Ser\textsuperscript{1928} Is a Common Site for Cav\textsubscript{1.2} Phosphorylation by Protein Kinase C Isoforms\textsuperscript{*}

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Voltage-dependent Ca\textsuperscript{2+} channel (Cav\textsubscript{1.2}, L-type Ca\textsuperscript{2+} channel) function is highly regulated by hormones and neurotransmitters in large part through the activation of kinases and phosphatases. Regulation of Cav\textsubscript{1.2} by protein kinase C (PKC) is of significant physiologic importance, mediating, in part, the cardiac response to hormonal regulation. Although PKC has been reported to mediate activation and/or inhibition of Cav\textsubscript{1.2} function, the molecular mechanisms mediating the response have not been definitively elucidated. We show that PKC forms a macromolecular complex with the α\textsubscript{1c} subunit of Cav\textsubscript{1.2} through direct interaction with the C terminus. This interaction leads to phosphorylation of the channel in response to activators of PKC. We identify Ser\textsuperscript{1928} as the residue that is phosphorylated by PKC in vitro and in vivo. Ser\textsuperscript{1928} has been identified previously as the site mediating, in part, the protein kinase A up-regulation of channel activity. Thus, the protein kinase A and PKC signaling pathways converge on the Cav\textsubscript{1.2} complex at Ser\textsuperscript{1928} to increase channel activity. Our results identify two mechanisms leading to regulation of Cav\textsubscript{1.2} activity by PKC: pre-association of the channel with PKC isoforms and phosphorylation of specific sites within the α\textsubscript{1c} subunit.

The influx of Ca\textsuperscript{2+} through Cav\textsubscript{1.2} is essential for activation of excitation-contraction coupling in the heart (1). Ca\textsuperscript{2+} influx also contributes to the plateau phase of the cardiac action potential, pacemaker activity in nodal cells, and the modulation of gene expression (2). A variety of diseases such as atrial fibrillation, heart failure, and ischemic heart disease have been associated with alterations in Cav\textsubscript{1.2} density and/or function (3, 4). The activation of members of the protein kinase C (PKC) family plays an important role in the signal transduction pathways within the cardiac myocyte leading to modulation of contraction and cell phenotype (5). Regulation of Cav\textsubscript{1.2} activity by PKC signaling pathways has been well established, with several studies demonstrating that activators of PKC increase Ca\textsuperscript{2+} channel currents in cardiac and smooth muscle (6–11). PKC is a serine/threonine kinase consisting of an N-terminal regulatory region and a C-terminal catalytic region and is activated by diacylglycerol (DAG) produced in response to hormones such as α-adrenergic agonists, angiotensin II, and endothelin-1 (12, 13). Three PKC subgroups have been identified and functionally distinguished: conventional PKC (εPKC), novel PKC (nPKC) and atypical PKC (αPKC) (14, 15).

The molecular basis for Cav\textsubscript{1.2} regulation by PKC has been widely studied, but remains incompletely elucidated and controversial. The cardiac Cav\textsubscript{1.2} α\textsubscript{1c} and β\textsubscript{2} subunits are phosphorylated by PKC on unidentified sites in vitro (16). Electrophysiologic studies of recombinant channels have attempted to identify specific mechanisms that mediate PKC regulation, but have yielded conflicting information, potentially due to differences in experimental conditions and/or sequences of α\textsubscript{1c}/β subunits (17–20).

Several studies have suggested that the N terminus of α\textsubscript{1c} is important for PKC up-regulation of channel function. Deletion of the first 46 or 139 amino acid residues of rabbit heart α\textsubscript{1c} causes a 5–10-fold increase in whole cell current in oocytes (21, 22), without affecting channel density. These findings suggest that the N terminus provides an inhibitory control, which can be relieved after PKC phosphorylation, potentially of a remote phosphorylation site(s) (23). To elucidate the mechanism(s) through which PKC isoforms regulate channel activity, we have utilized glutathione S-transferase (GST) fusion proteins to screen potential phosphorylation sites. Here, we demonstrate that the protein kinase A (PKA) phosphorylation site (Ser\textsuperscript{1928}) on the α\textsubscript{1c} subunit (24), which mediates, in part, Cav\textsubscript{1.2} activation (25), is a substrate for PKC in vitro and in vivo. The phosphorylation of Ser\textsuperscript{1928} is mediated, in part, through a direct association between the α\textsubscript{1c} subunit and PKC isoforms. The results suggest that both PKA and PKC converge on Ser\textsuperscript{1928} of the α\textsubscript{1c} subunit to mediate phosphorylation-dependent regulation of Ca\textsuperscript{2+} influx.

