Molecular basis of protein kinase C-induced activation of ATP-sensitive potassium channels

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Potassium channels that are inhibited by internal ATP (K_{ATP} channels) provide a critical link between metabolism and cellular excitability. Protein kinase C (PKC) acts on K_{ATP} channels to regulate diverse cellular processes, including cardioprotection by ischemic preconditioning and pancreatic insulin secretion. PKC action decreases the Hill coefficient of ATP binding to cardiac K_{ATP} channels, thereby increasing their open probability at physiological ATP concentrations. We show that PKC similarly regulates recombinant channels from both the pancreas and heart. Surprisingly, PKC acts via phosphorylation of a specific, conserved threonine residue (T180) in the pore-forming subunit (Kir6.2). Additional PKC consensus sites exist on both Kir and the larger sulfonylurea receptor (SUR) subunits. Nonetheless, T180 controls changes in open probability induced by direct PKC action either in the absence of, or in complex with, the accessory SUR1 (pancreatic) or SUR2A (cardiac) subunits. The high degree of conservation of this site among different K_{ATP} channel isoforms suggests that this pathway may have wide significance for the physiological regulation of K_{ATP} channels in various tissues and organelles.

Potassium channels that are inhibited by ATP (K_{ATP} channels) consist of a heterooctamer of four sulfonylurea receptor (SURx) and four inwardly rectifying K^+ channel (Kir6.x) subunits (1–5). The SUR is a member of the ATP-binding cassette (ABC) family of proteins and acts as a regulatory subunit, conferring ADP sensitivity and the distinctive pharmacological characteristics on the K_{ATP} channel complex (1–6). In contrast, the Kir6.x subunit forms the pore of the channel and mediates the defining ATP-dependent inhibition of K_{ATP} channels (6). Protein kinase-catalyzed phosphorylation is an important mechanism by which the activity of ion channels, including the K_{ATP} channel, can be controlled (7–9). For instance, another ABC protein ion channel, the cystic fibrosis transmembrane conductance regulator, is regulated by cAMP-dependent protein kinase-mediated phosphorylation, which, itself, may be permissively regulated by protein kinase C (PKC) (8, 10, 11). In addition, mounting evidence suggests the importance of PKC in activating K_{ATP} channels during both the protective mechanism of ischemic preconditioning (12, 13) and in regulating insulin secretion (14), although the site(s) and mechanism of action of PKC-mediated phosphorylation events have not been described. Therefore, we sought to determine: (*i*) the functional effects of PKC on the K_{ATP} channel, (*ii*) whether the action of PKC is mediated via the SUR or Kir6.2 subunit, and (*iii*) the identity of specific amino acid residue(s) phosphorylated by PKC.

Materials and Methods

Cell Culture and Transfection. tsA201 cells (an SV40-transformed variant of the HEK293 human embryonic kidney cell line) were maintained in DMEM supplemented with 10 mM glucose/2 mM L-glutamine/10% FCS/0.1% penicillin/streptomycin at 37°C (10% CO_2). Cells were plated at 30–40% confluence on 35-mm culture dishes 4 h before transfection. K_{ATP} channel subunit clones were generously provided by Lydia Aguilar-Bryan and Joseph Bryan (hamster SUR1; ref. 4) and Susumu Seino (mouse Kir6.2 and rat SUR2A; ref. 5). Clones were inserted into mammalian expression vectors (pCDNA3 or pCMV6) and were transfected into tsA201 cells by calcium phosphate precipitation. Transfected cells were identified by using coexpression of the K_{ATP} channel subunit clones with the green fluorescent protein [Green Lantern (pGL); Life Technologies, Rockville, MD]. Recordings were made from cells 48–72 h after transfection. Coexpression of pGL or pCDNA3 without any channel constructs inserted did not produce any measurable whole-cell current (data not shown).

Electrophysiology. The nystatin-perforated patch technique was used to measure whole-cell currents while maintaining the integrity of the intracellular environment (15). Block by external barium (2 mM) was used to reversibly indicate the amount of ΔC26 current present. For cells coexpressing the SUR isoforms (SUR1 and SUR2A), block by glibenclamide (10 μM) also was used to indicate the amplitude of K_{ATP} current.

All whole-cell recordings were made with symmetrical K^+ (140 mM), using 100-ms command steps from -90 to $+60$ mV at 10-mV intervals, to establish current–voltage relationships. Data were acquired and analyzed by using PCLAMP 5.5 and 6.0 software (Axon Instruments, Foster City, CA).

