed twice with PKD washing buffer containing 12.5 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 0.5 mM Na_3VO_4, 5 mM β-glycerophosphate, and 0.01% Triton X-100. The PKD assay was conducted in phosphorylation buffer by adding 2.5 mM DTT, 100 μM ATP, and 100 ng of PKD1 in washing buffer at 30 °C for 10−30 min.

Cardiac Perfusion. All animal care and procedures were approved by Columbia University College of Physicians and Surgeons Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health and institutional guidelines. Rats were injected with heparin and then anesthetized with pentobarbitol. The hearts were rapidly excised and placed in ice-cold Tyrode solution containing 134 mM NaCl, 5.4 mM KCl, 1.0 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, and 2 mM $CaCl_2$ (pH adjusted to 7.4 with NaOH). The aorta was cannulated and mounted on a Langendorff perfusion apparatus. The hearts were perfused for 5 min with Tyrode solution, followed by perfusion for 15 min with Tyrode solution containing 50 nM calyculin, 0.5 μM PMA and 50 nM calyculin, 0.5 μM 4α-phorbol and 50 nM calyculin, or a control solution (without PMA and calyculin). Perfusions were conducted at 36 °C.

Preparation of Heart Lysates. PKCα-overexpressing transgenic (C57), PKCα knockout (FVB), and corresponding littermate control hearts were obtained from 12-month-old mice (*25*). Hearts were homogenized in 1% Triton 100/RIPA buffer containing 1% (v/v) Triton X-100, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 10 mM EGTA, phosphatase inhibitor cocktail, and protease inhibitors (complete mini-tablet, calpain I and II inhibitors, Roche).

Immunoblots. Proteins were transferred to a nitrocellulose membrane and probed with the phospho specific and general antibodies, followed by anti-rabbit HRP-conjugated secondary antibody and ECL (Pierce). Detection was performed with a CCD camera (Carestream). Image quantification was performed using ImageQuant.

Immunoprecipitations. Immunoprecipitations were performed overnight in a modified RIPA buffer as previously described (*23*).

Statistical Analysis. Bar graphs with error bar data show means ± standard deviations. The sample size was at least three in all cases. Statistical analysis was performed with a Student's unpaired *t* test.

RESULTS

The brain and liver contain virtually all PKCs, but most other tissues express only certain PKC isoforms. It is known that different isoforms mediate diverse cellular responses, defined by different resting and stimulus-induced subcellular localization and different target substrates, based upon optimal phosphorylation consensus sequences (*26*). Cardiomyocytes coexpress conventional (PKCα), novel (PKCδ and PKCε), and atypical (PKCλ) isoforms; conventional PKCβ has also been variably detected by some investigators. Our prior work established that α_{1c} Ser^{1928} was phosphorylated by PKCα, PKCε, and PKCζ. We also showed that PKCα, but not PKCε and PKCζ, phosphorylated unidentified residue(s) within a GST fusion protein containing rabbit α_{1c} subunit amino acid residues 1509−1905 (*23*).

We utilized a panel of GST fusion proteins that encompassed all major intracellular regions as substrates for in vitro PKC phosphorylation. The small intracellular loops between transmembrane segments (S2−S3 and S4−S5) do not contain consensus PKC phosphorylation sites and were thus not included in the panel. The α_{1c}-containing GST fusion proteins were differentially phosphorylated by PKC isoforms (Figure 1). Many of the conventional PKC isoforms, namely, PKCα, PKCβI, PKCβII, and PKCγ, all phosphorylated the GST fusion protein I−II loop and residues 1509−1905 and 1906−2170. PKCα and PKCγ also phosphorylated GST fusion protein 70−154. The novel PKC isoforms phosphorylated the GST fusion proteins to

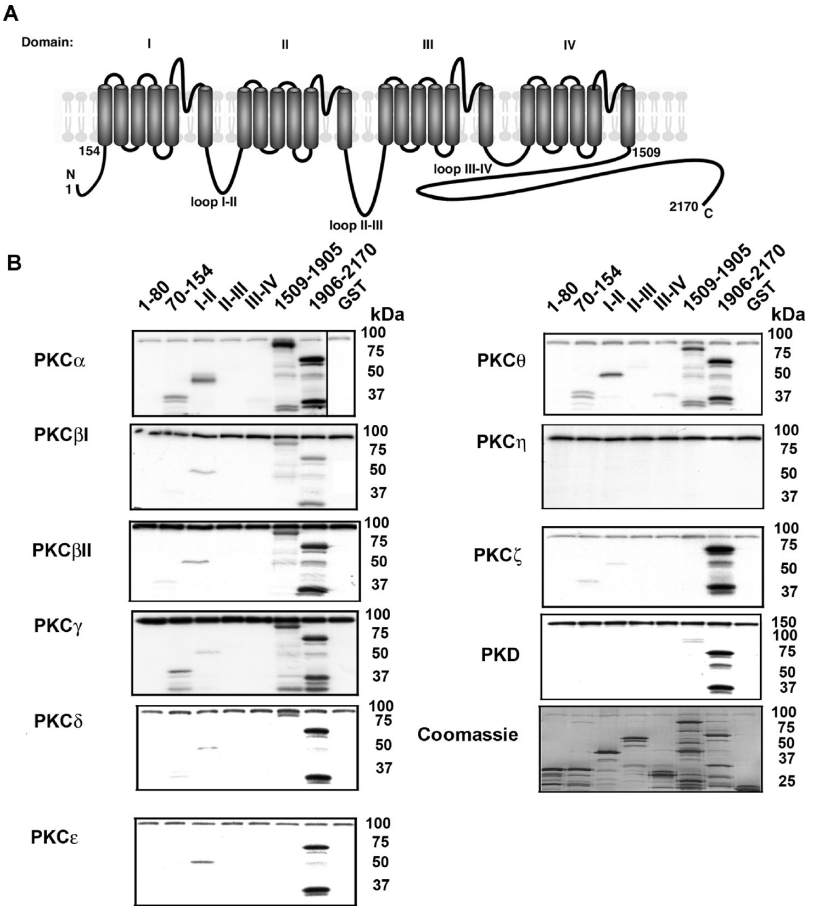

FIGURE 1: PKC isoforms phosphorylate the α_{1c} subunit. (A) Schematic of the α_{1c} subunit with the intracellular segments used to construct GST fusion proteins. (B) α_{1c} subunit−GST fusion proteins bound to glutathione-Sepharose were subjected to PKC kinase reaction with [γ-^{32}P]ATP. The amount of PKC isoform used for each kinase assay was normalized on the basis of the number of moles of phosphate transferred to test substrate. The amount of GST fusion protein utilized is shown in the Coomassie-stained gel. The bands at ~80−100 kDa are autophosphorylated PKC. All blots are representative of three or more similar experiments.

different extents. PKCθ phosphorylated 1906−2170, 1509−1905, the I−II loop, and 70−154. PKCδ phosphorylated 1906−2170 and 1509−1905 and to a modest extent the I−II loop. PKCε phosphorylated 1905−2170 and the I−II loop, but not 1509−1905. PKCη did not phosphorylate any GST fusion protein. The atypical PKC isoform, PKCζ, phosphorylated GST fusion protein 1905−2170, but not 1509−1905, and only weakly phosphorylated 70−154 and the I−II loop. Protein kinase D (PKD), which is activated by PMA and is downstream of PKC, primarily phosphorylated the 1906−2170 fusion protein. These results suggest that the individual PKC isoforms can phosphorylate distinct regions within the α_{1c} subunit. The differential phosphorylation of the α_{1c} subunit by PKC isoforms may represent an important regulatory control mechanism for fine-tuning the L-type Ca^{2+} channel response to distinct neurohormonal stimulation.

