Protein kinase C activation inhibits Ca\textsubscript{v}1.3 calcium channel at NH\textsubscript{2}-terminal serine 81 phosphorylation site

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L-TYPE VOLTAGE-GATED Ca\textsuperscript{2+} channels (VGCC) are widely expressed in various tissues and exert pivotal functions in excitation-contraction coupling in muscles, hormone secretion in endocrine cells, gene expression and regulation in neurons, and other cellular functions. Four types of L-type VGCC have been described (4). Whereas \(\alpha_{1S}\) and \(\alpha_{1P}\) are mainly restricted to skeletal muscle and retinal neuron, respectively (1, 4, 16, 22), the \(\alpha_{1C}\) is expressed in the heart, vascular smooth muscles, and neurons (10, 32). More recently, \(\alpha_{1D}\) has been implicated in the pathophysiology of \(\alpha_{1D}\) Ca\textsuperscript{2+} channel and in the development of PKC isozyme-targeted therapeutics.

Eleven isozymes of PKC have been identified and divided into three groups based on their sequence homology and biochemical properties (8, 13, 23). These include the conventional PKCs (\(\alpha, \beta I, \beta II,\) and \(\gamma\)), regulated by Ca\textsuperscript{2+} and phorbol esters, the novel PKCs (\(\epsilon, \delta, 6, \) and \(\eta\)), regulated by phorbol esters but not by Ca\textsuperscript{2+}, and the atypical PKCs (\(\zeta\) and \(\iota\)), insensitive to both Ca\textsuperscript{2+} and phorbol esters. Various PKC isozymes are shown to have different intracellular distribution, and therefore each isozyme is suggested to exert different cellular function (6) and have different effects and mechanisms of action on ion channels (12, 35, 36).

The specific role of each PKC isozyme on L-type Ca\textsuperscript{2+} currents (\(i_{\text{CaL}}\)) recorded from native myocytes or transfected cells has been made possible by the use of PKC modulator peptides that can inhibit individual endogenous PKC isozymes (31). We report for the first time that \(\alpha_{1D}\) VGCC is specifically regulated by \(\beta II\)- and ePKC isozymes. Molecular analysis of PKC-mediated regulation unraveled the implication of a serine residue located at position 81 of the \(\alpha_{1D}\) NH\textsubscript{2}-terminal domain as an important site for this regulation.

MATERIALS AND METHODS

Plasmids and site-directed mutagenesis. Rat pCMV6b/\(\alpha_{1D}\) plasmid was kindly provided by Dr. Susumu Seino (Kobe University, Kobe, Japan). Putative PKC phosphorylation sites with prediction score above the threshold were identified using NetPhos (http://www.cbs.dtu.dk/services/NetPhos) (3, 17). Site-directed mutagenesis was performed on pCMV6b/\(\alpha_{1D}\) using QuickChange TM site-directed mutagenesis kit from Stratagene (La Jolla, CA) according to the manufacturer’s instructions. Serine residues were replaced by alanine in all substitution mutants. The following mutations were introduced by mutagenic primers: S81A, S91A, S121A, and S98A/S100A to the \(\alpha_{1D}\) plasmid (Fig. 1). Two NH\textsubscript{2}-terminal truncations were also constructed: \(\alpha_{1D}\)/\(\Delta N(2-59)\) and \(\alpha_{1D}\)/\(\Delta N(1-123)\). To delete amino acids 2 to 59 and 2 to 123, we introduced restriction enzyme XbaI followed by the Kozak sequence and ATG starting codon, using site-directed mutagenesis in pGFP37 constructs at the following positions: 177tctagaCCACCATG178 for \(\Delta N(2-59)\) and 366tctagaCCACCATG367 for \(\Delta N(1-123)\). Fragment XbaI was then removed. The presence of mutations was confirmed using the automatic sequencing at Laval University sequencing facility. Constructs were purified using Qiagen columns (Qiagen, Chatsworth, CA).