Standard patch-clamp techniques were used to record single-channel currents in the inside-out patch configuration. The internal faces of the patches were exposed to test solutions via a multiinput perfusion pipette (solution changes complete in <2 s). Single-channel currents were recorded at fixed holding potentials, amplified (Axopatch 200; Axon Instruments), digitized (Neuro-corder DR-384; Neuro Data Instruments, New York), and then stored on videotape. Data were sampled at 500 Hz and filtered at 200 Hz, with the exception of the recordings shown in Fig. 4*D*, which were sampled at 2.5 kHz and filtered at 1 kHz.

The pipette solution used for all patch recordings contained the following: 140 mM KCl, 10 mM Hepes, 1.4 mM $MgCl_2$, 1 mM EGTA, and 10 mM glucose. The pH of the solution was adjusted to 7.4 with KOH. This solution also was used to superfuse the cells/patches for experiments using symmetrical K^+.

ATP (as MgATP; Sigma) was added as required from a 10-mM stock, which was prepared immediately before use. Glibenclamide (Sigma) was stored as a 10-mM stock solution in DMSO. 4-β-Phorbol 12-myristate 13-acetate (PMA) (Sigma) and 4-α-phorbol 12,13-didecanoate (PDD) (Calbiochem) were

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Abbreviations: K_{ATP} channel, ATP-sensitive potassium channel; PMA, phorbol 12-myristate 13-acetate; PDD, phorbol 12,13-didecanoate; PKC, protein kinase C; SUR, sulfonylurea receptor; Kir6, inwardly rectifying K^+ channel.

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stored in ethanol at a concentration of 1×10^{-4} M at −20°C. Chelerythrine chloride (Calbiochem) was stored at 5 mM in distilled H_2O at −20°C. All drugs were diluted to the required concentration immediately before use.

PKC was purified from rat brain and rendered constitutively active by proteolytic cleavage by using the techniques described previously (16). In patch-clamp experiments, PKC was used at a final concentration of 20 nM.

Statistical significance was evaluated by using Student's paired t test. Differences with values of probability $P < 0.05$ were considered to be significant. Values in the text are given as mean ± SEM.

Molecular Biology. Point mutations were introduced into the mouse Kir6.2 clone and the Kir6.2 ΔC26 construct by using the Unique Site Elimination (U.S.E.) Mutagenesis Kit (Amersham Pharmacia). The truncated Kir6.2 construct ΔC26 was made by the introduction of a stop codon at the appropriate position. The coding sequence for an N-terminal M5-FLAG fusion protein (Sigma) was introduced into the coding region of the ΔC26 construct immediately after the methionine start codon. Measurable glibenclamide-insensitive whole-cell currents were obtained, after dialysis with an ATP-free pipette solution, from cells expressing the ΔC26 and ΔC26/FLAG constructs (data not shown). The rabbit heart Kir6.2 clone was identified by PCR screening of a rabbit heart λgt10 cDNA library by using a probe based on the pore region of Kir6.1 and Kir6.2. *Kpn*I restriction site analysis and sequencing then were used to confirm the identity of the Kir6.2 clone.

Phosphorylation Assays. Membrane proteins from tsA201 cells cotransfected with either the ΔC26/FLAG or ΔC26(T180A/E)/FLAG constructs plus pGL were isolated by using procedures described previously (17). Protein phosphorylation was performed *in vitro* on membrane fractions, using [γ-^{32}P]ATP as a substrate for PKC and using the techniques described previously (17). ^{32}P-labeled C26Δ/FLAG proteins then were extracted from the crude membrane preparation by using M5-FLAG affinity beads (Sigma) and then subjected to autoradiographic analysis. Western blot analysis was performed in parallel by using the enhanced chemiluminescence procedure (Amersham Pharmacia) with M5-FLAG antibody. Neither significant phosphorylation nor FLAG antibody staining was detected at the molecular mass (45 kDa) corresponding to ΔC26/FLAG on gels or Western blots of crude membrane protein from the following: untransfected tsA201 cells or cells transfected with pGL alone (not shown). Nor was any significant phosphorylation detected in membrane preparations to which boiled PKC was added or to which no PKC was added. Labeling of a 45-kDa protein was detected in cells transfected with ΔC26/FLAG after application of PMA to intact cells to stimulate PKC activity in the presence of ^{32}P-labeled orthophosphate.

Using either crude membrane homogenates or purified ΔC26/FLAG protein, a reduction in the amount of PKC-catalyzed phosphorylation was always observed when the threonine residue at position 180 was substituted by an alanine or glutamate residue. The inability of PKC peptide inhibitor PKC(19–31) to completely abolish PKC-mediated phosphorylation probably can be attributed to its effective "quenching" by the excess protein in crude tissue homogenates used for the phosphorylation reaction (see ref. 18). In separate experiments using affinity bead-purified ΔC26/FLAG protein as substrate, PKC(19–31) appeared more effective in blocking the effects of PKC (data not shown). Also, in inside-out patch recordings, 5 μM PKC(19–31) completely inhibited up-regulation of channel activity by PKC (Fig. 1*A Inset*). Thus, PKC(19–31) effectively inhibited PKC action in the absence of excess protein.