Identifying Phosphorylated Residues in the N-Terminus and the I−II Loop. Several studies have suggested that the N-terminus of α_{1c} is important for PKC upregulation of channel function (*20*, *21*). Phosphorylation of α_{1c} Thr^{27} and Thr^{31} was proposed, on the basis of electrophysiological studies utilizing heterologous expression of mutant channels, to mediate PKC-induced inhibition of channel activity (*22*). Both of these residues are within GST fusion protein 1−80, which was not phosphorylated by any of the tested PKC isoforms in the in vitro kinase assays (Figure 1), although it is conceivable that the folding of the GST fusion protein is different in the full-length channel. GST fusion protein 70−154 was phosphorylated by several PKC isoforms, although in comparison to GST fusion protein 1905−2170, which is predominantly phosphorylated on a single residue (Ser^{1928}) (*23*), the amount of [γ-^{32}P]ATP incorporation was significantly smaller. This suggests that the equivalent of less than one site is phosphorylated within the 70−154 fusion protein. To test which site(s) was phosphorylated by PKCα, we created Ala substitution mutants at amino acid residues 107−109 (SST to AAA), 124−126 (STT to AAA), and 138 (T to A) and expressed the fusion proteins. For all three mutant GST fusion proteins, no significant change in the modest amount of phosphorylation was detected (data not shown). This suggests that multiple sites are weakly phosphorylated in this region.

An examination of the amino acid sequence within the I−II loop revealed several potential phosphorylation sites. We created, by site-directed mutagenesis, single and double Ala substitution mutants of the potential PKC phosphorylation residues within the I−II loop (Figure 2A). The level of radiolabeling of the GST I−II loop fusion protein was reduced with each of the single mutants and was nearly completely abrogated by the double Ala substitution of Ser^{528} and Ser^{533} (Figure 2B). We previously developed phospho-epitope specific antibodies for Ser^{528} and Ser^{533} (*24*), which were designed to report the phosphorylation of Ser^{528} (pS528) and Ser^{533} (pS533), respectively, by protein kinase G (PKG). The antibodies detected the appropriate phosphorylated residue (Figure 2C,D). The pS533 antibody weakly recognized the I−II loop under nonphosphorylated conditions, as

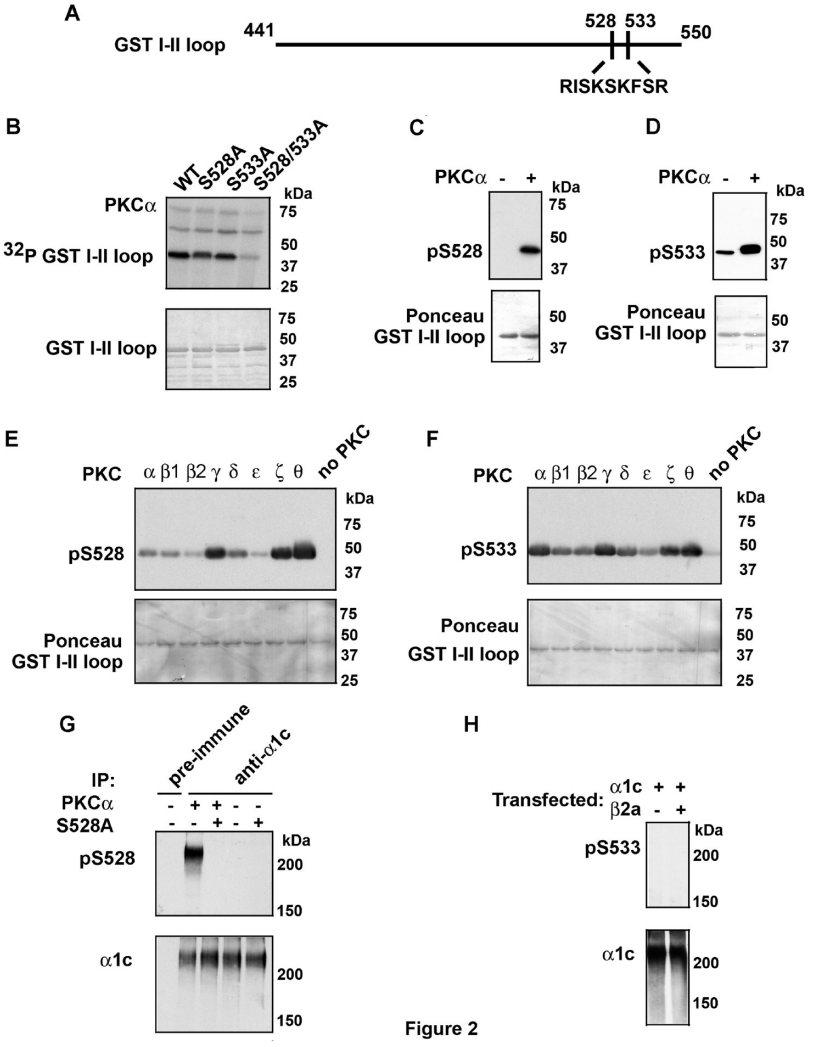

FIGURE 2: PKC phosphorylates α_{1c} Ser^{528} and Ser^{533}. (A) Schematic demonstrating the PKC phosphorylation sites in the α_{1c} I−II loop. (B) The top panel shows an autoradiogram of PKCα in vitro kinase reaction performed with [γ-^{32}P]ATP and GST-fused WT, S528A, S533A, and S528A/S533A I−II loop. PKC phosphorylated Ser^{528} and Ser^{533}. The bottom panel shows Coomassie staining of an autoradiogram demonstrating the amount of fusion protein used. (C and D) In the top panels, the WT GST fusion protein was phosphorylated with PKCα, size-fractionated on a SDS−polyacrylamide gel, transferred to nitrocellulose, and immunoblotted using anti-phospho-Ser^{528} and -Ser^{533} antibodies (pS528 and pS533, respectively). PKC phosphorylates Ser^{528} and Ser^{533} in the GST fusion protein I−II loop. The bottom panels show Ponceau staining which indicates equivalent loading of GST fusion proteins. (E and F) pS528 and pS533 immunoblots of in vitro kinase reactions of eight PKC isoforms. The bottom panels show Ponceau staining which indicates equivalent loading of GST fusion proteins. (G) Extracts from HEK cells transfected with WT or S528A were prepared, followed by preimmune or α_{1c} immunoprecipitation and PKCα kinase reaction as indicated. Samples were size-fractionated on a SDS−polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with anti-phospho-Ser^{528} antibody (top panel) or α_{1c} antibody (bottom panel). (H) Extracts from HEK cells transfected with WT α_{1c}, in the presence or absence of β_{2a} subunit, were prepared, followed by α_{1c} immunoprecipitation and PKCα kinase reaction as indicated. Samples were size-fractionated on a SDS−polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with anti-phospho-Ser^{533} antibody (top panel) or α_{1c} antibody (bottom panel). All blots are representative of three or more similar experiments.

previously reported (*24*). Having validated the specificity of the pS528 and pS533 antibodies, as reagents for tracking PKC phosphorylation, we used them to examine the phosphorylation of the GST I−II loop fusion protein by the different PKC isoforms. Consistent with our results using [γ-^{32}P]ATP incorporation (Figure 1B), we found that both Ser^{528} and Ser^{533} were phosphorylated by multiple PKC isoforms (Figure 2E,F). The specificity of the antibodies is demonstrated by the lack of significant signal in the nonphosphorylated lanes (right-most lanes, Figure 2E,F). These results suggest that Ser^{528} and Ser^{533} account for the PKCα phosphorylation of the GST fusion protein I−II loop (Figure 1B) and are potential PKC phosphorylation sites within the full-length α_{1c} subunit.