**EXPERIMENTAL PROCEDURES**

**Preparation of Adult Heart and Brain and Human Embryonic Kidney (HEK) 293 Cell Extracts/Membranes**—Membranes were prepared from rat ventricular tissue as described previously (26). All preparative procedures were performed at −4 °C. Rat ventricles were minced and homogenized in 10 ml Tris maleate (pH 6.8) containing 1% benzanilide, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 1 mM calpain I inhibitor, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Membranes were stored at −80 °C. Rat brain extracts were prepared in phosphate-buffered saline containing 1% sucrose and 0.9% NaCl. Membranes were stored at −80 °C.
PKC Phosphorylation of Ca,1.2

(v/v) Triton X-100, one Complete mini-tablet (Roche Applied Science), 44 μM calpain I inhibitor, 18 μM calpain II inhibitor, and 200 μM phenylmethylsulfonyl fluoride as described previously (27). Insoluble material was removed by centrifugation (twice at 14,000 rpm), and supernatants were collected. HEK cells were washed with 1x phosphate-buffered saline and lysed in 50 mM Tris- HCl (pH 7.4), 150 mM NaCl, and 1% Triton X-100 containing protease inhibitors. The lysates were centrifuged at 14,000 g for 10 min to clarify supernatants.

Isolation of Neonatal Cardiac Myocytes—Cardiomyocytes were isolated from 1-day-old Wistar rat hearts by trypsin dispersion using a differential attachment procedure to enrich for cardiomyocytes, followed by irradiation as described previously (28). Cells were plated on protamine sulfate-coated culture dishes at a density of 5 x 10^6 cells/100-mm dish. Experiments were performed on cultures grown for 5 days in minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum and then serum-deprived for the subsequent 24 h. Neonatal cardiac myocytes maintained in cultures were washed with 1x phosphate-buffered saline and then resuspended in kinase reaction buffer containing 8 mM MgCl2, 10 mM EGTA, 50 mM Tris, and 50 mM PIPES (pH 6.8) containing a protease and phosphatase inhibitor mixture (Calbiochem). Membranes were prepared according to methods described previously (29). The pellets were suspended in 100 μl of 1x sample buffer.

cDNA Clones and Site-directed Mutagenesis—The rabbit αc subunit (NCBI accession number X15539) and the rabbit PKA catalytic subunit (Sigma), 50 μg/ml Triton X-100, Complete mini-tablets (one/7 ml), and 200 μM phenylmethylsulfonyl fluoride using 2 μg of anti-αc, antibody (AC-0009, Alomone Laboratories) immune complexes were collected using protein A (Amersham Biosciences) for 2 h, followed by extensive washing. All immunoprecipitations included negative controls (peptide-blocked, preimmune serum, antibody alone). Additional antibodies included horse-radish peroxidase-conjugated anti-GST antibody (sc-138, Santa Cruz Biotechnology, Inc.) and a panel of PKC isoform-specific antibodies (BD Transduction Laboratories) or SuperSignal detection (Pierce). In all cases, data shown are representative of three or more similar experiments.

For PKA kinase reactions, full-length recombinant αc, was immunoprecipitated with the αc-specific antibody, and immune complexes were captured on protein A-Sepharose. GST fusion proteins were purified on glutathione-Sepharose. Sepharose beads were extensively washed and resuspended in kinase reaction buffer containing 8 mM MgCl2, 10 mM EGTA, 50 μM Tris, not 50 mM PIPES (pH 6.8). Phosphorylation reactions were done using the GST fusion proteins containing domains in the αc C terminus interaction were performed overnight in modified radioimmune precipitation assay buffer consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, Complete mini-tablets (one/7 ml), and 200 μM phenylmethylsulfonyl fluoride using 2 μg of anti-αc, antibody (AC-0009, Alomone Laboratories) immune complexes were collected using protein A (Amersham Biosciences) for 2 h, followed by extensive washing. All immunoprecipitations included negative controls (peptide-blocked, preimmune serum, antibody alone). Additional antibodies included horse-radish peroxidase-conjugated anti-GST antibody (sc-138, Santa Cruz Biotechnology, Inc.) and a panel of PKC isoform-specific antibodies (BD Biosciences). Blots were developed with the use of ECL (Amersham Biosciences) or SuperSignal detection (Pierce), and these non-overlapping regions of the αc, C terminus were generated (Fig. 2A). In each case, the PKC isoform interaction with Ca,1.2 were specific, as PKC isoforms were not recovered when immunoprecipitations were performed with irrelevant IgGs. Collectively, these results show a constitutive interaction between Ca,1.2 and certain PKC isoforms (primarily pPKCs) in cardiac and brain tissue.

