Vascular ATP-sensitive K⁺ channels are inhibited by multiple vasoconstricting hormones via the protein kinase C (PKC) pathway. However, the molecular substrates for PKC phosphorylation remain unknown. To identify the PKC sites, Kir6.1/SUR2B and Kir6.2/SUR2B were expressed in HEK293 cells. Following channel activation by pinacidil, the catalytic fragment of PKC inhibited the Kir6.1/SUR2B currents but not the Kir6.2/SUR2B currents. Phorbol 12-myristate 13-acetate (a PKC activator) had similar effects. Using Kir6.1-Kir6.2 chimeras, two critical protein domains for the PKC-dependent channel inhibition were identified. The proximal N terminus of Kir6.1 was necessary for channel inhibition. Because there was no PKC phosphorylation site in the N-terminal region, our results suggest its potential involvement in channel gating. The distal C terminus of Kir6.1 was crucial where there are several consensus PKC sites. Mutation of Ser-354, Ser-379, Ser-385, Ser-391, or Ser-397 to non-phosphorylatable alanine reduced PKC inhibition moderately but significantly. Combined mutations of these residues had greater effects. The channel inhibition was almost completely abolished when 5 of them were jointly mutated. In vitro phosphorylation assay showed that 4 of the serine residues were necessary for the PKC-dependent 32P incorporation into the distal C-terminal peptides. Thus, a motif containing four phosphorylation repeats is identified in the Kir6.1 subunit underlying the PKC-dependent inhibition of the Kir6.1/SUR2B channel. The presence of the phosphorylation motif in Kir6.1, but not in its close relative Kir6.2, suggests that the vascular K_ATP channel may have undergone evolutionary optimization, allowing it to be regulated by a variety of vasoconstricting hormones and neurotransmitters.

ATP-sensitive K⁺ (K_ATP) channels play an important role in vascular tone regulations (1–3). Such a function attributes to channel regulation by a variety of vasodilating and vasoconstricting hormones and neurotransmitters (3–7). Therefore, the understanding of the molecular basis for channel regulation has an impact on the design of therapeutic modalities by targeting at specific molecular substrates of the channel. It is known that the major isoform of K_ATP channels in vascular smooth muscles is composed of Kir6.1 and SUR2B (8–12). Genetic disruption of Kir6.1 or SUR2 indeed results in a phenotype of Prinzmetal angina with a high rate of sudden death (13, 14), consistent with the importance of the channel in vascular regulations.

Experimental evidence suggests that vasoconstrictors act on the vascular K_ATP channel through the PKC² signaling system. Our previous studies have shown that the Kir6.1/SUR2B channel and its counterpart in vascular smooth muscle cells are inhibited by vasopressin and that channel inhibition can be abolished by specific PKC blockers (6). Similar observations have been made by other groups with endothelin (15), muscarinic M3 receptor agonist (16), and angiotensin II (17). The effect of angiotensin II on the vascular K_ATP channel requires translocation of PKCe to plasma membranes (18, 19). By comparing the effects of the acetylcholine M3 receptor on Kir6.1/SUR2B and Kir6.2/SUR2B channels, Quinn et al. (16) suggest that Kir6.1/SUR2B channel inhibition is mediated via a direct effect of PKC rather than a change in phosphatidyl 4,5-bisphosphate concentrations. They also show evidence for Kir6.1 phosphorylation using in vitro biochemical assay (16). Consistently, purified PKC inhibits the cloned Kir6.1/SUR2B and vascular smooth muscle endogenous K_ATP channels in inside-out patches where cytosolic soluble components are absent (4, 20). Although these previous studies have significantly improved our understanding of vascular K_ATP channel regulation by vasoconstrictors, the molecular substrate of PKC remains unknown. Therefore, we performed these studies to identify the critical protein domain and amino acid residues for PKC phosphorylation.