Having validated the specificity of the antibodies in tracking PKC phosphorylation of the I−II loop, we used them to examine phosphorylation of full-length recombinant α_{1c}, coexpressed with the β_{2a} subunit in HEK293 cells. Recombinant channels were immunoprecipitated by an anti-α_{1c} antibody from HEK cell extracts and subjected to the PKCα in vitro kinase assay. In contrast to the GST fusion proteins, Ser^{528} (Figure 2G), but not Ser^{533} (Figure 2H), demonstrated significant PKC phosphorylation. The specificity of Ser^{528} phosphorylation was demonstrated by the lack of a pS528 signal in the preimmune serum lane, in the Ala-substituted α_{1c} lane, and in the lanes to which PKCα had not been added (Figure 2G). The lack of Ser^{533} phosphorylation in the full-length recombinant channel compared to the GST I−II loop fusion protein may be due to the coexpression of the β_{2a} subunit, which may sterically block access to PKC and/or change the I−II loop conformation. The α-interaction domain (AID) does not overlap with the phosphorylation sites on the I−II loop.

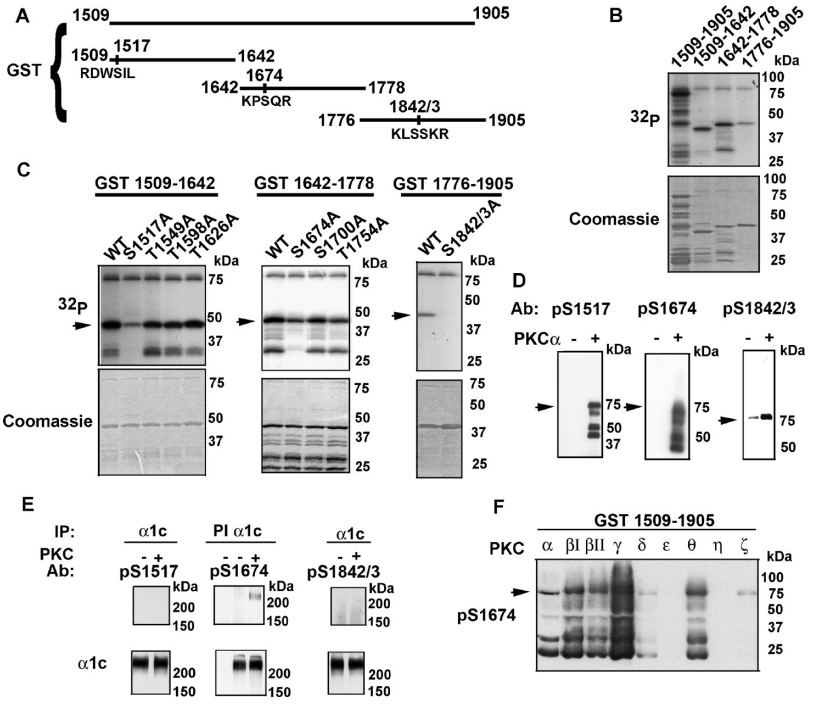

FIGURE 3: PKC phosphorylates α_{1c} Ser^{1517}, Ser^{1674}, and $Ser^{1842/1843}$. (A) Schematic demonstrating the PKC phosphorylation sites within the 1509−1905 GST fusion protein. (B) The top panel shows the autoradiogram of the PKCα in vitro kinase reaction performed with [γ-^{32}P]ATP and GST-fused 1509−1905, 1509−1642, 1642−1778, and 1776−1905. The bottom panel shows Coomassie staining of the autoradiogram demonstrating the amount of fusion protein used. (C) The top panels show autoradiograms of PKCα in vitro kinase reactions performed with [γ-^{32}P]ATP and WT and Ala-substituted GST-fused 1509−1642, 1642−1778, and 1776−1905 proteins. Arrowheads indicate full-length GST fusion protein; lower bands are truncated GST proteins. The bottom panel shows Coomassie staining of the autoradiogram demonstrating the amount of fusion protein used. (D) WT GST-fused 1509−1905 fusion proteins were phosphorylated with PKCα, size-fractionated on a SDS−polyacrylamide gel, transferred to nitrocellulose, and immunoblotted using anti-phospho-Ser^{1517}, -Ser^{1674}, and -$Ser^{1842/1843}$ antibodies (pS1517, pS1674, and pS1842/43, respectively). PKCα phosphorylates Ser^{1517}, Ser^{1674}, and $Ser^{1842/1843}$. (E) Extracts from HEK cells transfected with WT α_{1c} and β_{2a} subunits were prepared, followed by preimmune (PI) or α_{1c} immunoprecipitation and PKCα kinase reaction as indicated. Samples were size-fractionated on a SDS−polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with anti-phospho-Ser^{1517}, -Ser^{1674}, and -$Ser^{1842/1843}$ antibodies (top panel) or α_{1c} antibody (bottom panel). (F) pS1674 immunoblot of in vitro kinase reactions of eight PKC isoforms. All blots are representative of three or more similar experiments.

We found that Ser^{533} was not phosphorylated even in the absence of the β_{2a} subunit (Figure 2H), suggesting that the I−II loop adopts a different, more inaccessible conformation in the full-length channel compared to the GST fusion protein.

Identifying Phosphorylated Residues in the GST 1509−1905 Fusion Protein. We divided the 1509−1905 GST fusion protein into three fragments [1509−1642, 1642−1778, and 1776−1905 (Figure 3A)] to facilitate identification of the phosphorylation site(s), expressed each as a GST fusion protein in *Escherichia coli*, and purified the protein on glutathione-Sepharose. The purified fusion proteins were subjected to in vitro kinase assays with [γ-^{32}P]ATP. All three fusion proteins were labeled in the in vitro kinase assay by PKCα; however, the 1776−1905 protein demonstrated less ^{32}P incorporation than 1509−1642 and 1642−1778 (Figure 3B). An examination of the amino acid sequence within each of these GST fusion proteins revealed several potential phosphorylation sites. We created, by site-directed mutagenesis, single Ala substitution mutants of the potential PKC phosphorylation residues. The level of radiolabeling of the 1509−1642 GST fusion protein was reduced by Ala substitution of Ser^{1517}, as demonstrated by the lack of phosphorylation of the truncated GST fusion products and significant reduction of the level of phosphorylation of the full-length product (Figure 3C). In a similar fashion, we found that a single Ala substitution of Ser^{1674} substantially reduced the level of phosphorylation of 1642−1778 and that a double Ala substitution of Ser^{1842} and Ser^{1843} abrogated phosphorylation of 1776−1905. Ala substitution of Ser^{1700} and Thr^{1754} did not substantially affect incorporation of phosphate into the GST fusion protein. Therefore, our results suggest that Ser^{1517}, Ser^{1674}, Ser^{1842}, and Ser^{1843} are phosphorylated by PKCα within the GST 1509−1905 fragment. Mutation of these sites within GST 1509−1905 did not completely abrogate phosphorylation but reduced by >80% the level of ^{32}P incorporation (data not shown), suggesting that other, potentially minor, sites within this fragment are unidentified.

We developed three phospho-epitope specific antibodies, designed to report the phosphorylation of Ser^{1517} (pS1517), Ser^{1674} (pS1674), and $Ser^{1842/1843}$ (pS1842/3). The antibodies detected the appropriate phosphorylated residue (Figure 3D). The pS1842/3 antibody weakly recognized the full-length GST 1509−1905 fusion protein under nonphosphorylated conditions.