To determine whether the PKC isoforms directly interact with the αc, subunit (and to map the PKC interaction domains on αc, subunits), a panel of GST fusion proteins containing the αc, subunit N terminus, individual intracellular loops, and non-overlapping regions of the αc, C terminus were generated (Fig. 2A). Fig. 2B shows that cPKCa co-sedimented with GST fusion proteins containing domains in the αc, subunit C terminus. The interactions between cPKCa and these non-overlapping regions of the αc subunit C terminus were specific, as cPKCa did not co-sediment with GST alone or GST fused to other αc, subunit intracellular domains (including the N terminus or the intracellular loop sequences) (Fig. 2B). To determine whether the cPKCa-αc, C terminus interaction is direct (i.e., does not require another coprecipitating protein), additional in vitro studies were performed with the αc, C terminus-GST fusion proteins and recombinant cPKCa. Fig. 2C shows that both C terminus-GST fusion proteins pull-downed cPKCa. cPKCa was not recovered when the pull-down was performed with GST alone. Collectively, these results identify a direct interaction between cPKCa and the αc, subunit.

RESULTS

Ca,1.2 Forms a Macromolecular Complex with PKC in the Heart and Brain—Although significant evidence supports the concept that PKC signaling pathways can modulate Ca,1.2 activity in the heart, there is still little to no information on the identity of the PKC isoform(s) and the molecular mechanisms underlying this regulation. In keeping with the emerging role of protein-protein interactions as a mechanism to locally regulate channel activity by regulatory kinases (particularly in differentiated cells such as cardiomyocytes and neurons), the initial studies examined whether PKC isoforms interact with Ca,1.2 in cardiac or brain tissue. Cardiomyocytes coexpress conventional (cPKCα), novel (nPKCδ and nPKCe), and atypical (APKCα, β, γ) isoforms; cPKCβ has also been variably detected in cardiomyocytes by some investigators (30–32). Fig. 1 shows that Ca,1.2 co-immunoprecipitated with cPKCa, but nPKCe and nPKCe were not recovered in the anti-Ca,1.2 immune complexes (Fig. 1A). Similarly, all three cPKC isoforms expressed in the brain (PKCe, PKCβ, and PKCγ) were recovered in Ca,1.2 immune complexes (Fig. 1B). In contrast, nPKCδ (which is abundant in cardiac and brain tissue) did not co-immunoprecipitate with Ca,1.2 in either preparation under these conditions (Fig. 1, A and C). nPKCe also did not co-immunoprecipitate with Ca,1.2 from cardiomyocytes (Fig. 1A), although some nPKCe was detected in Ca,1.2 immune complexes from the brain (Fig. 1C), perhaps due to the higher levels of nPKCe expression in this tissue. Other PKC isoforms such as PKCe and nPKCδ (which are abundant in neuronal tissue and are not expressed at any appreciable level in cardiomyocytes) also were recovered with Ca,1.2 in the brain (Fig. 1C). In each case, the PKC isoform interaction with Ca,1.2 was specific, as PKC isoforms were not recovered when immunoprecipitations were performed with irrelevant IgGs. Collectively, these results show a constitutive interaction between Ca,1.2 and certain PKC isoforms (primarily cPKCs) in cardiac and brain tissue.
We next sought to determine whether the interaction between Cav1.2 and cPKCα/H9251 which we detected in native tissues and in vitro binding assays could be reconstituted in a mammalian expression system. To this end, we expressed Cav1.2 (αc and H9252a) in HEK293 cells, which have nearly undetectable levels of endogenous cPKCα upon immunoblotting. HEK293 cells demonstrated relatively high expression of cPKCα, cPKCβ, and aPKCα and weaker expression of nPKCδ, nPKCε, and aPKCγ upon immunoblotting (data not shown). Fig. 3A shows that αc specifically co-immunoprecipitated with endogenous cPKCα in unstimulated HEK293 cells (Fig. 3A, left panel). No cPKCα immunoreactivity could be detected when immunoprecipitation was performed with preimmune serum or with anti-αc antibody in the presence of blocking (immunogenic) peptide (Fig. 3A, left panel), consistent with the lack of immunoprecipitation of αc (right panel).