Transfection of tsA201 cell line. The mammalian cell line tsA201 is derived from human embryonic kidney HEK-293 cells by stable transfection with SV40 large-T antigen. Cells were grown in high-

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glucose DMEM supplemented with 10% fetal bovine serum, 1-glutamine (2 mM), penicillin G (100 U/ml), and streptomycin (10 mg/ml; GIBCO BRL Life Technologies). Cells were incubated in a 5% CO₂ humidified atmosphere. The tsA201 cells were transfected with EBO/CD8 plasmid was cotransfected with 7 μg of EBO/CD8 plasmid and 10 μg of each of α₁D, β, and α₁B cDNAs. Transfected cells that bind beads generally also express Ca²⁺ channels. For patch-clamp experiments, cells 2–3 days posttransfection were incubated for 2 min in medium containing anti-CD8-a coated beads (M-450 CD8-a-Dynabeads) (14). The unattached beads were removed by washing with extracellular solution. Beads were prepared according to the manufacturer’s instructions (Dynal Biotech, Brown Deer, WI). Cells expressing CD8-a, and therefore binding beads, were distinguished from nontransfected cells by light microscopy.

Solution, drugs, and peptides. For whole cell recordings, the pipette solution contained (in mM) 135 CsCl, 4 MgCl₂, 4 ATP, 10 HEPS, 10 EGTA, and 1 EDTA, adjusted to pH 7.2 with tetraethylammonium hydroxide (TEAOH). The bath solution contained (in mM) 135 choline chloride, 1 MgCl₂, 2 CaCl₂, and 10 HEPS, adjusted to pH 7.2 with TEAOH. PKC isozyme peptides were added in the pipette solution as previously described (12, 35, 36). PKC isozyme-specific inhibitor and control peptides used in this study include αC2-4 (αPKC), βIV5-3 (βIPKC), βIIV5-3 (βIPKC), εV1-2 (εPKC), δV1-7 (δPKC), scrambled εV1-2, and pentalysine. The sequence and properties of PKC isozyme peptides were reported previously (12, 35, 36). Two additional peptides (peptide I and peptide II) corresponding to the NH₂ terminus of α₁D were also used in this study for both patchclamp and in vitro phosphorylation studies. Peptide I corresponds to the NH₂ terminus of α₁D from amino acid 69 to 104 with all serine residues converted to alanines except for serine 81: N'-MATAAPPVGLSQKRQYYAKKKQGNAANARPA-C'. For peptide II, all serine including the serine 81 were substituted by alanines: N'-MATAAPPVGLAQKRQYYAKKKQGNAANARPA-C'. All peptides had >90% purity and were synthesized by Genemed Synthesis (South San Francisco, CA). PMA, 4α-phorbol 12,13-didecanoate (4αPDD), and GF 109203X were obtained from Sigma-Aldrich (St. Louis, MO).

Kinase assay. βIPKC or εPKC kinases. PKC kinase assays were performed in 25 μl of phosphorylation buffer containing (in mM) 200 HEPS, 1 CaCl₂ (for βIPKC), 2.5 PKC lipid activator, pH 7.4, 0.3% Triton X-100, and 8.5 μCi γ-[³²P]ATP. Reactions were incubated for 30 min at 30°C, separated on 20% SDS-polyacrylamide gel, extensively washed, stained with Coomassie blue, fixed, and dried. [γ-³²P]ATP was detected by autoradiography.

Electrophysiology. Ca²⁺ currents were recorded in whole cell configuration of the patch-clamp technique (9). Data were digitized at 5 kHz with an analog-to-digital converter (Digidata 1200 Axon Instruments, Axon Instrument CA). The recordings were filtered with a low-pass corner frequency of 2 kHz. Borosilicate glass electrodes (outer diameter 1.5 mm) with resistances of 0.8–1.0 MΩ when filled were connected to a patch-clamp amplifier (Axopatch 200B amplifier). A voltage error of 7 mV, attributable to liquid junction potential, was corrected. Data were analyzed using pCLAMP version 9.0 (Axon Instruments).

Statistical analysis. Data were expressed as means ± SE. Percent inhibition was calculated as the difference of the current amplitude and the intervention over the control value. When indicated, paired or unpaired t-test was performed. Differences were deemed significant at a P value < 0.05.