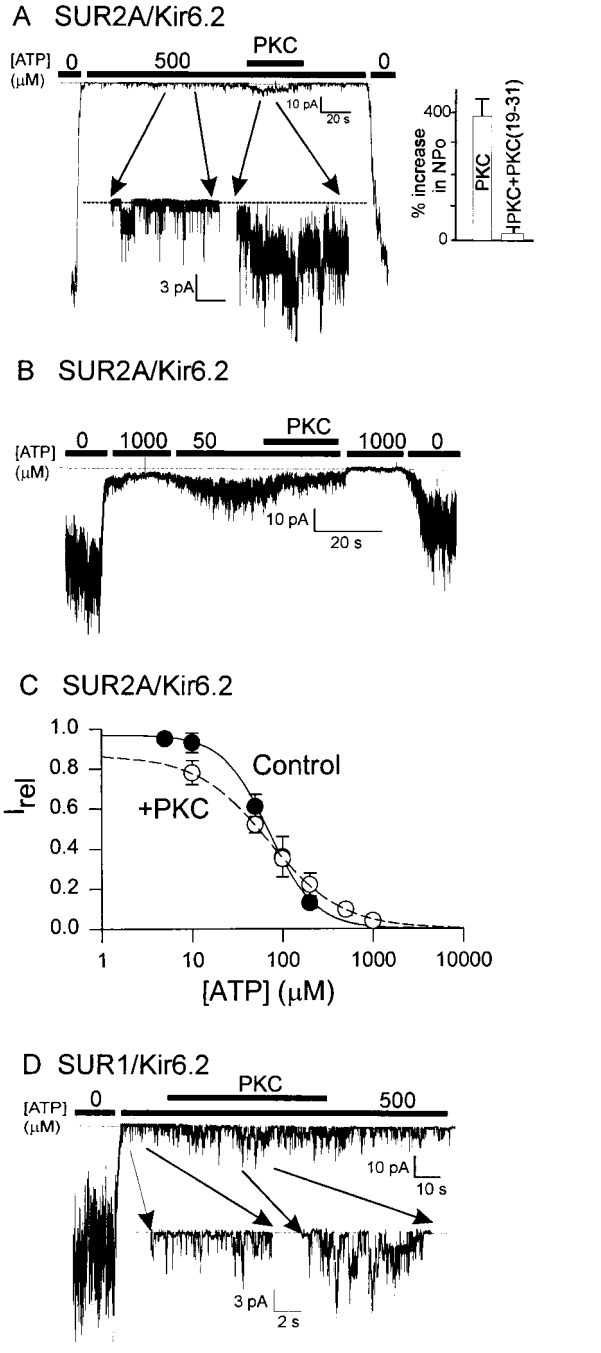

Fig. 1. PKC and mechanisms of action on the K_{ATP} channel. (*A* and *B*) Representative currents from an inside-out patch containing wild-type (WT) (SUR2A/Kir6.2) cardiac K_{ATP} channels. The patches were excised and held at −50 mV in symmetrical K^+ (140 mM) and then exposed to different internal ATP concentrations. The addition of constitutively active PKC (20 nM) is indicated by the solid bar. (*A*) Note that PKC activates SUR2A/Kir6.2 channel activity in the presence of 500 μM ATP. *Inset* shows cumulative data from five patches (PKC) or three patches [PKC(19–31)]. The PKC inhibitor peptide PKC(19–31) was used at a concentration of 5 μM. (*B*) Note that PKC inhibits SUR2A/Kir6.2 channel activity at low (50-μM) levels of ATP. (*C*) Estimates of IC_{50} were obtained by grouping data from between four and seven patches, at each ATP concentration, in the absence (●) or presence (○) of PKC. Data were fitted to the equation $I_{rel} = 1/\{1 + ([ATP]/IC_{50})^n\}$, where I_{rel} is the current relative to the maximal current observed in the absence of ATP and n is the Hill coefficient. IC_{50} values of 70 μM and 71 μM were determined in the absence and presence of PKC, respectively. (*D*) Representative WT (SUR1/Kir6.2) pancreatic beta cell K_{ATP} channel current response to PKC from an inside-out patch under the same conditions as in *A*.

Accession Numbers. Kir6.2 GenBank accession numbers for mouse, human, and rat amino acid sequences are Q61743, Q14654, and D8603, respectively. The rabbit Kir6.2 sequence is identical to the sequence entered into GenBank by E. Marban, M. Janecki, and B. O'Rourke (accession no. AAB61473).