A constitutive interaction between Cav1.2 and cPKCα could constitute a mechanism to optimize channel phosphorylation in the context of stimulatory conditions that are associated with the generation of Ca2+ and DAG. To determine whether αc subunits are phosphorylated by PKCα docked to the channel, we performed in vitro kinase assays. Immune complexes were
isolated with anti-α1c subunit antibody or preimmune serum and subjected to kinase assays with [γ-32P]ATP in the presence of sonicated vesicles containing the PKC-activating lipid cofactors and DAG. Although endogenous cPKCα co-immunoprecipitated with α1c subunits under these conditions, exogenous cPKCα (alone or with a PKC peptide inhibitor; Promega catalog no. V5691) was added to some incubations as indicated. Fig. 3 shows that α1c subunits were labeled in the in vitro kinase assays by both exogenous and endogenous PKCs. α1c subunit phosphorylation increased when excess exogenous PKCα was added to the assays (and it was blocked in the presence of a PKC inhibitor). These results indicate that the α1c subunit is phosphorylated by PKC, rather than another kinase(s) that also might co-immunoprecipitate with the Cav1.2 channel complex.

PKC Phosphorylation of Cav1.2—The sites for PKC phosphorylation of Cav1.2 α1c subunits have not been identified. Based upon electrophysiologic findings, PKC inhibitory modulation of channel activity has been mapped to several residues in the α1c subunit N terminus (Thr27 and Thr31) (33), although evidence that this region is not directly phosphorylated in vitro by PKC has also been presented (23), which we have confirmed (data not shown). Because cPKCα interacts directly with the C terminus, we hypothesized that regions

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**Fig. 2.** PKC isoforms associate with the C terminus of the α1c subunit. A: a schematic is shown of the α1c subunit with the intracellular segments used to construct GST fusion proteins indicated. B: upper panel, α1c subunit-GST fusion proteins bound to Sepharose were incubated with a cardiac membrane preparation (500 μg), washed, pelleted, size-fractionated on SDS-polyacrylamide gel, and immunoblotted with anti-PKCa antibody. An equivalent amount of GST fusion proteins (~1 μg) was used. Only the two C-terminal fragments (non-overlapping) bound PKCa in a pull-down assay. + cont represents 10% input. Lower panel, shown is an anti-GST immunoblot representing the amount of GST fusion proteins utilized for pull-down. C: upper panel, GST-fused region 1509–1905 or 1906–2170 or GST alone was incubated with 100 ng of recombinant cPKCα. The Sepharose beads were extensively washed, size-fractionated on SDS-polyacrylamide gel, and immunoblotted with anti-PKCa antibody. Recombinant cPKCa bound to the GST fusion proteins containing the C terminus of α1c. GST alone failed to associate with cPKCa. Lower panel, shown is an immunoblot representing the amount of GST fusion proteins utilized for pull-down.

**Fig. 3.** Reconstitution of PKCa-α1c association in HEK293 cells. A: the α1c and β2 subunits were expressed in HEK293 cells. Lysates were prepared, and α1c was immunoprecipitated (IP) with anti-α1c antibody. Left panel, cPKCα specifically co-immunoprecipitated with Ca1.2. Negative controls included preimmune serum and anti-α1c antibody alone (without lysate) and α1c peptide (pep)-blocked immunoprecipitation. Right panel, shown is the specificity of immunoprecipitation, in which preimmune serum and antigenic peptide blocked immunoprecipitation of α1c. The band at 50 kDa is the IgG heavy chain. cont, control. B: left panel, lysates from α1c (+) or vector (−)-expressing HEK cells were prepared. cPKCa phosphorylation of immunoprecipitated α1c with [γ-32P]ATP was performed. Right panel, immunoprecipitated α1c was phosphorylated with or without exogenous cPKCa, followed by extensive washing. α1c was specifically phosphorylated by both exogenous (first lane) and endogenous (third lane) PKCs, which were blocked by PKC inhibitor (Inh). The band at ~125 kDa was not identified.
within the C terminus may contain the authentic PKC phosphorylation sites. To map PKC phosphorylation sites on the \( \alpha_{1c} \) subunit, we utilized GST fusion proteins containing non-overlapping regions of the C terminus as substrates for \textit{in vitro} PKC phosphorylation. Whereas cPKCa phosphorylated both regions 1509–1905 and 1906–2170 of the C terminus, nPKCe and \( \alpha_{1c} \) phosphorylated primarily region 1906–2170 (Fig. 4A). These results suggest that individual PKC isoforms can exert distinct regulatory control on channel function by phosphorylating different regions of the \( \alpha_{1c} \) subunit. Previous studies mapped the PKA phosphorylation site on the \( \alpha_{1c} \) subunit to a PKA consensus motif (RRAS\(^{1928}\)) located in the C terminus (24). \textit{In vitro} \( \alpha_{1c} \) subunit phosphorylation assays are consistent with this conclusion, as PKA induced the radiolabeling of fragment 1906–2170 (which encompasses the Ser\(^{1928}\) site) (Fig. 4B).