MATERIALS AND METHODS

Molecular Biology—Rat Kir6.1 (GenBank™ accession number D42145), mouse Kir6.2 (D50581), and mouse SUR2B (D86038) cDNAs were used in the present study. The cDNAs were cloned in the eukaryotic expression vector pcDNA3.1 and used for mammalian cell expression (5, 6). Kir6.1-Kir6.2 chimeras were produced by overlap extension using PCR (Pfu DNA polymerase; Stratagene, La Jolla, CA) (21). Site-specific mutations were made using a site-directed mutagenesis kit (Stratagene). The orientation of the constructs and the correct muta-

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The abbreviations used are: PKC, protein kinase C; PKGI, PKC inhibitory peptide 19–31; cPKC, catalytic fragment of PKC; PMA, phorbol 12-myristate 13-acetate; MOPS, 4-morpholinepropanesulfonic acid; L, liter; WT, wild type; MBP, maltose-binding protein.
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In Vitro Phosphorylation—The fusion peptides were treated with the catalytic fragment of PKC (ePKC) (BIOMOL, Plymouth, PA) under the following condition: $\sim$5 $\mu$g of fusion peptides in 5 $\mu$L of modified elution buffer (in mmol/L) (200 NaCl, 30 Tris-HCl, 6 EDTA, 10 maltose, pH 7.4), 5 $\mu$L of 5X reaction buffer (125 Tris-HCl, 0.1 EGTA, pH 7.5), 5 $\mu$L of MgATP solution (20 MOPS, 25 $\beta$-glycerophosphate, 5 EGTA, 1 Na$_2$VO$_4$, 1 dithiothreitol, 75 MgCl$_2$, 0.5 ATP, pH 7.2), 10 ng of cPKC in 10 $\mu$L of H$_2$O, and 1 $\mu$L of 5 $\mu$Ci/$\mu$L [y-$^{32}$P]ATP (PerkinElmer Life Sciences). After 60 min of reaction, 5 $\mu$L of 5X protein loading buffer were added to each sample to terminate the reaction. The samples were subjected to electrophoresis in 10% SDS-polyacrylamide gel. The gel was stained with Coomassie Blue and photographed. The gel was then fixed and dried. Autoradiography was performed using a Fuji BAS 2500 imaging plate. The in vitro phosphorylation experiment was repeated twice.

Data are presented as the mean $\pm$ S.E. Differences in means were tested by an analysis of variance or Student’s t test and were accepted as significant if $p \leq 0.05$. RESULTS

PKC-dependent Inhibition of the Kir6.1/SUR2B Channel—Whole-cell currents were recorded with high K$^+$ in both bath and pipette solutions. The Kir6.1/SUR2B currents remained small in 8–10 min of base-line recording and were strongly activated by 10 $\mu$mol/L pinacidil and inhibited by 10 $\mu$mol/L glibenclamide (Fig. 1, A and B). At maximum activation, application of 100 $\mu$mol/L PMA strongly inhibited the Kir6.1/SUR2B currents (77.5 $\pm$ 3.7%, n = 19), which were blocked by PKCi, a specific PKC inhibitor (Fig. 1, C and F). The inactive phorbol ester, 4a-phorbol 12,13-didecanoate, did not affect the Kir6.1/SUR2B currents (Fig. 1F).

