

Protein kinase C modulation of recombinant ATP-sensitive K⁺ channels composed of Kir6.1 and/or Kir6.2 expressed with SUR2B

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The molecular identity of smooth muscle ATP-sensitive K⁺ channels (K_{ATP}) is not established with certainty. Patch clamp methods were employed to determine if recombinant K_{ATP} channels composed of Kir6.1 and SUR2B subunits expressed by human embryonic kidney (HEK293) cells share an identical modulation by protein kinase C (PKC) with the vascular K_{NDP} subtype of K_{ATP} channel. The open probability of Kir6.1/SUR2B channels was determined before and after sequential exposure to pinacidil (50 μM) and the combination of pinacidil and phorbol 12,13-dibutyrate (PdBu; 50 nM). Treatment with PdBu caused a decline in channel activity, but this was not seen with an inactive phorbol ester, 4α-phorbol 12,13-didecanoate (PdDe; 50 nM). Angiotensin II (0.1 μM) induced a similar inhibition of Kir6.1/SUR2B channels in cells expressing angiotensin AT₁ receptors. The effects of PdBu and angiotensin II were blocked by the PKC inhibitor, chelerythrine (3 μM). Purified PKC inhibited Kir6.1/SUR2B activity (in 0.5 mM ATP/0.5 mM ADP), and the inhibition was blocked by a specific peptide inhibitor of PKC, PKC(19–31). In contrast, PdBu increased the activity of recombinant K_{ATP} channels composed of Kir6.2 and SUR2B, or the combination of Kir6.1, Kir6.2 and SUR2B subunits. The results indicate that the modulation by PKC of Kir6.1/SUR2B, but not Kir6.2/SUR2B or Kir6.1-Kir6.2/SUR2B channel gating mimics that of native vascular K_{NDP} channels. Physiological inhibition of vascular K_{ATP} current by vasoconstrictors which utilize intracellular signalling cascades involving PKC is concluded to involve the modulation of K_{NDP} channel complexes composed of four Kir6.1 and their associated SUR2B subunits.

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Vasoconstrictors elicit contraction of vascular smooth muscle cells by enhancing Ca²⁺ influx through voltage-gated L-type Ca²⁺ channels, releasing Ca²⁺ from intracellular Ca²⁺ stores, and sensitization of contractile filaments to Ca²⁺ (Walsh *et al.* 1995). The influence of vasoconstrictors on Ca²⁺ influx involves direct effects on L-type Ca²⁺ channel gating via intracellular signalling cascades and channel phosphorylation, as well as an indirect voltage-dependent activation of Ca²⁺ channels due to depolarization of membrane potential. Depolarization of vascular smooth muscle cells in response to vasoconstrictors involves the activation of inward currents, such as non-selective cation and Cl⁻ currents, as well as the depression of outward K⁺ currents, such as delayed rectifier (Clément-Chomienne *et al.* 1996; Hayabuchi *et al.* 2001*b*), large conductance Ca²⁺-activated K⁺ (Toro *et al.* 1990) and ATP-sensitive K⁺ (K_{ATP}) currents (Nelson & Quayle, 1995; Kubo *et al.* 1997; Hayabuchi *et al.* 2001*a*).

Inhibition of K_{ATP} currents following exposure to constrictor agonists has been observed for myocytes isolated from arterial and venous blood vessels (Nelson & Quayle, 1995; Quayle *et al.* 1997; Cole & Clément-Chomienne, 2000), as well as airway (Nuttall & Farley, 1997), colonic (Jun *et al.* 2001), oesophageal (Hatakeyama *et al.* 1995), urinary bladder (Bonev & Nelson, 1993) and gall bladder (Firth *et al.* 2000) smooth muscle tissues. The involvement of protein kinase C (PKC) in the regulation of vascular K_{ATP} current by vasoconstrictor agonists is well-recognized (Nelson & Quayle, 1995; Quayle *et al.* 1997). Hayabuchi and co-workers (2001*a*) recently demonstrated a role for the Ca²⁺-independent isoform of PKC, PKCε, in the inhibition of rat mesenteric arterial K_{ATP} currents by angiotensin II, as well as the existence of a second regulatory pathway involving a suppression of adenylyl cyclase activity and loss of protein kinase A-mediated stimulation of channel activity. The molecular basis of the