\( \alpha_{1c} \) Ser\(^{1928}\) Is a PKC Phosphorylation Site—An examination of the amino acid sequence within fragment 1906–2170 revealed that Ser\(^{1928}\) represents a consensus PKC phosphorylation site (Fig. 5A) (34). PKA and cPKCa induced similar increases in \(^{32}\)P incorporation into GST-fused wild-type (WT) fragment 1906–2170; in each case, radiolabeling was nearly completely abrogated by a single substitution (S1928A) (Fig. 5B). Similarly, utilizing a phospho-specific antibody developed to detect Ser\(^{1928}\)-phosphorylation, prominent immunoreactive bands were detected (with a range of mobilities corresponding to the GST-fused full-length protein as well as the proteolytic/truncated fragments) in both PKA and cPKCa phosphorylation assays in GST-fused WT fragment 1906–2170 (Fig. 5C). In contrast, no anti-phospho-Ser\(^{1928}\)-antibody immunoreactivity was detected when a single substitution (S1928A) was introduced into the substrate sequence. Similar to cPKCa, nPKCe and \( \alpha_{1c} \) phosphorylated Ser\(^{1928}\) using GST-fused fragment 1906–2170 (Fig. 5D).

Because Ser\(^{1928}\) represents a common target for PKA and PKC \textit{in vitro} (Fig. 5, B and C) and PKA has been shown to form a macromolecular complex with Ca\(_{1.2}\), we sought to determine whether PKC directly phosphorylates Ser\(^{1928}\). The PKA inhibitor PKI-(5–24) specifically blocked phosphorylation of Ser\(^{1928}\) by PKA (but not cPKCa) of GST-fused fragment 1906–2170 (Fig. 5E) and full-length recombinant \( \alpha_{1c} \), expressed in HEK293 cells (Fig. 5F). Similar to cPKCa, nPKCe and \( \alpha_{1c} \) specifically phosphorylated Ser\(^{1928}\) \textit{in vitro} in full-length recombinant \( \alpha_{1c} \) (Fig. 5G). These results demonstrate that PKA and PKC can independently phosphorylate Ser\(^{1928}\), \( \alpha_{1c} \), Ser\(^{1928}\).

Having validated the specificity of the phospho-Ser\(^{1928}\)-specific antibody as a reagent to track kinase-dependent phosphorylation of the \( \alpha_{1c} \) C terminus, we used it to examine the phosphorylation status of full-length recombinant WT and S1928A \( \alpha_{1c} \) subunits expressed in HEK293 cells. Fig. 6A shows that WT \( \alpha_{1c} \) subunits (immunoprecipitated from quiescent HEK293 cells and subjected to immune complex kinase assays without lipid cofactors) displayed very low levels of Ser\(^{1928}\) phosphorylation (second lane); in contrast, Ser\(^{1928}\) phosphorylation was not detected when studies were performed on HEK293 cells expressing the S1928A mutant (first lane), despite equivalent expression and immunoprecipitation of WT and mutant \( \alpha_{1c} \) subunits (lower panels). WT \( \alpha_{1c} \) Ser\(^{1928}\) phosphorylation was increased by the addition of lipid cofactors to the \textit{in vitro} kinase assays (Fig. 6A, fourth lane), consistent with Fig. 3 results showing that \( \alpha_{1c}\) subunits were recovered in complexes with cPKCa. Exogenously added cPKCa or PKA further increased WT \( \alpha_{1c} \) Ser\(^{1928}\) phosphorylation (Fig. 6A, fifth and sixth lanes).