In inside-out patches, channel activity was low in the absence of nucleotides. The Kir6.1/SUR2B channel was activated in the presence of MgADP/ATP, which was further augmented with pinacidil application (Fig. 2A). When cPKC was applied to the internal patch membranes, the Kir6.1/SUR2B currents were markedly inhibited (61.1 $\pm$ 3.8%, n = 6) (Fig. 2, A and C). Thus, these results indicate that the Kir6.1/SUR2B channel is inhibited by PKC independently of cytosolic soluble components, consistent with previous reports (20). In contrast, PMA and cPKC had no effect on the Kir6.2/SUR2B channel in whole-cell recordings (Fig. 1, D–F) or in inside-out patches (Fig. 2, B and C), suggesting that the SUR2B subunit is not critical. Critical Protein Domains for PKC-dependent Channel Inhibition—The differential PKC sensitivity of Kir6.1/SUR2B compared with Kir6.2/SUR2B suggests that critical protein domains for PKC regulation are located on the Kir subunit. Therefore, we constructed Kir6.1–Kir6.2 chimeras, which were expressed in HEK293 cells (American Type Culture Collection, Manassas, VA) as detailed in our previous report (5). The Kir6.1/SUR2B currents were tested by an analysis of variance or Student’s t test and were accepted as significant if $p \leq 0.05$.
FIGURE 1. Kir6.1/SUR2B and Kir6.2/SUR2B channels expressed in HEK293 cells. A, whole-cell currents were recorded from a cell transfected with Kir6.1/SUR2B. Symmetric concentrations of K+ (145 mmol/L) were applied to the pipette and bath solutions. The cell was held at 0 mV, and pulse voltages from −120 to 80 mV with a 20-mV increment were applied. The current amplitude increased in response to pinacidil (Pin, 10 μmol/L). The pinacidil-induced currents were strongly inhibited by PMA (100 nmol/L) and completely inhibited by glibenclamide (Glib). B, shown is the time course for Kir6.1/SUR2B channel modulation. Whole-cell currents were recorded from a cell transfected with Kir6.1/SUR2B. Symmetric concentrations of K+ (145 mmol/L) were applied to the pipette and bath solutions. The cell was held at 0 mV, and pulse voltages from −80 to +80 mV every 3 s. After whole-cell configuration was formed, the cell was perfused with extracellular solution for a 2-min base-line (BL) recording. The currents were strongly activated by pinacidil (10 μmol/L), and the maximum activation was reached in 3–4 min of the exposure. The currents were inhibited by PMA (100 nmol/L) in ∼5 min and further inhibited by glibenclamide. The lower panel shows individual currents produced by a single command pulse. C, Kir6.1/SUR2B currents were recorded with PKCi (10 μmol/L) in the pipette solution. PKCi almost completely blocked Kir6.1/SUR2B channel inhibition by PMA. D and E, Kir6.2/SUR2B currents were recorded from transfected HEK293 cells with the same treatment as for Kir6.1/SUR2B. PMA did not affect the pinacidil-activated Kir6.2/SUR2B currents. F, shown is a summary of the effects of PMA on Kir6.1/SUR2B and Kir6.2/SUR2B currents activated by pinacidil. PMA inhibited Kir6.1/SUR2B currents strongly (77.5 ± 3.7%, n = 19). The nonactive PMA analog 4α-phorbol 12,13-diacepate (PDD, 100 nmol/L) had little effect on the Kir6.1/SUR2B currents (13.3 ± 8.2%, n = 4). In the presence of PKCi, the PMA effect was blocked (7.1 ± 6.0%, n = 4). PMA had no inhibitory effects on Kir6.2/SUR2B currents (∼0.7 ± 0.7%, n = 5).

We further divided the N terminus into two segments and the C terminus into three. These chimeras showed similar responses to pinacidil and glibenclamide as the WT channels. When the distal N terminus was replaced, the Kir6.1-21_111 channel responded to PMA to the same extent as Kir6.1 (71.1 ± 4.8%, n = 5) (Fig. 4A). However, replacement of the proximal N terminus significantly decreased PMA effects, although such an effect was somehow incomplete (36.7 ± 7.6%, n = 5) (Fig. 4B). A serine residue (Ser-40) was found in the proximal N terminus of Kir6.1 but not in Kir6.2. When Ser-40 was mutated to the corresponding residue (Lys-39) in Kir6.2, the Kir6.1-S40K channel was still inhibited in the same way as the WT Kir6.1 channel (Fig. 5A). Because no PKC site was found in the N terminus, how this protein domain is involved in the PKC action remains to be understood. When the proximal C terminus was replaced, Kir6.1-11_211 responded to PMA to the same degree as the WT channel (Fig. 4C). With the middle or distal segment swapped, the Kir6.1-11_121 and Kir6.1-11_112 channel responses to PMA were significantly diminished (Fig. 4, D and E). PMA inhibition was further decreased in a chimera in which both middle and distal segments were from Kir6.2 (Kir6.1-11_122) (Fig. 4F).