Having validated the specificity of these antibodies, we used them to examine phosphorylation of full-length recombinant α_{1c}, coexpressed with the β_{2a} subunit, in HEK293 cells. Recombinant channels were immunoprecipitated by an anti-α_{1c} antibody from HEK cell extracts and subjected to the PKCα in vitro kinase assay. In contrast to the GST fusion proteins, Ser^{1674}, but not Ser^{1517} or Ser^{1842}/Ser^{1843}, demonstrated significant PKCα phosphorylation. These results suggest that PKCα phosphorylates Ser^{1674} in full-length recombinant α_{1c}. The lack of phosphorylation of Ser^{1517} by PKCα in the full-length channel may be due to lack of accessibility of PKCα to Ser^{1517} in the full-length channel (Figure 3E).

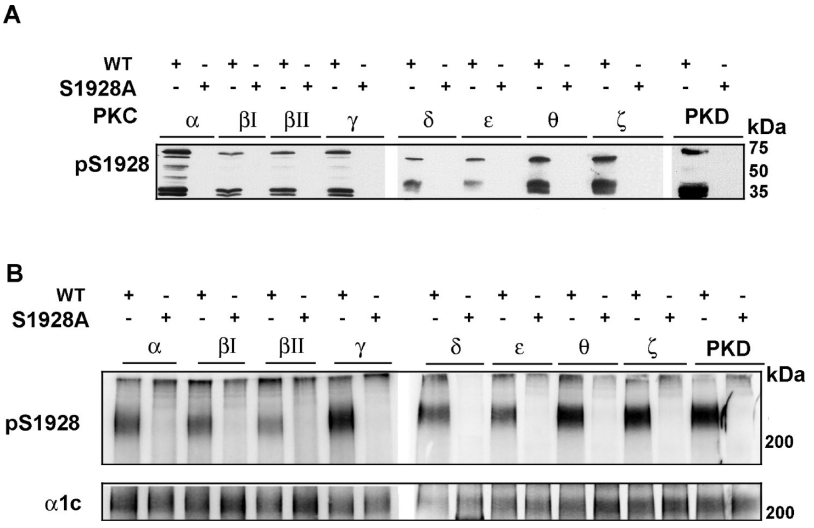

FIGURE 4: PKC isoforms phosphorylate α_{1c} Ser[1928]. (A) GST fusion proteins (WT and S1928A 1906−2170 fragment) were phosphorylated by PKC isoforms and PKD, size-fractionated, transferred to nitrocellulose, and immunoblotted with a phospho specific antibody recognizing phosphorylated Ser[1928] (pS1928). (B) Extracts from HEK cells transfected with WT or S1928A were prepared, followed by α_{1c} immunoprecipitation and kinase reaction by the different PKC isoforms and PKD. Samples were size-fractionated on a SDS−polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with anti-phospho-Ser1928 antibody (top panel) or α_{1c} antibody (bottom panel).

PKC isoforms can differentially phosphorylate GST 1509−1905; specifically, we showed that PKCα, PKCβI, PKCβII, PKCγ, PKCδ, and PKCθ, but not other PKC isoforms, can substantially phosphorylate GST 1509−1905 (Figure 1B) in an in vitro kinase assay. To test whether phosphorylation of Ser[1674] was PKC isoform specific, we performed an in vitro kinase assay for each PKC isoform (equivalent specific activity of 1500 nmol of phosphate transferred to substrate per minute per milligram of protein) and detected phosphorylation using the pS1674 antibody. We found that PKCα, -βI, -βII, -γ, and -θ phosphorylated Ser[1674]; PKCδ and PKCζ very weakly phosphorylated Ser[1674], and PKCε and PKCη did not phosphorylate Ser[1674]. These results demonstrate that α_{1c} Ser[1674] is differentially phosphorylated by PKC isoforms (Figure 3F).

Ser[1928] Is Phosphorylated by Conventional, Novel, and Atypical PKC Isoforms and PKD. We have previously reported that PKCα, PKCε, and PKCζ phosphorylated the C-terminus of α_{1c} at residue Ser[1928] (*23*). In Figure 1, we showed that PKD and all tested PKC isoforms except PKCη phosphorylated GST 1906−2170. To demonstrate whether Ser[1928] was phosphorylated by the other PKC isoforms, we utilized a phospho-epitope specific antibody developed to specifically detect Ser[1928] phosphorylation (*23*). Prominent immunoreactive bands were detected (with a range of mobilities corresponding to the GST-fused full-length protein as well as truncated/proteolytic fragments) using eight PKC isoforms and PKD (Figure 4A). No anti-phospho-Ser[1928] antibody immunoreactivity was detected when a single Ala substitution of Ser[1928] was introduced into the GST fusion protein.

Having determined that PKD and these PKC isoforms can phosphorylate Ser[1928] in GST fusion proteins, we next asked whether these kinases can phosphorylate Ser[1928] in full-length α_{1c}. We coexpressed β_{2a} and WT or Ala-substituted Ser[1928] α_{1c} in HEK cells. α_{1c} immunoprecipitates were subjected to immune complex kinase assays with PKD and PKC isoforms. PKD and PKC isoforms α, β1, βII, γ, δ, ε, θ, and ζ phosphorylated Ser[1928] in full-length α_{1c} (Figure 4B), thus indicating that Ser[1928] is a target for conventional, novel, and atypical PKC isoforms.

α_{1c} Ser[1674] and Ser[1928] Are Phosphorylated by PKC in HEK Cells. We assessed α_{1c} phosphorylation in HEK cells transfected with WT or the Ala-substituted Ser[528], Ser[1674], or Ser[1928] form, in the absence or presence of overexpressed PKCβI. Incubation of HEK cells transfected with α_{1c} and β_{2a} with PMA, prior to lysis, led to phosphorylation of Ser[1674] and Ser[1928] (Figure 5B,C), but not Ser[528] (Figure 5A). The PMA-induced phosphorylation of Ser[1674] was inhibited by bisindolylmaleimide (Bis), indicating that the phosphorylation was mediated by PKC (Figure 5B). The level of PMA-induced phosphorylation of Ser[1674] and Ser[1928] was increased by the overexpression of PKCβ1 in HEK cells (Figure 5B,C). In contrast, PMA-induced Ser[528] phosphorylation was not detected with the endogenous PKC isoforms expressed in HEK cells (data not shown) or after PKCβI overexpression (Figure 5A). Taken together, these results suggest that in HEK cells, Ser[1674] and Ser[1928] can be phosphorylated in a cellular context.