RESULTS

PKC and K_{ATP} Channel Activation. Our earlier studies on cardiac myocytes showed that PKC increases the open probability of K_{ATP} channels at physiological levels of ATP by reducing the Hill coefficient for the ATP dose–response curve, thus rendering the channel less sensitive to inhibition by high ATP concentrations (16). One possible interpretation of this observation is that phosphorylation reduces the positive cooperativity of ATP binding required for inhibition. To further understand the underlying mechanisms, we have studied the cardiac (SUR2A/Kir6.2) and pancreatic beta cell (SUR1/Kir6.2) isoforms of the K_{ATP} channel (1–3) expressed in mammalian cells. Use of the patch-clamp technique in the excised inside-out patch configuration showed that constitutively active PKC caused a 390 ± 78% increase in channel activity (measured as NP_o, the product of N, the number of channels in the patch, and P_o, the mean open probability) for SUR2A/Kir6.2 channels (Fig. 1*A*). The effect of PKC was blocked by concomitant treatment with the specific PKC inhibitor peptide PKC(19–31) (35 ± 14% increase, Fig. 1*A Inset*). PKC also induced a similar increase in NP_o (360 ± 31%, $n = 5$) for pancreatic beta cell K_{ATP} (SUR1/Kir6.2) channels (Fig. 1*D*). Construction of ATP dose-inhibition curves revealed that PKC reduced the Hill coefficient from 1.65 to a value of 1.10 in SUR2A/Kir6.2 channels (Fig. 1*C*). The calculated crossover point of the curves was 110 μM ATP, indicating that, at higher ATP concentrations, the effect of PKC will be excitatory (Fig. 1*A*), whereas at lower ATP concentrations (<110 μM), the effect of PKC will be inhibitory (Fig. 1*B*). These findings parallel those observed previously on native cardiac K_{ATP} channels (16) and indicate that the recombinant channels contain all of the mechanistic elements required for this behavior. In the majority of patches tested (13 of 19), the effect of PKC was partially or fully reversible upon washout of the kinase. This reversal was prevented by treatment with okadaic acid (10 nM), a protein phosphatase inhibitor (19), indicating the presence of an endogenous phosphatase (data not shown).

PKC Acts on the Kir6.2 Subunit. The recent discovery that Kir6.2 forms functional ATP-sensitive channels in the absence of SUR, when the C-terminal 26 aa are removed to yield the deletion mutant ΔC26 (6), provided a useful approach to determine whether PKC acts via the SUR or Kir6.2 subunit. We studied PKC action after expressing either ΔC26, in the absence of the associated SUR subunit, or the full-length Kir6.2 subunit with either SUR1 or SUR2A.

In cells expressing ΔC26 alone, application of the membrane-permeant phorbol ester PMA (100 nM), a PKC activator, caused a significant increase in whole-cell current ($P < 0.05$) of 376 ± 120% (Fig. 2*A* and *B*). PMA failed to activate any current (4.5 ± 14% increase, $P = 0.26$, Fig. 2*B*) when cells were pretreated for 10 min with chelerythrine (5 μM), a membrane-permeant PKC inhibitor (20). The inactive phorbol ester analog, PDD (100 nM), did not increase the ΔC26 current (6.1 ± 10% increase, $P = 0.67$, Fig. 2*A* and *B*). Thus, activation of PKC enhances K_{ATP} channel currents in the absence of the sulfonylurea receptor subunit to a similar extent as with the wild-type, heterooctameric channels (Fig. 2*B*).

Coexpression of the β-pancreatic SUR isoform, SUR1, with Kir6.2 also yielded currents that were enhanced by PMA. A significant increase in current of 440 ± 210% was observed ($P < 0.05$). The PMA-induced current was sensitive to glibenclamide (increment reduced to 42 ± 40%). No significant increase in