Similar \textit{in vitro} studies were performed on endogenous \( \alpha_{1c} \) subunits recovered from adult rat hearts. In native tissues (heart, brain, and skeletal muscle), the \( \alpha_{1c} \) subunit displayed a slight increase in mobility upon SDS-PAGE (relative to the mobility of full-length \( \alpha_{1c} \) subunits expressed in HEK293 cells) due to the previously described C-terminal truncation (35–37). The common PKA and PKC phosphorylation site (Ser\(^{1928}\)) is present only in the full-length-form of \( \alpha_{1c} \) (37) and is removed by calpain, a Ca\(^{2+}\)-activated protease in the brain (38) and an unidentified protease in the heart (39). Fig. 6B demonstrates that \( \alpha_{1c}\) recovered from adult rat heart was phosphorylated at Ser\(^{1928}\) by PKCa in an \textit{in vitro} kinase assay. Ser\(^{1928}\) was partially phosphorylated under basal conditions in adult rat heart by either PKA or PKC (Fig. 6B, second lane).

In HEK cells overexpressing WT Ca\(_{1.2}\) channels, incubation with phorbol 12-myristate 13-acetate (PMA; 500 nM) prior to lysis led to phosphorylation of Ser\(^{1928}\) as assessed using the phospho-Ser\(^{1928}\)-specific antibody (Fig. 7A). The PMA-induced phosphorylation was inhibited by preincubating the cells with bisindolylmaleimide (500 nM; GF109203X), indicating that the phosphorylation was mediated by PKC. Exposure of the cells to the protein phosphatase (PP) inhibitor calyculin A (50 nM) prior to stimulation with PMA caused increased phosphorylation of Ser\(^{1928}\) \textit{in vivo}, which was also significantly inhibited by bisindolylmaleimide (Fig. 7A). Calyculin A-induced phosphorylation of Ser\(^{1928}\) was partially inhibited by H-89 (500 nM) and bisindolylmaleimide (500 nM), but completely inhibited by both inhibitors (Fig. 7B). This suggests that both PKA and PKC con-
tribute to the calyculin A-induced phosphorylation of Ser$^{1928}$ in HEK293 cells overexpressing Cav1.2. Similarly, phosphorylation of Ser$^{1928}$ by PKC was also observed in neonatal cardiomyocytes treated with PMA and calyculin A (Fig. 7C). The phosphorylation was inhibited by the PKC inhibitor bisindolylmaleimide. Taken together, these findings demonstrate that Ser$^{1928}$ is phosphorylated by PKC in vivo. Phosphorylation of α1c at Ser$^{1928}$ is believed to lead, in part, to increased channel.

Fig. 5. PKC phosphorylates Ser$^{1928}$. A: the schematic demonstrates the phosphorylation site in fragment 1906–2170. B: shown is an autoradiogram of PKA and cPKCa in vitro kinase reaction performed using [$\gamma$-32P]ATP with GST-fused WT fragment 1906–2170 and S1928A. PKA and cPKCa phosphorylated Ser$^{1928}$. C: GST fusion proteins (WT and S1928A 1906–2170 fragment) were phosphorylated by either PKA or cPKCa, size-fractionated on SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with a phospho-specific antibody recognizing phosphorylated Ser$^{1928}$. Both PKA and cPKCa phosphorylated Ser$^{1928}$. Bands below the full-length fusion protein are proteolytic/truncation products of the fusion protein. D: shown is an anti-phospho-Ser$^{1928}$ antibody immunoblot of PKC isoform in vitro kinase reaction with GST-fused WT fragment 1906–2170 and S1928A. cPKCa, nPKCε, and aPKCδ phosphorylated Ser$^{1928}$ equivalently. E: upper panels, immunoblotting using anti-phospho-Ser$^{1928}$ antibody demonstrates that PKA phosphorylation of GST-fused fragment 1906–2170 was inhibited by the PKA inhibitor PKI-(5–24), whereas cPKCa phosphorylation was not affected. Lower panels, Ponceau staining of nitrocellulose membrane indicates equivalent loading of GST fusion proteins. A dark exposure was selected to highlight differences between PKI inhibition of PKA and PKC. F: shown are immunoblots with anti-phospho-Ser$^{1928}$ (upper panel) and anti-α1c (lower panel) antibodies of in vitro kinase reaction of α1c with the indicated kinase/inhibitor. Ca,1.2 was expressed in HEK293 cells, and α1c was immunoprecipitated, followed by the kinase reaction. Ser$^{1928}$ was phosphorylated by PKA and cPKCa, but PKI inhibited PKA (but not cPKCa) phosphorylation. G: shown are immunoblots with anti-phospho-Ser$^{1928}$ antibody (upper panels) and anti-α1c antibody (lower panel) demonstrating cPKCa, nPKCε, and aPKCδ phosphorylation of WT (but not S1928A) α1c. Recombinant α1c was recovered by immunoprecipitation. In each case, phosphorylation conditions were optimized for the indicated isoform, and equivalent kinase units were utilized. Lower panels, α1c, immunoblot demonstrating equivalent α1c input into phosphorylation assay.