α_{1c} Ser[1674] Is Phosphorylated by PKC in Cardiomyocytes. We have previously shown that α_{1c} Ser[1928] is phosphorylated by PKC in cardiomyocytes (*23*). We tested whether Ser[528], which is not PKC phosphorylated in HEK cells (Figure 5A) but can be phosphorylated by PKC isoforms in vitro, and Ser[1674] could be phosphorylated by PKC isoforms in cardiomyocytes. To induce PKC phosphorylation, we mounted rat hearts on a Lagendorff apparatus and perfused through the aortic root for 15 min calyculin A, calyculin A and PMA, or calyculin A and 4α-phorbol (which does not activate PKC). The hearts were then frozen in liquid nitrogen and extracts prepared. To ensure that under these conditions infusion of PMA induced PKC activation and subsequent phosphorylation of targets within cardiomyocytes, we first examined the phosphorylation of multiple targets using a phospho-Ser PKC substrate antibody, which detects many cellular proteins only when phosphorylated at serine residues surrounded by Arg or Lys at the −2 and 2 positions and a hydrophobic residue at the 1 position. PMA (Figure 6A), but not 4α-phorbol (Figure 6B), increased the level of phosphorylation of many PKC targets in the heart, assessed using the PKC phospho-Ser antibody. Of six hearts treated with PMA, we excluded two hearts because a significant increase in phosphoproteins was not

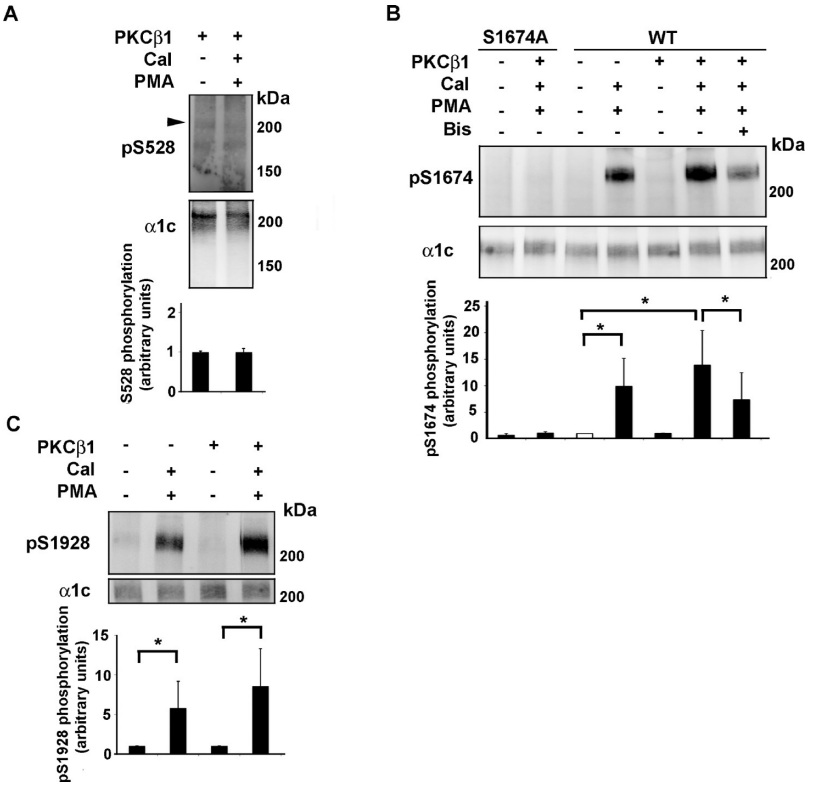

FIGURE 5: Reconstitution of PMA- and PKC-mediated phosphorylation of Ser^{1674} and Ser^{1928} in HEK cells. (A) Recombinant WT α_{1c} was transiently coexpressed with β_{2a} and PKCβ1 in HEK293 cells. Cells were exposed to PMA (1 μM) and calyculin A (Cal, 50 nM) for 10 min. Bisindolylmaleimide (Bis, 0.5 μM) was preincubated for 1 h at 37 °C. Cells were harvested 24−48 h after transfection and lysed in the presence of phosphatase inhibitors. Lysates were size-fractionated on a SDS−polyacrylamide gel, transferred to nitrocellulose membranes, and blotted with anti-phospho-Ser^{528} [pS528 (top panel)] or α_{1c} (middle panel) antibodies. The arrowhead denotes the size of the anticipated phosphorylated band. Representative of three similar experiments. The bottom panel shows activation of PKC by PMA had no effect on Ser^{528} phosphorylation. Mean ± standard deviation. (B) Recombinant WT or Ala-substituted Ser^{1674} (S1674A) α_{1c} was transiently coexpressed with β_{2a} in HEK293 cells, in the absence or presence of PKCβ1 as indicated. The methodology is identical to that described for panel A except nitrocellulose membranes were blotted with anti-phospho-Ser^{1674} [pS1674 (top panel)] or α_{1c} (middle panel) antibodies. The specificity of the pS1674 antibody is shown using the S1674A α_{1c} mutant. Representative of three similar experiments. The bottom panel shows activation of PKC by PMA increased the level of phosphorylation of α_{1c} Ser^{1674}. Phosphorylation of Ser^{1674} was caused by PKC because bisindolylmaleimide (Bis) prevented Ser^{1674} phosphorylation. (C) Recombinant WT or Ala-substituted Ser^{1928} (S1928A) α_{1c} was transiently coexpressed with β_{2a} in HEK293 cells, in the absence or presence of PKCβ1 as indicated. The methodology is identical to that described for panel A except nitrocellulose membranes were blotted with anti-phospho-Ser^{1928} [pS1928 (top panel)] or α_{1c} (middle panel) antibodies. Representative of three similar experiments. The bottom panel shows activation of PKC by PMA increased the level of phosphorylation of α_{1c} Ser^{1928}. The asterisk indicates $p < 0.05$.

observed (data not shown). α_{1c} immunoprecipitates of the untreated and calyculin A-, PMA-, and 4α-phorbol-treated heart extracts were probed with pS528 and pS1674 antibodies. Exposure of the heart to the combination of PMA and calyculin A induced phosphorylation of Ser^{1674} ($p = 0.02$, $n = 4$), whereas Ser^{528} demonstrated only a modest increase in signal (p = NS, $n = 4$). Calyculin A and the combination of 4α-phorbol and calyculin A had no effect on the phosphorylation of Ser^{1674} (Figure 6D).

To further support the idea that Ser^{1674} is phosphorylated in heart, we studied the hearts from PKCα knockout mice and transgenic mice overexpressing PKCα (*25*). There are no compensatory changes in the expression and function of other PKC isoforms in the PKCα null mouse (*25*). Heart extracts were prepared from littermate control, knockout, and TG mice. α_{1c} immunoprecipitates were size-fractionated via SDS−PAGE and probed with pS1674 and pS1928 antibodies. PKCα TG mice demonstrated markedly increased levels of Ser^{1674} phosphorylation compared to littermate controls (Figure 7A; $p < 0.05$, $n = 3$). Similarly, PKCα TG mice had increased levels of phosphorylation of Ser^{1928} compared to littermate control mice (Figure 7C; $p < 0.05$, $n = 3$). Ser^{1928} is phosphorylated under basal conditions (Figure 7C,D); the basal phosphorylation may be due to PKC phosphorylation, since the PKCα null mice have decreased levels of Ser^{1928} phosphorylation compared to the littermate control (Figure 7D). In comparison, Ser^{1674} demonstrates minimal basal phosphorylation (Figure 7A,B). Ser^{528} phosphorylation is not present in these mice (data not shown).

DISCUSSION

The regulation of Ca^{2+} influx through $Ca_v1.2$ phosphorylation is important for the modulation of E−C coupling in the heart. Despite prior electrophysiological characterization of the modulation of $Ca_v1.2$ by phosphorylation, the underlying molecular mechanisms remain largely unknown (*2*). This has been exemplified recently with the findings that an Ala substitution at Ser^{1928} in knockin mice retained β-adrenergic agonist upregulation of Ca^{2+} current (*27*). Ser^{1928} has been postulated to be one of the residues in $Ca_v1.2$ responsible for PKA upregulation of channel activity (*28*−*31*). The scarcity of this transmembrane protein, the difficulties performing biochemical experiments, and reconstituting regulation in heterologous expression systems (oocyte and mammalian cells) have led to limited progress (*2*).