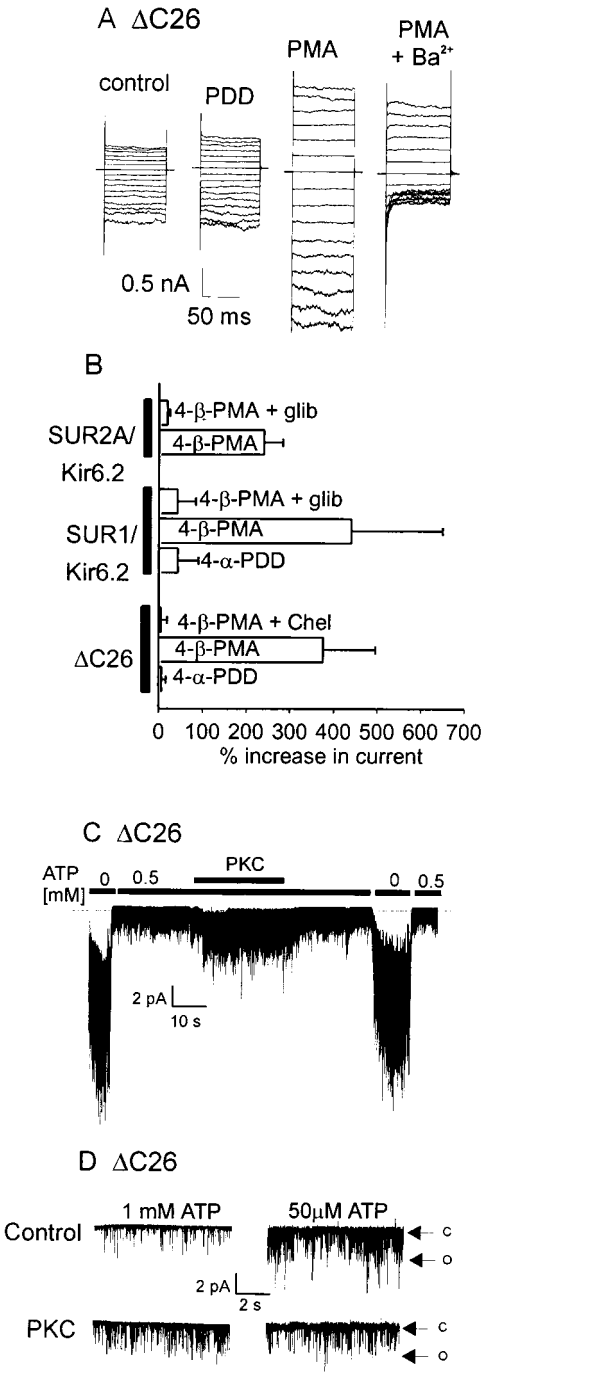

Fig. 2. PKC activates K_{ATP} channels in the presence and absence of the SUR subunit. (*A*) Representative whole-cell current recordings from a tsA201 cell expressing the Kir6.2 ΔC26 truncation mutant by using the nystatin-perforated patch technique (20). Recordings were made 6 min after the application of either the inactive phorbol analog (PDD, 100 nM) or the active phorbol ester PMA (100 nM). Block by external barium (2 mM) was used to indicate K_{ATP} current amplitude. (*B*) Cumulative data from whole-cell current experiments using ΔC26 alone, SUR1/Kir6.2, or SUR2A/Kir6.2 ($n = 4–8$ cells for each group). (*C*) Recording from an inside-out patch containing multiple ΔC26 channels that shows an increase in activity on application of constitutively active PKC (20 nM). (*D*) Inside-out patch recordings from ΔC26 channels showing the effects of constitutively active PKC on channel activity at an internal [ATP] of either 1 mM or 50 μM. Note that PKC activates ΔC26 in the presence of high (1 mM) ATP but inhibits ΔC26 activity at low (50 μM) ATP. Holding potential in both *C* and *D* was −50 mV using symmetrical, 140 mM K^+. C and O indicate closed and open levels, respectively.

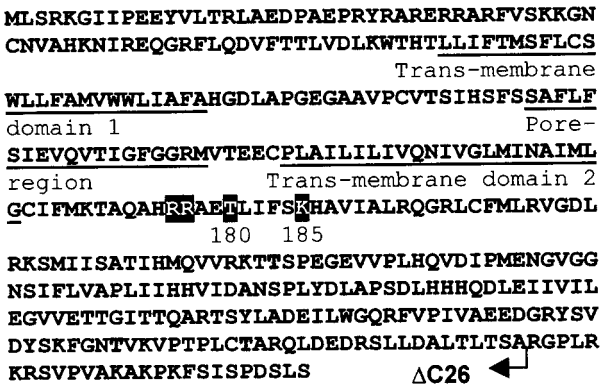

Fig. 3. Amino acid sequence of the rabbit heart Kir6.2 subunit. T180, situated within the best consensus site for PKC phosphorylation, is highlighted white on black. Other potential PKC phosphorylation sites are shaded. The position of the ΔC26 truncation is denoted by the arrow.

current (43 ± 47%, $P = 0.5$) resulted from application of PDD. In addition, coexpression of Kir6.2 with the cardiac SUR isoform, SUR2A, yielded currents that showed a significant increase in response to PMA (240 ± 43%, $P < 0.05$). This increment was glibenclamide-sensitive (reduced to 18 ± 6%, Fig. 2*B*). Excised, inside-out patches from cells expressing the ΔC26 construct consistently revealed an ATP-sensitive current with fast-gating kinetics (Fig. 2 *C* and *D*). In the recording illustrated in Fig. 2*D* (1 mM ATP), PKC increased P_o by ≈300% (0.01 to 0.043). The mean open time, however, remained unchanged after addition of PKC (1.93 ms, control vs. 2.06 ms, PKC). A similar stimulatory effect of PKC on ΔC26 channels was observed in each of five patches tested at ATP concentrations of 0.5 and 1 mM. At low concentrations of ATP (50 μM), PKC caused an inhibition of ΔC26 current in a similar manner to that observed with SUR2A/Kir6.2 channels (36 ± 12% decrease, $n = 3$ patches, $P < 0.05$, Fig. 2*D*).