RESULTS

PMA inhibits α₁D Ca²⁺ current. Representative inward α₁D IC₅₀ traces recorded before and after application of the general PKC activator PMA (10 nM) are shown in Fig. 2, A and B, respectively. PMA consistently inhibited α₁D IC₅₀ and a steady-state inhibition was reached in all cells studied. For easy comparison, reduction in IC₅₀ density (pA/pF) is represented as a percentage value throughout the text. Averaged current-voltage relations show that PMA reduced α₁D IC₅₀ density by 50.5 ± 5.0% (n = 9, P < 0.05; Fig. 2C) without significantly affecting steady-state activation [control: −25.0 ± 1.8 mV, n = 7 vs. PMA: −23.8 ± 2.2 mV, P = not significant (NS), n = 12; Fig. 2D] and inactivation curves [control: −35.4 ± 1.6 mV, n = 9 vs. PMA: −32.9 ± 1.4 mV, P = NS, n = 10; Fig. 2D]. Slope factors were −5.7 ± 0.3 for control (n = 7, P =
compared with -5.5 ± 0.4 for PMA (n = 12) for activation and 7.6 ± 0.8 for control (n = 9, P = NS) compared with 7.0 ± 0.8 for PMA (n = 10) for inactivation. The time course for PMA-induced inhibition of α1D ICaL elicited at -10 mV is shown in Fig. 2E. For easy comparison, α1D ICaL are normalized to maximal amplitudes. To exclude the possibility that the current decay observed with PMA is due to a rundown, we carried out a set of control experiments. The time course of α1D ICaL recorded in the absence of PKC modulators shows no significant current reduction (n = 3; Fig. 3A). To confirm the specificity of PMA on α1D ICaL, we compared its effect to that of an inactive phorbol ester analog, 4αPDD, which does not activate PKC. Superfusion of tsA201 cells with 10 nM 4αPDD did not significantly affect α1D ICaL (reduction of 4.0 ± 1.7%,
To further substantiate the role of PKC in the PMA inhibitory effect, we tested the ability of a general PKC inhibitor (GF 109203X) to antagonize the effect of PMA. Preincubation of 1D-expressing cells with GF 109203X (15 μM) for 10–15 min before superfusion with PMA resulted in only 7.0 ± 2.0% inhibition (n = 3, P = NS) of ICaL after PMA application (Fig. 3C). In these experiments, GF 109203X (15 μM) alone did not affect ICaL amplitude (data not shown). Because conventional PKC (cPKC) isozymes depend on Ca2+ for their activity (25), PMA effect was also assessed using a lower concentration of the Ca2+ chelator EGTA (0.1 mM) in the pipette (Fig. 3D). The results show that the PMA-induced inhibitory effect on ICaL was not significantly affected (42.1 ± 7%, n = 5, P = NS).

PMA inhibition of α1D ICaL is mediated through serine 81 in NH2 terminus.

Previous studies have shown that the NH2-terminal domain of α1C channel is critical for the PKC-mediated modulation (21, 30). In the present study, 11 putative PKC phosphorylation sites were predicted throughout α1D NH2-terminal domain as described in MATERIALS AND METHODS. To assess the role of these potential sites, deletion [ΔN(2–59)] and point mutation (S81A, S91A, S98A/S100A, and S121A) constructs were synthesized and studied in tsA201 cells (Fig. 1). No current could be detected from 1D/N(2–123) construct (data not shown). 1D/N(2–59) deletant channel yielded a macroscopic ICaL. This deletion did not prevent inhibition of ICaL following superfusion with PMA (n = 10; Fig. 4A). This led us to rule out the existence of a functional PKC target within the segment of amino acid 2 to 59. We next investigated the role of five key potential phosphorylation residues in the remaining NH2-terminal portion. To this end, four α1D VGCC mutations were tested: one double substitution (S98A/S100A) and three single mutations (S81A, S91A, and S121A). The PMA effect on α1D ICaL was not significantly altered in α1D/S98A/S100A, α1D/S91A, and α1D/S121A constructs compared with the wild type (WT). The average percentages of α1D ICaL inhibition by PMA are 57.6 ± 4.8% (n = 5), 52.3 ± 5.1%