We have identified several new PKC phosphorylation sites within the α_{1c} subunit of the L-type Ca^{2+} channel. These sites are

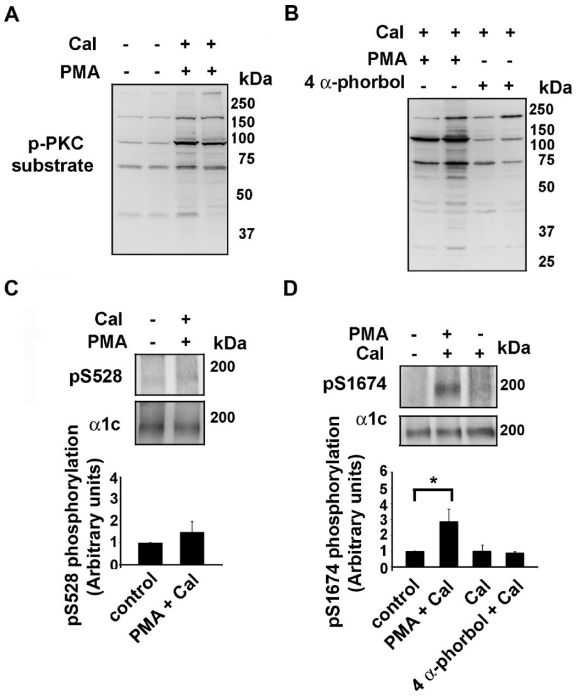

FIGURE 6: Ser[1674] is phosphorylated in cardiomyocytes. Rat hearts were perfused on a Langendorff apparatus with Tyrode solution in the absence or presence of PMA (0.5 μM) with calyculin A (Cal, 50 nM) or 4 α-phorbol (0.5 μM) with calyculin A as indicated. Hearts were flash-frozen in liquid nitrogen and lysates prepared. (A and B) Lysates were size-fractionated via SDS−PAGE, transferred to nitrocellulose, and blotted with anti-phospho-PKC substrate antibody. (C and D) α_{1c} immunoprecipitates were size-fractionated via SDS−PAGE, transferred to nitrocellulose, and blotted with anti-pS528 (top panel) and anti-pS1674 (top panel) antibodies or anti-α_{1c} antibody (middle panels). The bottom panels show bar graphs of densitometric quantification of Ser[528] and Ser[1674] phosphorylation (normalized to control; $n = 3-5$). The asterisk indicates $p < 0.025$ for PMA with calyculin compared to control or $p < 0.05$ for PMA with calyculin compared to calyculin A.

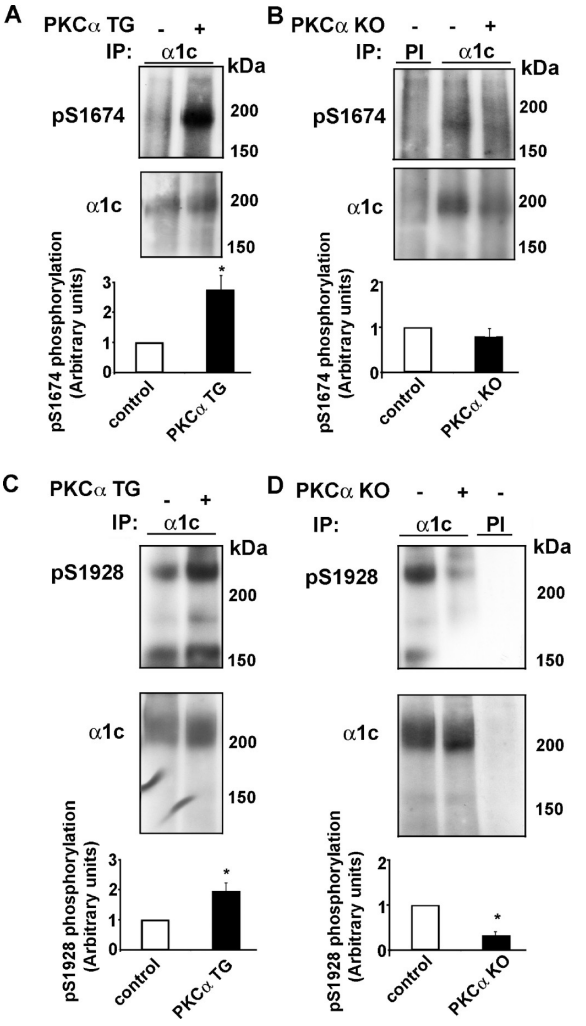

FIGURE 7: Ser[1674] and Ser[1928] phosphorylation is altered in PKCα transgenic and knockout mice. Heart extracts were prepared from PKCα transgenic and knockout mice, and α_{1c} or preimmune (PI) immunoprecipitates were size-fractionated via SDS−PAGE, transferred to nitrocellulose, and blotted with (A and B) anti-pS1674 or (C and D) anti-pS1928 antibodies (top panels) or anti-α_{1c} antibody (middle panel). Bar graphs of densitometric quantification of relative levels of pS1674 and pS1928 phosphorylation (normalized to α_{1c} immunoprecipitation; littermate control normalized to 1; $n = 3$; $^*p < 0.05$).

distinctly phosphorylated by PKC isoforms, suggesting that the L-type Ca^{2+} channel function may be differentially regulated. The rabbit α_{1c} subunit has many consensus PKC phosphorylation sites in the intracellular, transmembrane, and extracellular domains. Using GST fusion proteins incorporating only the intracellular regions, which are exposed to cellular kinases and phosphatases, we avoided studying sites that cannot be modulated in a cellular context. The disadvantage of this approach is that the fusion proteins may not fold correctly. For these in vitro kinase assays, we used nine PKC isoforms, representing conventional, novel, and atypical forms, as well as PKD. We found that the first portion of the amino-terminal segment of the rabbit α_{1c} (residues 1−80) was not phosphorylated by any PKC isoform; two residues within this segment were proposed to be responsible for PKC-induced inhibition of channel activity, based upon cellular electrophysiology experiments (*22*). We found that the second portion of the amino-terminal segment of the α_{1c} subunit could be weakly phosphorylated by several PKC isoforms; mutagenesis of all potential sites, either as single, double, or triple Ala substitutions, failed to significantly reduce the level of phosphorylation. This suggests that residues within this fragment do not represent significant phosphorylation sites in vitro. Significant phosphorylation by several PKC isoforms was found for the I−II loop and for two segments within the C-terminus, 1509−1905 and 1905−2170.

Within the I−II loop, we identified Ser[528] and Ser[533] as PKC phosphorylation sites. In addition to Ser[1928] in the 1905−2170 fusion protein (*23*), we identified residues within the 1509−1905 fusion protein, Ser[1517], Ser[1674], and Ser[1842/1843], that are PKC-phosphorylated. We generated phospho-epitope specific antibodies for each of these sites and found that Ser[1674] and Ser[1928] are phosphorylated in HEK cells and cardiomyocytes in response to direct PKC activators. Whereas Ser[1928] is strongly phosphorylated by all PKC isoforms tested except PKCη, Ser[1674] demonstrates variable PKC phosphorylation and is primarily phosphorylated by PKCα, -βI, -βII, -γ, and -θ.