PKC Functionally Phosphorylates a Single Residue in Kir6.2. The above findings provide strong evidence that the stimulatory effect of PKC occurs via the Kir6.2 subunit, because it is observed in the absence of the SUR subunit. Therefore, we set out to identify which amino acid residue(s) in Kir6.2 may be phosphorylated by PKC. Recent work indicates that ATP binding occurs in the Kir6.2 subunit and mutations at sites in the proximal C terminus of the Kir6.2 subunit can alter gating and ATP binding (6, 21). For example, K185 is important in determining the ATP sensitivity of the Kir6.2 subunit expressed alone (as ΔC26) (6). PKC consensus sites have at least one basic residue (arginine or lysine) several positions upstream or downstream (or preferably both) from the phosphorylated residue (22). Four highly conserved, potential PKC phosphorylation sites were identified in Kir6.2 (Fig. 3). Of these four sites, T180

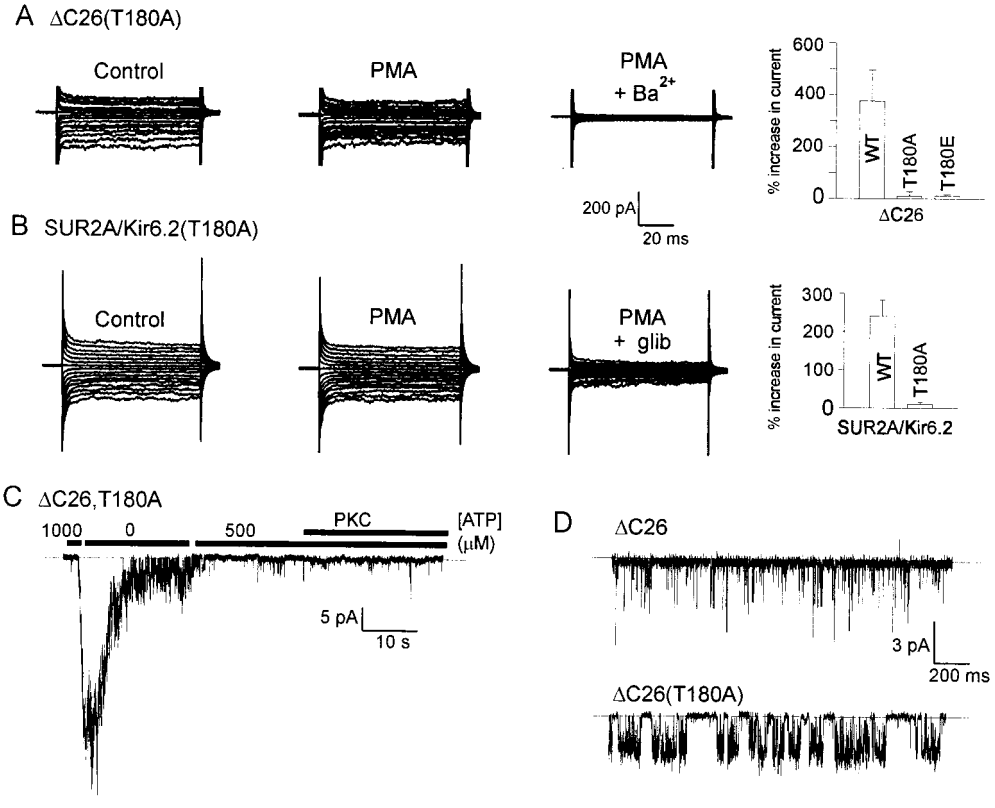

Fig. 4. Phorbol ester (PMA) does not increase currents in cells expressing K_{ATP} channels with the T180A Kir6.2 mutation. (*A*) Representative whole-cell current recordings of ΔC26(T180A) channel activity in response to 100 nM PMA (6 min) by using the nystatin-perforated patch technique. (*B*) Whole-cell perforated patch current recording from a cell coexpressing SUR2A and a full length Kir6.2(T180A) mutant showing the effect of 100 nM PMA (6 min). The histograms represent cumulative data from between four and six cells; wild-type (WT) data are replotted from Fig. 2*B* for comparison. (*C*) Representative inside-out patch recording of ΔC26(T180A) channel activity showing the response to ATP and constitutively active PKC. No effect of PKC on ΔC26(T180A) channels was observed in any of three patches tested. (*D*) Representative inside-out patch, single-channel recordings from ΔC26 and ΔC26(T180A) channels. Longer open times than for the wild-type ΔC26 were observed in each of three patches containing the ΔC26(T180A) mutant channel. Recordings were made in the absence of ATP at a holding potential of −50 mV in symmetrical, 140 mM K^+.