We then used chimeras with the Kir6.2 core to search for the necessary protein domain in the C terminus. Kir6.2–21_211 showed full PMA sensitivity (69.9 ± 6.7%, n = 4) (Fig. 4H), and Kir6.2–21_221 was partially inhibited by PMA (33.7 ± 3.1%, n = 5) (Fig. 4G). With a slight extension of the distal segment, the Kir6.2–21_221′ channel showed full PMA response (78.8 ± 2.5%, n = 5) (Fig. 4I), suggesting that the distal segment contains the essential C-terminal elements for PKC phosphorylation.
the Kir6.1-4A and Kir6.1-5A peptides was almost completely eliminated in comparison with the WT, consistent with our mutational analysis in this region (S403A, S404A, and T414A) (Fig. 5E), suggesting that residues other than Ser-379, Ser-385, Ser-391, and Ser-397 have very little effect, if any, on PKC-dependent channel inhibition.

**DISCUSSION**

The present studies show evidence for the molecular basis underlying Kir6.1/SUR2B channel inhibition by PKC. The channel inhibition relies on a short motif consisting of four phosphorylation repeats in the Kir6.1 subunit. Graded channel inhibitions are produced by phosphorylation of different numbers of these serine residues. The presence of the phosphorylation motif in Kir6.1, but not in its close relative Kir6.2, suggests that the vascular KATP channel may have undergone evolutionary optimization allowing it to be regulated by a variety of vasoconstrictors.

PKC acts on several isoforms of KATP channels. Previous studies indicate that PKC activation leads to inhibition of the vascular Kir6.1/SUR2B channel, and this effect is likely mediated by direct phosphorylation of the channel protein (16, 20). PKC activates the pancreatic isoform (Kir6.2/SUR1) and striated muscular isoform (Kir6.2/SUR2A) of KATP channels (22).

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**FIGURE 2. Effects of recombinant PKC on Kir6.1/SUR2B and Kir6.2/SUR2B currents in inside-out patches.** A, at base line (BL) without nucleotides, the Kir6.1/SUR2B channels were closed. Application of 1 mmol/L ATP and 0.5 mmol/L ADP to the internal patch membrane slightly activated the currents, which were strongly activated by pinacidil (Pin, 10 μmol/L). Application of cPKC inhibited the Kir6.1/SUR2B currents. Glib, glibenclamide. B, without nucleotides, the Kir6.2/SUR2B channel was open. The currents were inhibited by 1 mmol/L ATP and 0.5 mmol/L ADP and activated by pinacidil. Unlike Kir6.1/SUR2B, cPKC had no effects on Kir6.2/SUR2B currents under the same experimental conditions. washout. C, shown is a summary of cPKC effects on Kir6.1/SUR2B and Kir6.2/SUR2B currents that were activated prior by pinacidil. The Kir6.1/SUR2B currents were significantly inhibited by cPKC (61.1 ± 3.8%, n = 6), whereas the Kir6.2/SUR2B currents were not affected (−4.9 ± 6.3%, n = 3).

**FIGURE 3. Responses of Kir6.1-Kir6.2 chimeras to PMA.** All chimeric channels were expressed with SUR2B. A, when the N terminus of Kir6.1 was replaced with that of Kir6.2, PMA failed to inhibit the 211 channel. BL, base line; Pin, pinacidil; Glib, glibenclamide. B, when the C terminus of Kir6.1 was replaced with that of Kir6.2, the 112 channel lost response to PMA. C, construction of both Kir6.1 N and C termini in the Kir6.2 core sequence resulted in PMA sensitivity as in WT Kir6.1/SUR2B. D, shown is a summary of PMA inhibition of chimeras. Kir6.1 N terminus, core, and C terminus refer to residues 1–71, 72–186, and 187–424, respectively. Kir6.2 N terminus, core, and C terminus refer to residues 1–70, 71–176, and 177–390, respectively.

Expressed with SUR2B, the Kir6.2 channel is activated by PKC or shows no response, depending on recording conditions (16, 20). A critical PKC phosphorylation site (Thr-180) has been found in Kir6.2 mediating the fast channel activation (22). However, it is unclear whether a corresponding site in Kir6.1 (Thr-190) plays a similar role, as mutation of the residue leads to a nonfunctional channel (5, 20). PKC also causes Kir6.2/SUR1 and Kir6.2/SUR2A channel internalization (23). Because the effect of PKC on Kir6.1/SUR2B (inhibition) is clearly in contrast to that on Kir6.2-containing channels (activation or no effect), the Kir subunit is likely to be targeted by PKC regulation. The differential responses of Kir6.1 and Kir6.2 to PKC allow chimeric dissections of critical protein domains. Indeed, our studies on the Kir6.1-Kir6.2 chimeras have shown that the proximal N terminus and distal C terminus of Kir6.1 are critical for PKC-dependent channel inhibition.