Both Ser[1674] and Ser[1928] are modulated in the PKCα transgenic and knockout mice. Mouse animal models with altered cardiomyocyte PKC isoforms, induced by either transgenic or gene ablation approaches, have demonstrated important roles for PKC isoforms in the regulation of cardiac contractility and the development of cardiac hypertrophy (reviewed in ref *5*). Hemodynamic overload can produce significant changes in PKC activity (*32*, *33*); for instance, aortic banding in Sprague-Dawley

rats caused an ~3-fold increased level of expression of PKCα, which correlated with the degree of left ventricular hypertrophy (LVH). PKCδ levels increased ~6-fold at 24 weeks, and its level of autophosphorylation increased in LVH and heart failure (*34*). In the failing human heart, the expression and activity of Ca^{2+} sensitive PKCα and -β isoforms are elevated (*35*). Postnatal cardiac specific expression of PKCβ2 caused a cardiomyopathy characterized by LVH, myocardial fibrosis, and reduced LV function (*36*). In contrast, mice with cardiac specific PKCε overexpression demonstrated concentric hypertrophy with normal cardiac function, implying that PKC isoforms may play different roles in cardiac hypertrophy and failure (*37*). Whereas transgenic overexpression of PKCα causes reduced cardiac contractility, PKCα deficient mice demonstrated enhanced cardiac contractility (*25*). Therefore, PKCα, the most strongly expressed of the myocardial PKC isoforms, may be more important as a regulator of myocardial contractility than cardiac hypertrophy.

L-Type Ca^{2+} channel currents recorded from the cardiomyocytes from PKCα null mice demonstrated a rightward shift in the current−voltage relationship, compared to that of littermate control mice (*25*). The molecular mechanism responsible for this shift in channel characteristics is not clear. PKCα directly phosphorylates protein phosphatase inhibitor-1 (I-1), which alters its inhibitory activity protein phosphatase 1 (PP1). PKCα null mice undergo a > 30% decrease in PP1 specific activity, but no change in PP2A activity (*25*). In contrast, the PKCα TG mice experience an increase in PP1 activity in the heart. Thus, our findings of a decreased level of phosphorylation of Ser^{1674} and Ser^{1928} in the PKCα null hearts and an increased level of phosphorylation of these residues in the PKCα TG hearts are not due to a change in phosphatase activity, but rather most likely due to a direct effect of PKCα phosphorylation of the channel.

The role of phosphorylation of Ser^{1928} in mediating β-adrenergic agonist upregulation of L-type Ca^{2+} current has recently been explored using adenoviral-mediated overexpression in cardiomyocytes and a knockin mouse. Ala substitution of Ser^{1928} did not prevent β-agonist upregulation of current, implying that other molecular mechanisms are responsible. It is not known whether Ser^{1928} plays a role in mediating PKC modulation of L-type Ca^{2+}channel function in the heart. The assessment of the functional effects of PKC phosphorylation of these newly identified sites will likely require either overexpression in cardiomyocytes or generation of knockin animals.

The L-type Ca^{2+} channel α and β subunits are phosphorylated by several kinases, including Ca^{2+}/calmodulin-dependent kinase (CamKII) (*38*, *39*), PKA (*40*), PKC (*23*, *40*), and PKG (*24*, *41*). Many of the phosphorylation sites for these kinases have been identified, although in some cases, the sites can be phosphorylated by several of these kinases [Ser^{1928} can be phosphorylated by PKA (*42*), PKC (*23*), and PKG (*24*)]. Further work is required to determine whether in a cellular context, specificity can be imparted by differential phosphorylation of these sites. Taken together, our findings identify additional PKC regulatory sites within the α_{1c} subunit of the L-type Ca^{2+} channel. The sites are differentially phosphorylated by PKC isoforms, suggesting a molecular mechanism that could lead to highly specific fine-tuning of channel activity.

ACKNOWLEDGMENT

We thank Jeffrey Molkentin for the PKCα knockout and transgenic mice hearts.

REFERENCES

1. Fabiato, A., and Fabiato, F. (1979) Calcium and cardiac excitation-contraction coupling. *Annu. Rev. Physiol. 41*, 473–484.
2. Kamp, T. J., and Hell, J. W. (2000) Regulation of cardiac L-type calcium channels by protein kinase A and protein kinase C. *Circ. Res. 87*, 1095–1102.
3. Dempsey, E. C., Newton, A. C., Mochly-Rosen, D., Fields, A. P., Reyland, M. E., Insel, P. A., and Messing, R. O. (2000) Protein kinase C isozymes and the regulation of diverse cell responses. *Am. J. Physiol. 279*, L429–L438.
4. Dorn, G. W.II, Tepe, N. M., Lorenz, J. N., Koch, W. J., and Liggett, S. B. (1999) Low- and high-level transgenic expression of β2-adrenergic receptors differentially affect cardiac hypertrophy and function in Gαq-overexpressing mice. *Proc. Natl. Acad. Sci. U.S.A. 96*, 6400–6405.
5. Dorn, G. W.II, and Force, T. (2005) Protein kinase cascades in the regulation of cardiac hypertrophy. *J. Clin. Invest. 115*, 527–537.
6. Puceat, M., Hilal-Dandan, R., Strulovici, B., Brunton, L. L., and Brown, J. H. (1994) Differential regulation of protein kinase C isoforms in isolated neonatal and adult rat cardiomyocytes. *J. Biol. Chem. 269*, 16938–16944.
7. Aiello, E. A., and Cingolani, H. E. (2001) Angiotensin II stimulates cardiac L-type Ca^{2+} current by a Ca^{2+}- and protein kinase C-dependent mechanism. *Am. J. Physiol. 280*, H1528–H1536.
8. Robu, V. G., Pfeiffer, E. S., Robia, S. L., Balijepalli, R. C., Pi, Y., Kamp, T. J., and Walker, J. W. (2003) Localization of functional endothelin receptor signaling complexes in cardiac transverse tubules. *J. Biol. Chem. 278*, 48154–48161.
9. He, J. Q., Pi, Y., Walker, J. W., and Kamp, T. J. (2000) Endothelin-1 and photoreleased diacylglycerol increase L-type Ca^{2+} current by activation of protein kinase C in rat ventricular myocytes. *J. Physiol. 524* (Part 3), 807–820.
10. Cheng, T. H., Chang, C. Y., Wei, J., and Lin, C. I. (1995) Effects of endothelin 1 on calcium and sodium currents in isolated human cardiac myocytes. *Can. J. Physiol. Pharmacol. 73*, 1774–1783.
11. Thomas, G. P., Sims, S. M., and Karmazyn, M. (1997) Differential effects of endothelin-1 on basal and isoprenaline-enhanced Ca^{2+} current in guinea-pig ventricular myocytes. *J. Physiol. 503* (Part 1), 55–65.
12. O-Uchi, J., Sasaki, H., Morimoto, S., Kusakari, Y., Shinji, H., Obata, T., Hongo, K., Komukai, K., and Kurihara, S. (2008) Interaction of α1-adrenoceptor subtypes with different G proteins induces opposite effects on cardiac L-type Ca^{2+} channel. *Circ. Res. 102*, 1378–1388.
13. Dosemeci, A., Dhallan, R. S., Cohen, N. M., Lederer, W. J., and Rogers, T. B. (1988) Phorbol ester increases calcium current and simulates the effects of angiotensin II on cultured neonatal rat heart myocytes. *Circ. Res. 62*, 347–357.
14. Lacerda, A. E., Rampe, D., and Brown, A. M. (1988) Effects of protein kinase C activators on cardiac Ca^{2+} channels. *Nature 335*, 249–251.
15. Tseng, G. N., and Boyden, P. A. (1991) Different effects of intracellular Ca and protein kinase C on cardiac T and L Ca currents. *Am. J. Physiol. 261*, H364–H379.
16. Schreur, K. D., and Liu, S. (1996) 1,2-Dioctanoyl-*sn*-glycerol depresses cardiac L-type Ca^{2+} current: Independent of protein kinase C activation. *Am. J. Physiol. 270*, C655–C662.
17. Zhang, Z. H., Johnson, J. A., Chen, L., El-Sherif, N., Mochly-Rosen, D., and Boutjdir, M. (1997) C2 region-derived peptides of β-protein kinase C regulate cardiac Ca^{2+} channels. *Circ. Res. 80*, 720–729.
18. Nishikawa, K., Toker, A., Johannes, F. J., Songyang, Z., and Cantley, L. C. (1997) Determination of the specific substrate sequence motifs of protein kinase C isozymes. *J. Biol. Chem. 272*, 952–960.
19. Puri, T. S., Gerhardstein, B. L., Zhao, X. L., Ladner, M. B., and Hosey, M. M. (1997) Differential effects of subunit interactions on protein kinase A- and C-mediated phosphorylation of L-type calcium channels. *Biochemistry 36*, 9605–9615.
20. Wei, X., Neely, A., Olcese, R., Lang, W., Stefani, E., and Birnbaumer, L. (1996) Increase in Ca^{2+} channel expression by deletions at the amino terminus of the cardiac α 1C subunit. *Recept. Channels 4*, 205–215.
21. Shistik, E., Ivanina, T., Blumenstein, Y., and Dascal, N. (1998) Crucial role of N terminus in function of cardiac L-type Ca^{2+} channel and its modulation by protein kinase C. *J. Biol. Chem. 273*, 17901–17909.
22. McHugh, D., Sharp, E. M., Scheuer, T., and Catterall, W. A. (2000) Inhibition of cardiac L-type calcium channels by protein kinase C phosphorylation of two sites in the N-terminal domain. *Proc. Natl. Acad. Sci. U.S.A. 97*, 12334–12338.