Fig. 3. Time course of α1D ICaL recordings from tsA201 cells using 2 mM Ca2+ as a charge carrier. Currents were elicited at a test potential of −10 mV pulse protocol. A: rundown test time course of α1D ICaL (A), the effect of 4α-phorbol 12,13-didecanoate (4αPDD; 10 nM) (B), and the effect of PMA (C) are shown in the presence of the general PKC inhibitor GF 109203X (15 μM). D: time course showing α1D ICaL inhibition following PMA (10 nM) superfusion when low EGTA (0.1 mM) concentration was used in the patch pipette. Peak currents are plotted against time before and during superfusion. Insets illustrate α1D ICaL traces recorded at time points indicated by arrows a and b.
(n = 5), and 52.6 ± 5.9% (n = 7) vs. 50.5 ± 5.0% (n = 9, P = NS), respectively (Fig. 4, B–D). Interestingly, PMA-induced α1D \( I_{\text{Ca,L}} \) inhibition was antagonized in the presence of S81A mutation (15.0 ± 8.2%, n = 9, P = NS), suggesting a critical role for this particular site in PKC-mediated modulation of the α1D subunit (Fig. 4E). Averaged data from all mutations are summarized in Fig. 4F. It is noteworthy that the presence of mutations altered neither the kinetics nor the gating properties of the α1D VGCC.

To substantiate the role of serine 81 in the PMA inhibitory effect, we tested the ability of a peptide of 35 amino acids (peptide I) that corresponded to the NH2-terminal region comprising residues 69–104 and where all serine residues, except for serine 81, were converted to alanines (see MATERIALS AND

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**Fig. 4. Effects of PMA (10 nM) on α1D mutant channels.** Time courses of α1D \( I_{\text{Ca,L}} \) were recorded from a tsA201 cell expressing α1D/ΔN12–59 (A), α1D/S98A/S100A (B), α1D/S91A (C), α1D/S121A (D), and α1D/S81A mutants (E). Insets illustrate current traces recorded at time points indicated by arrows a and b. F: histogram showing percentage of inhibition of α1D \( I_{\text{Ca,L}} \) in the presence of PMA (10 nM) recorded from different mutants. Currents amplitudes are normalized against the control (before PMA). Data are means ± SE. *P < 0.05, statistically significant difference compared with α1D/WT after superfusion of PMA (10 nM).
METHODS) to antagonize the effect of PMA on α1D channels. Cells were dialyzed with 10 μM peptide-I for at least 5 min to allow for the peptide to access the cytoplasm. PMA perfusion of cells initially dialyzed with peptide I resulted in only 30.7 ± 5.8% inhibition (n = 7, P < 0.05) of basal α1D ICaL compared with control (no peptides were dialyzed into cells) (Fig. 5A). However, when similar experiments were repeated using a control peptide (peptide II) in which all the serine residues were replaced with alanines, the effect of PMA on α1D ICaL was not prevented by the presence of peptide II (10 μM). PMA inhibited α1D ICaL by 53.3 ± 7.2% (n = 8, P = NS) in cells dialyzed with peptide II.

To determine the mechanism by which serine 81 is involved in α1D modulation by PKC, we substituted this neutral residue with the negatively charged aspartate, to mimic the negative charge introduced during naturally occurring phosphorylation. In the absence of PMA, conversion of serine (neutral amino acid) to aspartate (negative amino acid) at position 81 resulted in a significant reduction of α1D ICaL by 42.2 ± 7.8% (n = 20, P < 0.05 compared with WT). In the presence of a negative charge at position 81, PMA did not have significant inhibitory effect on α1D ICaL (6.23 ± 4.5%, n = 9) (Fig. 5B). These data suggest that gain of negative charge at position 81 in the NH2-terminal domain could be the molecular mechanism by which α1D subunit is modulated by PKC.