The proximal N terminus has only 7 residues different between Kir6.1 and Kir6.2 in their primary sequences, none of which can be phosphorylated. Previous studies have indeed shown that the proximal N terminus is important for channel gating by multiple channel regulators. Kir6.2 channels are sensitive to ATP and phosphatidyl 4,5-bisphosphate, and both N
and C termini are important for the sensitivities (24–27). A notable difference between Kir6.1 and Kir6.2 channels is that in the absence of nucleotides the Kir6.2 channels are spontaneously open, whereas the Kir6.1 channels are completely closed in the absence of nucleotides the Kir6.2 channels are spontaneously open, whereas the Kir6.1 channels are completely closed.

**FIGURE 4. Dissection of critical protein domains for PKC-dependent channel inhibition.** The N terminus was further divided into two segments at residue 35 in Kir6.1 and at residue 34 in Kir6.2. The C terminus was divided into three segments at residues 275 and 363 in Kir6.1 and at residues 265 and 354 in Kir6.2.

A notable difference between Kir6.1 and Kir6.2 channels is that in the absence of nucleotides the Kir6.2 channels are spontaneously open, whereas the Kir6.1 channels are completely closed (28), a phenomenon that is also explained as fast rundown of the Kir6.1 channels (29). Using Kir6.1-Kir6.2 chimeras, Kondo et al. (28) found that both the N and C termini are important for this difference, whereas the N terminus seems to be more critical; 5 residues in the proximal N terminus are found to play a major role in the difference. The Kir6.2 channels are also activated by intracellular H+ (30). The pH-dependent channel gating requires both N and C termini, although there is only one protonation site (His-175) located in the C terminus (21, 31). The importance of the proximal N terminus in channel gating has been studied with different ligands, indicating that this region is indeed involved in Kir6.2 channel gating rather than ligand binding (32, 33). In addition to Kir6.2, the Kir2.3 channel is gated by acidic pH in an N terminus-dependent manner, and there is no protonation site in the N terminus (34). The requirement of the N terminus for PKC-dependent channel inhibition found in our current studies is thus consistent with these previous reports, suggesting that this protein domain is likely to be involved in interactions with other protein domain(s) in channel gating.

The distal C terminus (residues 346–424) of Kir6.1 is critical for PKC-dependent channel inhibition. This protein domain is a direct target of PKC, as multiple PKC phosphorylation sites are found in this narrow region. Our results indicate that there are at least four phosphorylation sites in this region, i.e., residues 379, 385, 391, and 397. Our patch clamp study suggests that Ser-354 is another, a result that is not supported by our in vitro phosphorylation assay. Therefore it is unclear whether Ser-354 can be phosphorylated in vivo. Of them, Ser-397 in human Kir6.1 has been previously reported to be phosphorylated by an unknown kinase in a global phosphorylation screening study (35). None of the rest has been studied previously. Interestingly, the effects of phosphorylation of these residues are additive or cumulative. Mutation of each individual residue reduces PKC effects by ~20%. Combined mutations of multiple residues have greater effects. Channel inhibition is almost completely abolished with mutations of all five (including Ser-354). Multiple phosphorylation sites often result in sequential phosphorylation, which does not seem to be the case in
Kir6.1/SUR2B, as our results suggest that none of the residues shows a dominant effect over others. Of the five putative phosphorylation sites, 4 serine residues appear in a clear pattern of repeats (SXRKRAN/SXRKRAN). To our knowledge, this is the first demonstration regarding such a phosphorylation motif in ion channels.