23. Yang, L., Liu, G., Zakharov, S. I., Morrow, J. P., Rybin, V. O., Steinberg, S. F., and Marx, S. O. (2005) Ser1928 is a common site for Cav1.2 phosphorylation by protein kinase C isoforms. *J. Biol. Chem. 280*, 207–214.
24. Yang, L., Liu, G., Zakharov, S. I., Bellinger, A. M., Mongillo, M., and Marx, S. O. (2007) Protein kinase G phosphorylates Cav1.2 α1c and β2 subunits. *Circ. Res. 101*, 465–474.
25. Braz, J. C., Gregory, K., Pathak, A., Zhao, W., Sahin, B., Klevitsky, R., Kimball, T. F., Lorenz, J. N., Nairn, A. C., Liggett, S. B., Bodi, I., Wang, S., Schwartz, A., Lakatta, E. G., DePaoli-Roach, A. A., Robbins, J., Hewett, T. E., Bibb, J. A., Westfall, M. V., Kranias, E. G., and Molkentin, J. D. (2004) PKC-α regulates cardiac contractility and propensity toward heart failure. *Nat. Med. 10*, 248–254.
26. Dobransky, T., Doherty-Kirby, A., Kim, A. R., Brewer, D., Lajoie, G., and Rylett, R. J. (2004) Protein kinase C isoforms differentially phosphorylate human choline acetyltransferase regulating its catalytic activity. *J. Biol. Chem. 279*, 52059–52068.
27. Toni, L., Andrea, W., Johannes, C. C., Anne, B., Dominik, B., Peter, L., Franz, H., and Sven, M. (2008) Unchanged β-adrenergic stimulation of cardlac L-type calcium channels in Cav1.2 phosphorylation site S1928A mutant mice. *J. Biol. Chem. 283*, 35738–34744.
28. Hall, D. D., Davare, M. A., Shi, M., Allen, M. L., Weisenhaus, M., McKnight, G. S., and Hell, J. W. (2007) Critical role of cAMP-dependent protein kinase anchoring to the L-type calcium channel Cav1.2 via A-kinase anchor protein 150 in neurons. *Biochemistry 46*, 1635–1646.
29. Hulme, J. T., Westenbroek, R. E., Scheuer, T., and Catterall, W. A. (2006) Phosphorylation of serine 1928 in the distal C-terminal domain of cardiac CaV1.2 channels during β1-adrenergic regulation. *Proc. Natl. Acad. Sci. U.S.A. 103*, 16574–16579.
30. Bunemann, M., Gerhardstein, B. L., Gao, T., and Hosey, M. M. (1999) Functional regulation of L-type calcium channels via protein kinase A-mediated phosphorylation of the β_2 subunit. *J. Biol. Chem. 274*, 33851–33854.
31. De Jongh, K. S., Murphy, B. J., Colvin, A. A., Hell, J. W., Takahashi, M., and Catterall, W. A. (1996) Specific phosphorylation of a site in the full-length form of the α1 subunit of the cardiac L-type calcium channel by adenosine 3′,5′-cyclic monophosphate-dependent protein kinase. *Biochemistry 35*, 10392–10402.
32. Jalili, T., Takeishi, Y., Song, G., Ball, N. A., Howles, G., and Walsh, R. A. (1999) PKC translocation without changes in Gαq and PLC-β protein abundance in cardiac hypertrophy and failure. *Am. J. Physiol. 277*, H2298–H2304.
33. Takeishi, Y., Jalili, T., Ball, N. A., and Walsh, R. A. (1999) Responses of cardiac protein kinase C isoforms to distinct pathological stimuli are differentially regulated. *Circ. Res. 85*, 264–271.
34. Bayer, A. L., Heidkamp, M. C., Patel, N., Porter, M., Engman, S., and Samarel, A. M. (2003) Alterations in protein kinase C isoenzyme expression and autophosphorylation during the progression of pressure overload-induced left ventricular hypertrophy. *Mol. Cell. Biochem. 242*, 145–152.
35. Murphy, S., and Frishman, W. H. (2005) Protein kinase C in cardiac disease and as a potential therapeutic target. *Cardiol. Rev. 13*, 3–12.
36. Wakasaki, H., Koya, D., Schoen, F. J., Jirousek, M. R., Ways, D. K., Hoit, B. D., Walsh, R. A., and King, G. L. (1997) Targeted overexpression of protein kinase C β2 isoform in myocardium causes cardiomyopathy. *Proc. Natl. Acad. Sci. U.S.A. 94*, 9320–9325.
37. Takeishi, Y., Ping, P., Bolli, R., Kirkpatrick, D. L., Hoit, B. D., and Walsh, R. A. (2000) Transgenic overexpression of constitutively active protein kinase Cε causes concentric cardiac hypertrophy. *Circ. Res. 86*, 1218–1223.
38. Hudmon, A., Schulman, H., Kim, J., Maltez, J. M., Tsien, R. W., and Pitt, G. S. (2005) CaMKII tethers to L-type Ca^{2+} channels, establishing a local and dedicated integrator of Ca^{2+} signals for facilitation. *J. Cell Biol. 171*, 537–547.
39. Grueter, C. E., Abiria, S. A., Dzhura, I., Wu, Y., Ham, A. J., Mohler, P. J., Anderson, M. E., and Colbran, R. J. (2006) L-Type Ca^{2+} channel facilitation mediated by phosphorylation of the β subunit by CaMKII. *Mol. Cell 23*, 641–650.
40. Catterall, W. A. (2000) Structure and regulation of voltage-gated Ca^{2+} channels. *Annu. Rev. Cell Dev. Biol. 16*, 521–555.
41. Jiang, L. H., Gawler, D. J., Hodson, N., Milligan, C. J., Pearson, H. A., Porter, V., and Wray, D. (2000) Regulation of cloned cardiac L-type calcium channels by cGMP-dependent protein kinase. *J. Biol. Chem. 275*, 6135–6143.
42. Hell, J. W., Yokoyama, C. T., Breeze, L. J., Chavkin, C., and Catterall, W. A. (1995) Phosphorylation of presynaptic and postsynaptic calcium channels by cAMP-dependent protein kinase in hippocampal neurons. *EMBO J. 14*, 3036–3044.