lies in the best PKC recognition sequence, with R176 and R177 lying upstream and K185 downstream. In addition, R176 and R177 are thought to interact with anionic phospholipids, which modulate the ATP sensitivity of the channel (23, 24). To determine whether these residues are conserved in the rabbit [the species used for our earlier studies on native K_{ATP} channels (16)], we cloned the full-length coding region of rabbit heart Kir6.2. Sequence analysis revealed a high degree of identity (>93%) of Kir6.2 from rat, mouse, human, and rabbit. Both T180 and K185 are conserved in these species, as are the two positively charged arginines at positions 176 and 177. These residues are also conserved in Kir6.1, which may form the pore of other K_{ATP} channel isoforms.

To test the hypothesis that T180 is necessary for PKC-induced modulation, we examined the effects of replacing this residue with alanine (T180A) or glutamate (T180E) in both full-length Kir6.2 and ΔC26 constructs. In whole-cell, perforated-patch recordings, application of 100 nM PMA to cells expressing either the ΔC26(T180A) or ΔC26(T180E) constructs had no significant effect on current [7.2 ± 6.0% and 13 ± 4.6% increments, respectively ($P > 0.05$); see Fig. 4*A*]. To determine whether the SUR subunit confers any additional PKC-induced activation of current, cells were cotransfected with SUR2A and a full-length Kir6.2(T180A) mutant. Application of PMA had no significant effect on the SUR2A/Kir6.2(T180A) whole-cell current (8.7 ± 7.4%, $P > 0.05$, Fig. 4*B*). Excised inside-out macropatch recordings from cells expressing the ΔC26(T180A) construct revealed no effect of PKC on channel open probability in any of three patches tested (Fig. 4*C*). Thus, T180 appears to be essential for the PKC-induced increase of K_{ATP} currents, regardless of whether Kir6.2 is coassembled with SUR.

In single-channel recordings, the ΔC26(T180A) construct gave rise to channels showing a markedly longer open state than the ΔC26 construct. The mean open time was increased from ≈1 ms in ΔC26 channels to ≈40 ms in ΔC26(T180A) channels (Fig. 4*D*). Similar results were observed with T180E, suggesting that charge at this location is not the sole determinant of gating behavior. Nonetheless, these observations reinforce the argument that this region of the Kir subunit is a critical determinant of channel-gating behavior. It should be noted that the ΔC26(T180A) mutant lost activity or "ran down" faster in excised patches than wild-type ΔC26 or SUR2A/Kir6.2 channels. We were unable to record activity from the ΔC26(T180E) mutant in the inside-out patch configuration.

PKC Directly Phosphorylates T180 in Kir6.2. To test biochemically whether PKC phosphorylates residue T180 in the Kir6.2 subunit, we engineered an M5-FLAG epitope onto the N terminus of the ΔC26 Kir6.2 truncation mutant (ΔC26/FLAG) and also onto the ΔC26/FLAG construct in which T180 was replaced by alanine [ΔC26(T180A)/FLAG] to remove the putative phosphoacceptor site. Membrane protein fractions from cells transfected with either the ΔC26/FLAG or ΔC26(T180A)/FLAG construct were subjected to *in vitro* PKC phosphorylation assays by using [γ-^{32}P]ATP as the phosphate donor. Western blot analysis of the purified (ΔC26/FLAG) proteins (using an M5-FLAG antibody) showed a distinct protein band at ≈45 kDa, the predicted molecular mass of one subunit of Kir6.2 (Fig. 5*B*). Autoradiographs of the purified (ΔC26/FLAG) proteins revealed that the single amino acid substitution of T180 by alanine almost completely abolished the PKC-catalyzed phosphorylation in the corresponding band (see Fig. 5*A*, lanes 1 and 2). Similar results were obtained in experiments with ΔC26(T180E)/FLAG. Pretreatment with chelerythrine (20), at a concentration that specifically blocks PKC action, largely prevented ^{32}P labeling (Fig. 5*A*, lanes 5 and 6). The PKC pseudosubstrate inhibitor peptide, PKC(19–31) (25), reduced the level of phosphorylation to a variable extent (Fig. 5*A*, lanes 3 and 4; see *Materials and Methods*, *Phosphorylation Assays* section, for discussion).