K_{ATP} channels of smooth muscle that participate in this regulation of tone by vasoconstrictors via PKC is not established. A range of values has been reported for the unitary conductance of smooth muscle K_{ATP} channels (Nelson & Quayle, 1995; Quayle *et al.* 1997); in general, these values fall into two populations including small conductance channels of < 50 pS and intermediate to large conductance channels of > 65 pS. We reported that the small conductance (37–41 pS), nucleoside diphosphate-activated (K_{NDP}) subtype, but not the larger conductance (70 pS), cardiac-like LK subtype of K_{ATP} channel in vascular myocytes was inhibited by PKC activation (Cole *et al.* 2000). A similar modulation by PKC of small conductance K_{ATP} channels in murine colonic myocytes was recently identified and shown to involve PKC ϵ (Jun *et al.* 2001). Significantly, the inhibition by PKC of smooth muscle K_{ATP} currents and single channels (Cole *et al.* 2000; Hayabuchi *et al.* 2001a; Jun *et al.* 2001) occurs at an intracellular concentration of ATP at which cardiac K_{ATP} channels exhibit an increased open probability following activation of the kinase (Light *et al.* 1995, 1996).

The basis for the divergent modulation of cardiac and vascular K_{ATP} channels by PKC is not established, but it may be due to a tissue-specific expression of different pore-forming (Kir6.1 and Kir6.2) and/or regulatory sulphonylurea receptor (SUR1, SUR2A and SUR2B) subunits (Seino, 1999; Fujita & Kurachi, 2000). Several lines of evidence indicate that cardiac K_{ATP} channels are octameric complexes of four Kir6.2 subunits and four associated SUR2A subunits (Seino, 1999; Fujita & Kurachi, 2000). Light *et al.* (2000) demonstrated that the activity of recombinant K_{ATP} channels due to the expression of cardiac Kir6.2 and SUR2A subunits is increased in response to PKC activation, similar to the modulation of native cardiac K_{ATP} channels (Light *et al.* 1995, 1996). In contrast, the molecular identity of vascular K_{ATP} channels is not established with certainty (Clapp & Tinker, 1998). Kurachi and co-workers (Yamada *et al.* 1997; Satoh *et al.* 1998) showed that recombinant K_{ATP} channels consisting of Kir6.1 and SUR2B subunits share several biophysical and pharmacological properties with vascular K_{NDP} channels, including a similar unitary conductance and sensitivity to nucleoside diphosphates, as well as K_{ATP} channel openers and channel inhibitors. However, vascular and non-vascular smooth muscles may express Kir6.2 (Isomoto *et al.* 1996; Koh *et al.* 1998; Gopalakrishnan *et al.* 1999) in addition to Kir6.1 and SUR2B. Indeed, Cui *et al.* (2001) recently suggested that the diverse range of unitary conductances reported for smooth muscle K_{ATP} channels may be due to the co-assembly of Kir6.1 and Kir6.2 to form channels with different combinations of the two pore-forming subunits and their associated SUR2B subunits. Whether channels composed of the combination of Kir6.1 and SUR2B, or alternatively Kir6.2- and/or Kir6.1–Kir6.2-containing channels contribute to the physiological vascular

K_{ATP} currents regulated by vasoconstrictors via PKC is unknown.