Fig. 6. Ser$^{1928}$ is phosphorylated by PKC in full-length recombinant channels and rat heart. A, extracts from HEK cells transfected with WT or S1928A α1c, + β$_{2a}$ were prepared, followed by α1c, immunoprecipitation and kinase reaction as indicated. Samples were size-fractionated on SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and probed with anti-phospho-Ser$^{1928}$ antibody (pS1928). Under basal conditions, the majority of Ser$^{1928}$ was not phosphorylated (second lane), compared with maximal phosphorylation with PKA and cPKCa (fifth and sixth lanes). cPKCa associated with channels phosphorylated Ser$^{1928}$ (fourth lane). B, rat heart lysates were prepared in the presence of phosphatase inhibitors, and α1c was immunoprecipitated (IP) and probed with anti-phospho-Ser$^{1928}$ (upper panel) or α1c (lower panel) antibody. In the last lane, the immunoprecipitates were phosphorylated with PKCa in vitro. Native α1c demonstrated partial C-terminal truncation as demonstrated by the broad band, with a significant portion below 200 kDa (compared with recombinant channels in A). The signal for anti-phospho-Ser$^{1928}$ antibody was present only in the full-length channel because truncation was N-terminal to the phosphorylation site.
activity (25, 40, 41), thereby potentially accounting for the activation of the channel observed after stimulation with agents that activate PKC signaling pathways.

**DISCUSSION**

This study provides the identification of a molecular mechanism that mediates the regulation of Ca\(_{1.2}\) in response to activators of PKC. We have mapped specific regions/residues within the Ca\(_{1.2}\) channel complex that are sites for PKC-dependent phosphorylation in vitro and in vivo. Observed differences in Ca\(^{2+}\) channel regulation by individual PKC isoforms serve to highlight the complexity that likely exists in vivo, where regulatory controls are through the orchestrated effects of a series of stimulatory and inhibitory kinases and phosphatases that regulate channel activity in a highly specific manner in response to a given physiologic stimulus.

A common theme in signal transduction is the close association of signaling molecules (such as kinases and phosphatases) with effectors (such as ion channels) to achieve specific and localized regulation. Indeed, our findings that specific PKC isoforms can associate with Ca\(_{1.2}\) are consistent with this premise. Moreover, the ability to bind different PKC isoforms may lead to variable regulation of channel activity, given the findings that several PKC isoforms demonstrate different abilities to phosphorylate GST-fused C-terminal fragment 1509–1905. Several PKC-binding proteins have been identified; for instance, RACK1s (receptors for activated C kinase) bind activated forms of PKC and enhance its activity (42). In non-stimulated rat heart, RACK1 did not bind Ca\(_{1.2}\) (data not shown). Interestingly, AKAP15-PKA also associates with Ca\(_{1.2}\) in the distal C terminus of the \(\alpha_1c\) subunit (43). Prior studies have established that PKC isoforms can bind directly with ion channels/substrates; for instance, PKC binds directly to the glutamate receptor (44), the \(\gamma\)-aminobutyric acid receptor (45), and phospholipases D1 and D2 (46).

The Ca\(_{1.2}\) channel complex is an important PKC target in the heart, as it plays a critical role in excitation-contraction coupling and potentially in myocyte survival and growth (13). Angiotensin II, \(\alpha_1\)-adrenergic receptors, and endothelin-1 receptors are members of a G protein-coupled receptor family that lead to stimulation of PKC isoforms through activation of phospholipase C and production of inositol 1,4,5-trisphosphate and DAG. Angiotensin II and endothelin-1 have been reported to increase Ca\(^{2+}\) entry and thus contractility in mammalian cardiac myocytes (13, 47). However, conflicting findings regarding the effects on Ca\(_{1.2}\) have been generated for several direct activators of PKC, including phorbol esters (6, 48–50), 1,2-dioctanoyl-sn-glycerol (51), and 1-oleoyl-2-acetyl-sn-glycerol (52).