PMA inhibition of α1D ICaL is mediated through βII- and εPKC isoforms. To dissect the role of individual PKC isoforms in the regulation of α1D VGCC, we tested the ability of PKC isozyme-specific inhibitor peptides (31) to antagonize the effect of PMA. Six PKC isozyme-specific inhibitor peptides were used: αC2-4, βIV5-3, βIV5-3, δV1-2, εV1-2, and ηV1-2 targeting α-, βI-, βII-, δ-, ε-, and εPKCs, respectively. For each set of experiments, cells were dialyzed with a different inhibitor (0.1 μM) for at least 5 min to allow for peptides to access the cytoplasm. These peptides alone did not have any effect on α1D ICaL. Subsequently, the PMA inhibitory effect on α1D ICaL was assessed in the presence of each of these six inhibitor peptides, respectively. PMA superfusion of cells initially dialyzed with αC2-4, βIV5-3, δV1-2, or ηV1-2 (0.1 μM) did not prevent α1D ICaL inhibition. Average percentages of inhibition were: 43.0 ± 4.0% for αC2-4 (n = 9, P = NS), 50.0 ± 6.0% for βIV5-3 (n = 4, P = NS), 47.6 ± 5.0% for δV1-2 (n = 9, NS), and 48.3 ± 5% for ηV1-2 compared with control (where cells were not dialyzed with any peptide, 50.5 ± 5.0%, n = 7). In contrast, PMA superfusion of cells dialyzed with βIV5-3 (0.1 μM) resulted in 22.0 ± 7.0% inhibition (n = 7, P < 0.05) of α1D ICaL (Fig. 6A). Similarly, in cells dialyzed with εV1-2 peptide inhibitor, PMA resulted in only 11.0 ± 5.0% inhibition (n = 5, P < 0.05) of α1D ICaL (Fig. 6B). Next, we tested whether βIV5-3 and εV1-2 peptides have an additive antagonizing effect of α1D ICaL inhibition by PMA. Simultaneous application of both βIV5-3 (0.1 μM) and εV1-2 (0.1 μM) in pipette solution completely abolished the inhibitory effect of PMA (0.5 ± 0.1%, n = 5, P < 0.05; Fig. 6C). In vitro phosphorylation reactions using NH2-terminus-derived peptides II and I showed that only peptide I, containing serine 81, was phosphorylated by βIIPKC and εPKC (Fig. 6D). These results indicate that serine 81 is required for α1D subunit phosphorylation by βIIPKC and εPKC.

Additional patch-clamp experiments were performed in the presence of pentalysine or a scrambled εV1-2 as negative controls (Fig. 7). The inhibitory effect of PMA on α1D ICaL was not prevented in the presence of scrambled εV1-2 (0.1 μM; 47.0 ± 2.8, n = 5, P < 0.05) or pentalysine (0.1 μM; 46.5 ± 3.9, n = 5, P < 0.05). These findings demonstrate that βIIPKC and εPKC isoforms are selectively involved in the modulation of α1D VGCC expressed in tsA201 cells. Data from different peptides are summarized in the histogram of Fig. 7.

DISCUSSION

In the present study we provide novel functional and biochemical evidence for α1D VGCC regulation by PKC through a serine residue located at position 81 in the NH2-terminal domain of the α1D subunit. Using the HEK-293-derived tsA201 cells known to endogenously express most of phorbol ester-sensitive PKC isoforms, including α, βI, βII, δ, and ε (18), we have shown that α1D ICaL is inhibited by 50% following superfusion with the general PKC activator PMA.
This inhibitory effect is specifically attributed to the activation of PKC given the fact that similar effect could not be reproduced using the biologically inactive phorbol ester analog 4αPDD, which does not activate PKC. In addition, the α1D IC\textsubscript{CaL} inhibition by PMA was prevented by a known general PKC inhibitor, GF 109203X. A time-dependent IC\textsubscript{CaL} rundown effect (2) has been ruled out, because no variations in IC\textsubscript{CaL} amplitudes were observed in control experiments. Together, these data confirm that α1D VGCC is regulated by PKC.

Phosphorylation of NH\textsubscript{2}-terminal serines by PKC. One of the main novel observations in the present work is that a single serine mutation (S81A) at the NH\textsubscript{2}-terminal domain of α1D subunit readily confers a dramatic lack of PMA effect on α1D IC\textsubscript{CaL}. To determine whether the NH\textsubscript{2}-terminal domain of α1D subunit is involved in channel phosphorylation by PKC, deletions [ΔN\textsubscript{(2-59)} and ΔN\textsubscript{(2-123)}] and individual point mutations of putative phosphorylation sites in which serines were mutated to alanine residues (S81A, S91A, S121A, and S98A/S100A) were tested in the presence of PMA. Interestingly, only the substitution of serine at position 81 by an alanine (S81A) prevented most of the PMA effect. Furthermore, intracellular application of peptide I, corresponding to the α1D NH\textsubscript{2}-terminal segment that contains serine 81, significantly reduced the inhibitory effect of PMA on α1D/WT channels, consistent with data obtained in the presence of S81A mutation. This finding suggests that peptide I directly competes with the corresponding NH\textsubscript{2}-terminal sequence on α1D/WT subunits expressed in tsA201 cells. Furthermore, introducing a negatively charged residue at this site (S81D) resulted in smaller α1D IC\textsubscript{CaL} amplitudes compared with WT, therefore mimicking the inhibitory effect of PKC activation on intact α1D channel. These data support the conclusion that serine 81 is a crucial site for α1D subunit phosphorylation by PKC and suggest that the gain of a negative charge at position 81 could be the molecular mechanism by which α1D subunit is modulated by PKC.