In this study, we tested the hypothesis that the K_{NDP} subtype of vascular K_{ATP} channel is due to the combination of Kir6.1 and SUR2B subunits. We reasoned that if this combination of subunits constitutes the native channel complex, then recombinant Kir6.1/SUR2B channels should exhibit an identical inhibition by PKC and angiotensin II as was demonstrated for the K_{NDP} channels of rabbit portal vein (Cole *et al.* 2000). PKC activation was achieved by treating HEK293 cells expressing Kir6.1/SUR2B channels with PdBu, or angiotensin II in cells co-expressing AT₁ receptors, and the participation of PKC was confirmed using a selective PKC inhibitor, chelerythrine and the inactive phorbol ester, PdDe. Additionally, I-O membrane patches were exposed to purified PKC and the effect on Kir6.1/SUR2B channel activity determined in the absence and presence of the specific peptide inhibitor of PKC, PKC(19–31). For comparative purposes, and to address the issue of the relevance of channels composed of SUR2B with Kir6.2 or the combination of Kir6.1 and Kir6.2 as candidates for PKC-sensitive vascular K_{ATP} channels, the effect of PdBu on recombinant Kir6.2/SUR2B and Kir6.1–Kir6.2/SUR2B channels was also assessed. Our results provide the first evidence that recombinant K_{ATP} channels due to the combination of Kir6.1 and SUR2B alone share functional identity with native vascular K_{NDP} channels with respect to their modulation by PKC. This finding has important implications regarding the molecular identity of the K_{ATP} channel subtype affected by constrictor agonists, such as angiotensin II, which activate PKC in smooth muscle, as well as the basis for differences in the modulation by PKC of K_{ATP} channels expressed by various cell types. Part of this work was published in abstract form (Thorneloe *et al.* 2001).

METHODS

HEK cell culture and transient transfection

HEK293 (American Type Culture Collection, Manassas, VA, USA) or HEK293T (DuBridge *et al.* 1987) cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen (Gibco-BRL), Burlington, ON, Canada) supplemented with 10% FBS (Invitrogen, Canada) under a 10% CO₂ atmosphere. Cells were plated on fresh culture dishes every 5–6 days by mechanical disruption. HEK293 or 293T cells were transfected with cDNA encoding a green fluorescent protein (GFP) (obtained from Dr K. Moriyoshi, Kyoto University, Japan) in pcA vector, as well as cDNAs encoding mouse Kir6.1 and SUR2B (obtained from Dr Y. Kurachi, University of Osaka, Japan) and/or mouse Kir6.2 (obtained from Dr S. Seino, Chiba University, Japan). Transient transfection was optimized using FuGENE 6 (Roche, Laval, PQ, Canada). Briefly, 80% confluent cultures of HEK293 or HEK293T cells in 30 mm dishes containing acid-washed coverslips were incubated with cDNAs encoding GFP and K_{ATP} channel subunits and FuGENE 6. For control experiments, cells were incubated with the GFP and pcDNA3 vector or not transfected. Transiently transfected cells

were stored at 37°C and used within 72 h. Voltage clamp recordings revealed the presence of pinacidil-induced channel activity in approximately 85% of the cells expressing GFP. Control cells transfected with GFP alone, or untransfected cells, did not display pinacidil-sensitive channel activity (data not shown).

Electrophysiological measurements

HEK293 or HEK293T cells on glass cover slips were placed in a 300 μ l constant flow bathing chamber containing bathing solution (at 20–22°C) on the stage of a Diaphot-TMD epifluorescence inverted microscope (Nikon, Mississauga, ON, Canada). Cells expressing GFP were detected using an HMX Lamphouse (Nikon) with a blue excitation filter (B2, 450–490 nm), a dichroic mirror cutting at 510 nm and a barrier filter at 520 nm. Single channel currents were measured using cell-attached (C-A) and inside-out (I-O) membrane patch clamp techniques (Hamill *et al.* 1981). Pipettes were prepared from capillary glass with a Sutter P-87 puller (Sutter Instrument Co., Novato, CA, USA) and MF-83 microforge (Narishige Scientific Instrument Laboratory, Tokyo, Japan). Pipette and bath solutions contained, respectively (mM): 140 KCl, 1 CaCl₂, 1 MgCl₂, 5.5 glucose, 10 Hepes and 140 KCl, 2.3 MgCl₂, 10 glucose, 1 EGTA, 10 Hepes (pH 7.4 with KOH). For I-O patches, the bath solution contained MgATP (0.5 mM) and MgADP (0.5 mM). Recordings were obtained using an Axopatch 200A amplifier (Axon Instruments, Union City, CA, USA) and a Digidata 1200 A–D convertor (Axon Instruments). Pipette potential and capacitance were nulled and an 8–15 G Ω seal formed with the cell membrane. Single channel activity was recorded at –40 mV (transmembrane potential) using AxoTape 2.0 software (Axon Instruments). The data were filtered at 2 kHz by an on-board eight-pole Bessel filter before digitization at 10–15 kHz and storage to the hard drive of a Pentium II computer. Single channel current records were displayed and analysed using pCLAMP 6.0 software (Axon Instruments).