The distal C termini of inward rectifier K⁺ channels play a critical role in channel trafficking and thus control the number of functional channels on the cell membrane (36–39). There is an endoplasmic reticulum retention sequence (RKR, residues 381–383 in Kir6.1 and 369–371 in Kir6.2) (40), which warrants the Kir6 subunit targeting the membrane together with the SUR subunit. A dileucine motif (Leu-355/Leu-356) in Kir6.2 is involved in channel endocytosis (23). Our results indicate that the distal C terminus of Kir6.1 is also involved in channel activity control (gating). In the narrow C-terminal region where the last four PKC sites are found, there are 9 alkaline residues, including the RKR endoplasmic reticulum retention signal, making this region extremely positively charged. The positive charges appear to favor the open state in the presence of pinacidil or other channel openers. These charges are largely neutralized following phosphorylation of the serine repeats, which may attenuate the channel opening.

It is noteworthy that although we used PMA and pinacidil to optimize the experimental condition, similar PKC-dependent channel inhibition has been shown to play a role in the channel modulation by vasopressin in our previous studies (6). Consistently, endothelin (15), muscarinic M3 receptor agonist (16), and angiotensin II (17) also inhibit the vascular KATP channels in a PKC-dependent manner. Because some of these previous studies were performed in mesenteric arteries and dissociated vascular smooth muscles cells, the channel modulation mechanism demonstrated in the present study is of physiological significance.

Why does the PKC phosphorylation motif exist in Kir6.1 but not in Kir6.2? The phosphorylation motif renders 16 phosphorylation sites in a KATP channel with four Kir6.1 subunits. This, as well as the cumulative nature of each phosphorylation, may allow the channel to be elaborately modulated according to the levels of PKC activation, which is consistent with the functional needs of the vascular KATP channel targeted by a variety of vasoconstricting hormones and neurotransmitters. The presence of the phosphorylation motif in Kir6.1, but not in its close

FIGURE 5. Mutagenesis analysis of potential PKC phosphorylation sites. A, a mutation Ser-403 of Kir6.1 to alanine did not affect the PMA inhibition. BL, base line; Pin, pinacidil; Glib, glibenclamide. B, mutation of Ser-385 decreased PMA effects. C and D, when four and five potential PKC sites were mutated to alanine simultaneously, the channel inhibition was largely eliminated. E, shown is a summary of the mutations. 3A, Kir6.1-S385A/S391A/S397A; 4A, Kir6.1-S379A/S385A/S391A/S397A; 5A, Kir6.1-S354A/S379A/S385A/S391A/S397A. Data were obtained from four to nine patches. White bars, p > 0.05; black bars, p < 0.05.

FIGURE 6. In vitro phosphorylation on MBP fusion proteins. A, four MBP fusion proteins were constructed by linking the distal C-terminal fragment of Kir6.1 (residues 346–424) to MBP with or without mutations of critical serine residues. The sequence underlined is the antigen for Western detection. B, all the constructs showed two bands on a Western blot. The band at ~52 kDa represented the intact fusion proteins (arrows), whereas the band at ~49 kDa was a degraded protein fragment (arrowheads). These bands were weakly detected in Kir6.1-3A (S385A/S391A/S397A), Kir6.1-4A (S379A/S385A/S391A/S397A), and Kir6.1-5A (S354A/S379A/S385A/S391A/S397A) by Western blotting because mutations were made in the antigen region. The lower panel shows protein input stained with Coomassie Blue. C, in vitro phosphorylation showed strong 32P incorporation in both 52-kDa and 49-kDa bands in the WT. The 32P incorporation was markedly reduced in Kir6.1-3A, Kir6.1-4A, and Kir6.1-5A were only weakly phosphorylated in the 52-kDa band. By comparing Kir6.1-3A and Kir6.1-4A, the phosphorylation of the 49-kDa band on Kir6.1-3A must occur at Ser-379. That the 49-kDa band of Kir6.1-4A was not phosphorylated also suggested that Ser-354 was not phosphorylated. In addition, phosphorylation on the 52-kDa band of Kir6.1-4A and Kir6.1-5A suggests that there are unidentified phosphorylation site(s) located C-terminal to Ser-379, but they were not functionally important according to mutational analysis in Fig. 5E.
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Relative Kir6.2, suggests that the vascular $K_{ATP}$ channel may have undergone evolutionary optimization serving for the vascular regulation under various physiological and pathophysiological conditions.

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