Because expression of α1D subunit alone in tsA201 cells yielded no functional current in the present study and a previous study (28), α1D subunit was coexpressed with the β and α2β accessory subunits in tsA201 cells. In this regard, we do not completely rule out the potential phosphorylation of these accessory subunits (essentially β subunit) by PKC. However, the resulting effect must be minimal, because S81 mutation of the pore-forming α1D subunit antagonized the majority of the PMA effect on α1D IC\textsubscript{CaL}.

Fig. 6. Effect of PMA on α1D IC\textsubscript{CaL} in the presence of PKC isoform-specific inhibitor peptides. Time courses of α1D IC\textsubscript{CaL} were recorded from tsA201 cells dialyzed with peptide inhibitors for βIPKC (βIIV5-3, 0.1 μM) (A), εPKC (εV1-2, 0.1 μM) (B), and βIPKC + εPKC (βIIV5-3, 0.1 μM + εV1-2, 0.1 μM) (C). Insets illustrate α1D IC\textsubscript{CaL} traces recorded at time points indicated by arrows a and b. D: in vitro phosphorylation of α1D NH\textsubscript{2} terminus-derived peptides I and II in the presence of βIPKC and εPKCs.
Similar effects were observed at either concentration. Indeed, E and 0.1 mM EGTA (Figs. 2

the effects of the corresponding inhibitors were studied at 10

involved in

the inhibition of

VGCC inhibition by PMA. Because members of cPKC (II, and

1D Ca2+

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its broad distribution in the brain (19) and insulin-secreting β

cells of the pancreas (29). The function of α1D Ca2+

channel in the heart had long been ignored until recent studies showed that

α1D Ca2+

channel knockout mice unexpectedly exhibited intrinsic sinoatrial and AV node dysfunction (20, 26, 39). At the whole animal level, significant prolongation of the R-R and PR intervals in surface ECG were observed in α1D knockout mice (20, 26, 39). More recently, atrial arrhythmias, mainly atrial fibrillation, were induced in all α1D Ca2+

channel knockout mice, indicating a role of α1D IC\textsubscript{cal} in the sinoatrial tissue (38). Furthermore, we also recently showed that α1D IC\textsubscript{cal} recorded from tsA201 cells is modulated by protein kinase A (PKA) (20, 26–28, 39) and may play a role in the pathophysiology of autoimmune-associated congenital heart block (27). Altogether, the above recent data demonstrate that α1D Ca2+

channels play an important role in the heart, and thus the mechanisms of its regulation by kinases such as PKA and PKC are relevant. Because α1D IC\textsubscript{cal} in native cardiac myocytes cannot be distinguished pharmacologically from α1C IC\textsubscript{cal} because of the absence of selective blockers (20, 26–28, 39), the characterization of α1D Ca2+

channel modulation by PKA/ PKC is limited to mammalian tsA201 cells.

In conclusion, the results establish that α1D VGCC is regulated by PKC. This regulation involves phosphorylation of α1D NH\textsubscript{2}-terminal domain. Specifically, we have shown that serine 81 represents a critical site for PKC-mediated regulation of α1D VGCC and pointed to βIIPKC and ePKC as the key isozyme players in this regulation. Understanding the molecular mechanism of α1D VGCC regulation through PKC provides novel insights in the development of new drugs that interfere with specific components of the channel protein and/or its regulatory pathways. The results also suggest that ePKC and βIIPKC isozymes may constitute suitable candidates for the development of targeted therapeutic for cardiac pathophysiology involving α1D Ca2+

channels, such as atrial fibrillation or autoimmune-associated congenital heart block.