Open probability of recombinant channels in the different treatment groups was determined based on amplitude histograms (bin width 0.1–0.5 pA) obtained using identical duration recording periods of 15–60 s; preliminary experiments showed that 15 s was the minimal period that permitted accurate determination of open probability due to the bursting behaviour of the channels. The number of channels in each patch was not known with certainty, and for this reason values of open probability (P_o) were expressed as NP_o (number of channels (N) \times mean P_o of the single channels) determined according to the following equation (Kajioka *et al.* 1991):

$$NP_o = (A_1 + 2A_2 + 3A_3 + \dots + nA_n)/(A_0 + A_1 + A_2 + A_3 + \dots + A_n)$$

where A_0 , A_1 , A_2 , A_3 and A_n are the areas under each histogram peak with the channels closed, one open, and simultaneous openings of 2 to n channels, respectively, assuming that all channels in the patch have the same open probability under the given condition and that they behave independently. Analyses of open dwell time, burst duration and inter-burst interval were made using pCLAMP software (Axon Instruments). A burst was defined as a train of openings of more than five transitions in duration. For statistical analysis, more than 200 individual transitions within at least five bursts, and more than 100 individual bursts from three to four patches were considered for each condition in the determination of intra-burst open and closed times, and burst duration. No patches of cells expressing Kir6.1 and SUR2B contained only a single channel and for this reason a determination of inter-burst interval was not possible.

Drugs and chemicals

PdBu, PdDe, chelerythrine and glibenclamide were obtained from Sigma–Aldrich (Oakville, ON, Canada). Pinacidil and angiotensin II were purchased from Calbiochem–Novabiochem (La Jolla, CA, USA). cDNA encoding the angiotensin AT₁ receptor in pRc vector (Burns *et al.* 1993) was obtained from Dr K. Burns (University of Ottawa, Canada). Phorbol esters and PKC inhibitors were added directly to the bath solution. Pinacidil and glibenclamide were prepared fresh each day in dimethylsulphoxide and added to the bath solution immediately prior to use. Constitutively active PKC was prepared from rat brain PKC (rat brains were purchased from Pel-Freez, Rogers, AR, USA) as previously described (Parente *et al.* 1992). The PKC inhibitor peptide, PKC (19–31), was synthesized in the Peptide Synthesis Core Facility at the University of Calgary and purified as previously described (Light *et al.* 1995).

Statistics

Average values of NP_o for the different treatment groups were compared by paired Student's t test or repeated measures ANOVA followed by Student–Newman–Keuls test for multiple comparisons. A level of $P < 0.05$ was considered to be statistically significant.