Our findings that Ca\(_{1.2}\) pre-associates with specific PKC isoforms in the heart and brain are consistent with this hypothesis and provide a potential molecular mechanism for the differences observed between PKC activators and effectors such as endothelin-1 and angiotensin II.

The identification of Ser\(^{1928}\) as a PKC phosphorylation site has important implications regarding the study of phosphorylation-dependent modulation of Ca\(_{1.2}\). PKA increases channel activity in the heart and brain. The full-length \(\alpha_1c\) subunit is phosphorylated by PKA at the single residue (Ser\(^{1928}\)) in the C terminus, which is missing in the truncated form of the channel (24). However, the C terminus remains associated with the channel, potentially modulating its activity (39, 54). Similar to PKA, our data demonstrate that three representative PKC isoforms (cPKCo, nPKCe, and aPKCz) specifically phosphorylate Ser\(^{1928}\) in vitro and in vivo. Because Ser\(^{1928}\) is the main and probably only PKA site on the \(\alpha_1c\) subunit (24), our findings suggest that Ser\(^{1928}\) phosphorylation plays an important role in mediating the PKA and PKC modulation of Ca\(_{1.2}\). Thus, Ser\(^{1928}\) phosphorylation may represent a target for the convergence of the two signal transduction pathways, with specificity imparted by the PKC-mediated phosphorylation of other sites within the \(\alpha_1c\) and/or \(\beta\) subunit.

Our findings also demonstrate the importance of phospa-
tase regulation of Ser\(^{1928}\) phosphorylation. Recent studies have suggested that PKs may regulate, in part, the activity of the Ca\(_{1.2}\) channel. PP2A has been shown to associate with Ca\(_{1.2}\) in a macromolecular complex in the brain (55). The PP1/PP2A inhibitor calyculin A (125 nM) increases Ca\(_{1.2}\) activity by 70% in murine ventricular myocytes (10). The calyculin A effect is believed to be due primarily to its effects on PP1 rather than PP2A activity because okadaic acid (10–100 nM) or fostriecin (500 nM) fails to increase steady-state Cav1.2 current (11). The PP2A activity because okadaic acid (10–100 nM) or fostriecin (500 nM) fails to increase steady-state Cav1.2 current (11). The PP2A activity because okadaic acid (10–100 nM) or fostriecin (500 nM) fails to increase steady-state Cav1.2 current (11). The PP2A activity because okadaic acid (10–100 nM) or fostriecin (500 nM) fails to increase steady-state Cav1.2 current (11). The PP2A activity because okadaic acid (10–100 nM) or fostriecin (500 nM) fails to increase steady-state Cav1.2 current (11). The PP2A activity because okadaic acid (10–100 nM) or fostriecin (500 nM) fails to increase steady-state Cav1.2 current (11). The PP2A activity because okadaic acid (10–100 nM) or fostriecin (500 nM) fails to increase steady-state Cav1.2 current (11). The PP2A activity because okadaic acid (10–100 nM) or fostriecin (500 nM) fails to increase steady-state Cav1.2 current (11). The PP2A activity because okadaic acid (10–100 nM) or fostriecin (500 nM) fails to increase steady-state Cav1.2 current (11). The PP2A activity because okadaic acid (10–100 nM) or fostriecin (500 nM) fails to increase steady-state Cav1.2 current (11). The PP2A activity because okadaic acid (10–100 nM) or fostriecin (500 nM) fails to increase steady-state Cav1.2 current (11). The PP2A activity because okadaic acid (10–100 nM) or fostriecin (500 nM) fails to increase steady-state Cav1.2 current (11). The PP2A activity because okadaic acid (10–100 nM) or fostriecin (500 nM) fails to increase steady-state Cav1.2 current (11). The PP2A activity because okadaic acid (10–100 nM) or fostriecin (500 nM) fails to increase steady-state Cav1.2 current (11). The PP2A activity because okadaic acid (10–100 nM) or fostriecin (500 nM) fails to increase steady-state Cav1.2 current (11). The PP2A activity because okadaic acid (10–100 nM) or fosteri
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