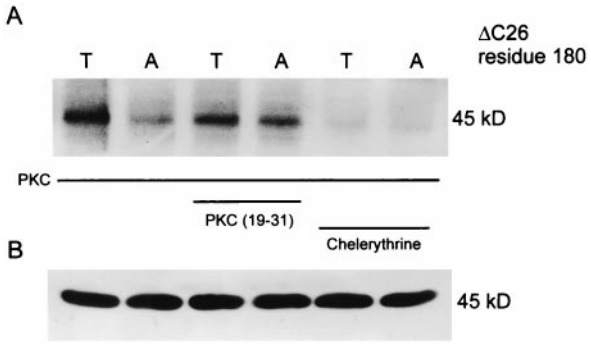

Fig. 5. Autoradiographic analysis of PKC-mediated phosphorylation of the K_{ATP} channel. (*A*) Autoradiograph showing an assay of PKC-catalyzed phosphorylation of the band corresponding to antibody-purified ΔC26/FLAG proteins with either threonine (T) or alanine (A) at position 180 (lanes 1 and 2). Phosphorylation of membrane proteins from tsA201 cells expressing either ΔC26/FLAG or ΔC26(T180A)/FLAG was performed by using [γ-^{32}P]ATP as the phosphate donor. The PKC concentration in the reaction mixture was 250 nM. For the indicated bands, the PKC inhibitor PKC(19–31) (500 nM) or chelerythrine (5 μM) was added to the phosphorylation reaction mixture before the addition of PKC. Under these conditions, the specific inhibitor peptide PKC(19–31) (lanes 3 and 4) caused a significant reduction in phosphorylation (see *Materials and Methods*, Phosphorylation Assays section) whereas chelerythrine (lanes 5 and 6) almost completely prevented PKC-catalyzed phosphorylation. (*B*) Corresponding Western blot of the same protein samples as in *A*, indicating comparable levels of ΔC26/FLAG protein in all lanes.

Discussion

Our electrophysiological data reveal that removal of the phosphoacceptor site at T180 eliminates the stimulatory effects of PKC on the K_{ATP} channel; the biochemical results show that the level of PKC-catalyzed Kir6.2 phosphorylation is markedly reduced by mutations at this site. Other residues in the Kir6.2 subunit also may be phosphorylated to a lesser extent, but do not significantly influence channel activity in our electrical recordings. Thus, we conclude that the up-regulation by PKC of K_{ATP} channel activity can be assigned to phosphorylation at T180 in the Kir6.2 subunit. In contrast, it has been shown recently that protein kinase A phosphorylates the serines at positions 372 in the Kir6.2 subunit and 1,571 in the SUR1 receptor (26).

The sensitivity of the K_{ATP} channel to sulfonylureas, K_{ATP} channel openers, and ADP, is conferred upon the channel by the SUR protein, with some of these effects being isoform-specific (1–3). In contrast, the K^+-conducting, inward rectification, and characteristic ATP-dependent inhibition of the K_{ATP} channel resides in the Kir6.2 subunit (6). Single-channel recordings from the ΔC26(T180A) mutant show that this mutation also affects gating kinetics, stabilizing the open state of the channel (Fig. 4*D*). Together, our findings underline the importance of the proximal C terminus in determining the ATP sensitivity and the gating behavior of the channel. The proximity of residue T180 to identified regions (R176, R177) of phospholipid binding (23, 24), the increased run-down of the ΔC26(T180A) mutant, and the lack of ΔC26(T180E) activity in inside-out patches combine to suggest that this region is important in controlling both phospholipid sensitivity and run-down of the K_{ATP} channel complex (23, 24). Moreover, interactions between PKC- and phospholipid-mediated modulation would raise the possibility of up- or down-regulation of activity. Thus, phospholipids and PKC could alter the shape or position of the ATP dose–response relation to allow channel activity over the range of physiological (mM) ATP concentrations.

K_{ATP} channels are expressed in many tissues, and Kir6.x subunits are the pore-forming subunits that associate with different isoforms of the SUR protein to constitute the various K_{ATP} subtypes that are characteristic of different tissues and organelles (1–3). The amino acid sequence of Kir6.2, including the phosphorylation site, is also highly conserved among several mammalian species. The implications of K_{ATP} channel regulation by PKC for ischemic preconditioning are potentially important, but the details remain open to vigorous debate and investigation (12, 13, 27). For example, the relative importance of sarcolemmal vs. mitochondrial K_{ATP} channels in the process of ischemic preconditioning mediated by PKC is still unresolved (28, 29). The homologous PKC consensus site is also highly conserved in Kir6.1, which may form the pore of smooth muscle (30) and mitochondrial (31) isoforms of the K_{ATP} channel. However, the molecular basis for the inhibitory effects of PKC on the smooth-muscle K_{ATP} channel isoform remains to be investigated. Activation of PKC has been linked specifically to the protective role of mitochondrial K_{ATP} channels (13, 32). Thus, the site of modulation by PKC that we have identified may provide a common mechanism for the up- or down-regulation of several isoforms of the K_{ATP} channel, with wide implications under both physiological and pathological conditions.

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