RESULTS

Unitary conductance of recombinant K_{ATP} channels

Initial experiments confirmed the presence of recombinant K_{ATP} channels with different levels of unitary conductance in patches of HEK293 cells that were transfected with cDNAs encoding Kir6.1 and SUR2B, Kir6.2 and SUR2B, or SUR2B with the combination of Kir6.1 and Kir6.2, as previously reported (Isomoto *et al.* 1996; Yamada *et al.* 1997; Satoh *et al.* 1998; Fujita & Kurachi, 2000; Seino, 1999; Cui *et al.* 2001). Recombinant K_{ATP} channel activity was induced in C–A patches by pinacidil (50 μ M) and inhibited by glibenclamide (3 μ M). Figure 1 shows the diversity of single channel currents due to Kir6.1/SUR2B, Kir6.2/SUR2B, and Kir6.1–Kir6.2/SUR2B channels. Kir6.1/SUR2B channels displayed a conductance of 36.5 ± 0.4 pS ($n = 22$) and 36.5 ± 0.6 pS ($n = 13$) in C–A and I–O patches (symmetrical 140/140 mM KCl recording conditions), respectively, compared to 71.3 ± 1.1 pS ($n = 9$ C–A patches) for Kir6.2/SUR2B channels. Co-expression of Kir6.1 and Kir6.2 with SUR2B produced channels with five distinct levels of unitary current: in addition to channels containing only Kir6.1 or Kir6.2 with SUR2B at approximately 36 and 71 pS, three additional channel subtypes of intermediate levels of conductance of approximately 43, 55 and 63 pS were resolved. This result is consistent with the observations of Cui *et al.* (2001), and indicates the presence of channels with respective Kir6.1 and Kir6.2 subunit distributions of 3:1, 2:2 and 1:3 in the patches. The number of recombinant K_{ATP} channels per patch varied substantially (two to eight) in this study, and for this reason, large differences in the calculated NP_o values in the presence of pinacidil were apparent (Table 1). Despite this potential complication, normalization of NP_o values following activation of PKC to the level in pinacidil alone was not required to resolve the decrease in Kir6.1/SUR2B, or increase in Kir6.2/SUR2B, channel activity associated with PKC activation. However,

Table 1. Effect of PKC activation on NP_O of recombinant Kir6.1/SUR2B channels

Experiment	<i>n</i>	Treatment	NP_O
1. PdBu	6	Control	0.026 ± 0.022
		Pinacidil ($50 \mu\text{M}$)	0.616 ± 0.240
		PdBu (50 nM) + Pinacidil	$0.017 \pm 0.030^*$
2. PdDe	5	Control	0.000 ± 0.000
		Pinacidil ($50 \mu\text{M}$)	0.191 ± 0.089
		PdDe (50 nM) + Pinacidil	0.392 ± 0.135
3. Chelerythrine + PdBu	5	Control	0.000 ± 0.000
		Pinacidil ($50 \mu\text{M}$)	0.083 ± 0.027
		PdBu (50 nM) + Pinacidil	0.141 ± 0.066
4. Angiotensin II	5	Control	0.000 ± 0.000
		Pinacidil ($50 \mu\text{M}$)	0.455 ± 0.175
		Ang II ($0.1 \mu\text{M}$) + Pinacidil	$0.114 \pm 0.099^*$
5. Chelerythrine + angiotensin II	6	Control	0.000 ± 0.000
		Pinacidil ($50 \mu\text{M}$)	0.666 ± 0.348
		Ang II ($0.1 \mu\text{M}$) + Pinacidil	0.780 ± 0.466

* Significantly different from value in pinacidil alone.

the presence of multiple conductance levels in nine of 11 patches of cells expressing both Kir6.1 and Kir6.2 precluded a quantitative analysis of the magnitude of increase in open probability of the three Kir6.1–Kir6.2/SUR2B-containing subtypes due to PdBu treatment (see below).

Effect of PKC activation with phorbol ester on Kir6.1/SUR2B channel activity

Figure 2 shows the effect of phorbol ester treatment on Kir6.1/SUR2B channel activity induced by pinacidil ($50 \mu\text{M}$). PdBu (50 nM) caused a time-dependent decrease