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PKC isozyme-specific modulation of α1D Ca2+ channels. We dissected the role of individual PKC isozymes in the regulation of α1D VGCC using the established PKC isozyme-specific inhibitor peptides (31). Two PKC isozymes were found to be involved in α1D IC\textsubscript{cal} inhibition by PMA: βIIPKC and ePKC. Whereas inhibition of ePKC antagonized ~78% of the PMA effect (inhibition of α1D IC\textsubscript{cal} was reduced from 50.5 to 11%), the inhibition of βIIPKC reduced it by 56% (inhibition of α1D IC\textsubscript{cal} was reduced from 50.5 to 22%). A combination of both βIIPKC and ePKC inhibitors into the pipette prevented almost 100% of the inhibitory effect of PMA. None of the other PKC isozyme-specific inhibitor peptides (α-, βI-, δ-, and γPKCs) significantly altered the PMA-induced inhibition of α1D IC\textsubscript{cal}. These results indicate that βIIPKC and ePKC mediate the α1D VGCC inhibition by PMA. Because members of cPKC (α, βII, and γ) are known to depend on Ca2+ in their activity (25), the effects of the corresponding inhibitors were studied at 10 and 0.1 mM EGTA (Figs. 2E and 3D) in the pipette solution. Similar effects were observed at either concentration. Indeed, under our experimental conditions, ePKC activation in the presence of EGTA can be attributed to the extracellular Ca2+ that we used as charge carrier and to the fact that phorbol esters are known to increase the affinity of ePKC for Ca2+ (24).

Study limitations. Because we used the whole cell configuration of the patch-clamp experiments, we cannot rule out the possibility that the quantitative aspects of the response to PMA in the presence of peptide PKC isozyme modulators might differ in a nondialyzed cell system. Introduction of mutation(s) to a channel may interfere with channel protein trafficking. In this study, we did not assess all the factors that may affect expression of a mutated channel, including trafficking. Therefore, we cannot completely rule out potential effects these factors may have on the expression of the mutated channel in this study.

Pathophysiological significance. In the cardiovascular system, voltage-gated L-type Ca2+ channels are essential for the generation of normal cardiac rhythm, for induction of rhythm propagation through the atrioventricular (AV) node, and for the contraction of the atrial and ventricular muscle. In diseased myocardium, L-type Ca2+ channels can contribute to abnormal impulse generation and cardiac arrhythmias (11). The α1D Ca2+ channel is generally termed neuroendocrine because of its broad distribution in the brain (19) and insulin-secreting β cells of the pancreas (29). The function of α1D Ca2+ channel in the heart had long been ignored until recent studies showed that α1D Ca2+ channel knockout mice unexpectedly exhibited intrinsic sinoatrial and AV node dysfunction (20, 26, 39). At the whole animal level, significant prolongation of the R-R and PR intervals in surface ECG were observed in α1D knockout mice (20, 26, 39). More recently, atrial arrhythmias, mainly atrial fibrillation, were induced in all α1D Ca2+ channel knockout mice, indicating a role of α1D IC\textsubscript{cal} in the sinoatrial tissue (38). Furthermore, we also recently showed that α1D IC\textsubscript{cal} recorded from tsA201 cells is modulated by protein kinase A (PKA) (20, 26–28, 39) and may play a role in the pathophysiology of autoimmune-associated congenital heart block (27). Altogether, the above recent data demonstrate that α1D Ca2+ channels play an important role in the heart, and thus the mechanisms of its regulation by kinases such as PKA and PKC are relevant. Because α1D IC\textsubscript{cal} in native cardiac myocytes cannot be distinguished pharmacologically from α1C IC\textsubscript{cal} because of the absence of selective blockers (20, 26–28, 39), the characterization of α1D Ca2+ channel modulation by PKA/ PKC is limited to mammalian tsA201 cells.

In conclusion, the results establish that α1D VGCC is regulated by PKC. This regulation involves phosphorylation of α1D NH\textsubscript{2}-terminal domain. Specifically, we have shown that serine 81 represents a critical site for PKC-mediated regulation of α1D VGCC and pointed to βIIPKC and ePKC as the key isozyme players in this regulation. Understanding the molecular mechanism of α1D VGCC regulation through PKC provides novel insights in the development of new drugs that interfere with specific components of the channel protein and/or its regulatory pathways. The results also suggest that ePKC and βIIPKC isozymes may constitute suitable candidates for the development of targeted therapeutic for cardiac pathophysiology involving α1D Ca2+ channels, such as atrial fibrillation or autoimmune-associated congenital heart block.

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