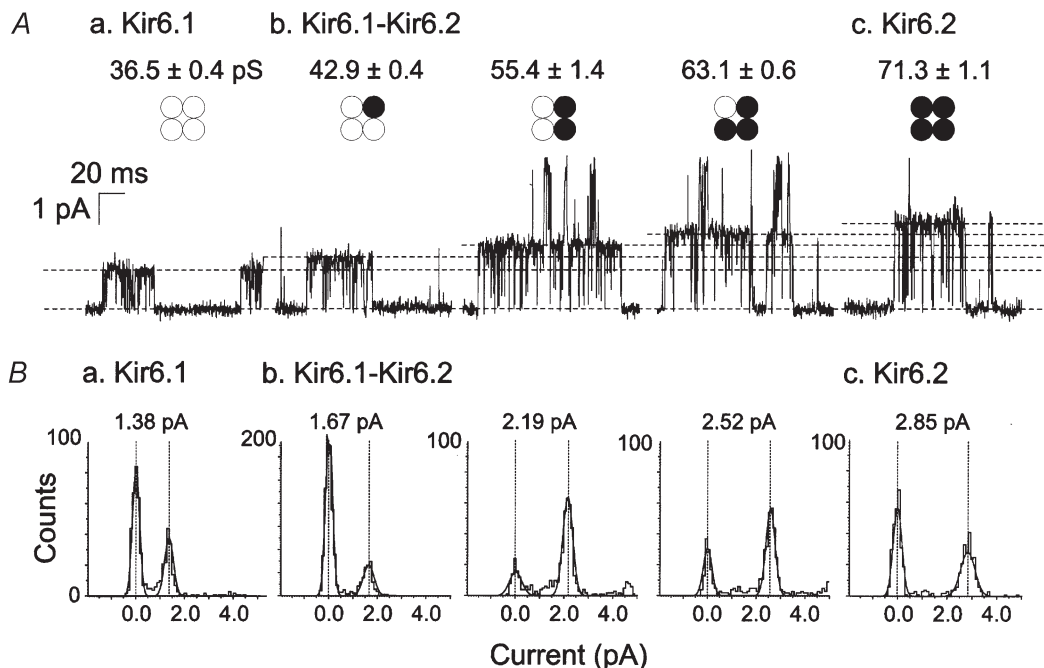


Figure 1. Representative single channel activity due to recombinant K_{ATP} channels composed of SUR2B with Kir6.1 alone, Kir6.2 alone or the combination of Kir6.1 and Kir6.2

A, representative single channel recordings and average unitary conductance for cells expressing (a) Kir6.1/SUR2B channels ($n = 22$), (b) Kir6.1–Kir6.2/SUR2B channels ($n = 9$) and (c) Kir6.2/SUR2B channels ($n = 9$). The respective stoichiometries of Kir6.1 and Kir6.2 forming the tetrameric pore complex for each combination of subunits studied is indicated by \circ and \bullet , respectively. B, representative amplitude histograms determined from 1–2 s segments of channel activity corresponding to the channels shown in (a) to (c) of A. Vertical dashed lines indicate the current amplitudes of the peaks of closed and open states of the solid line fits of the histograms. The single channel current amplitude of each subunit combination is indicated at the top of each histogram.

in channel activity that required between 5 and 15 min to achieve a steady-state level of inhibition. NP_o values for Kir6.1/SUR2B channels during treatment with PdBu and pinacidil were significantly lower than in pinacidil alone (Table 1). In contrast, prolonged exposure of cells expressing Kir6.1 and SUR2B subunits to pinacidil in the absence of phorbol ester failed to show any change in channel activity for more than 15 min ($n = 3$; data not shown). Figure 3 shows that the inactive phorbol ester, PdDe (50 nM), failed to cause a decline in Kir6.1/SUR2B channel activity; sustained activity at a similar level of open probability was observed after 15 min of treatment with this inactive phorbol ester. On average, the value for NP_o in the presence of PdDe was not different after 15 min treatment compared to the value in pinacidil alone (Table 1).

This indicates that a non-specific effect of phorbol ester treatment was not responsible for the decline in open probability in the presence of PdBu.

Suppression of the inhibition of Kir6.1/SUR2B channel activity by PdBu following pretreatment with the PKC inhibitor, chelerythrine, is demonstrated in Fig. 4. HEK293 cells were pretreated with chelerythrine ($3 \mu\text{M}$) for 10–15 min after C-A patch configuration was achieved and then sequentially exposed to pinacidil followed by pinacidil in combination with PdBu. Chelerythrine pretreatment did not affect the activation of Kir6.1/SUR2B channel activity by pinacidil. However, subsequent exposure to PdBu failed to affect the open probability of the channels in cells exposed to chelerythrine (Fig. 4 and Table 1).

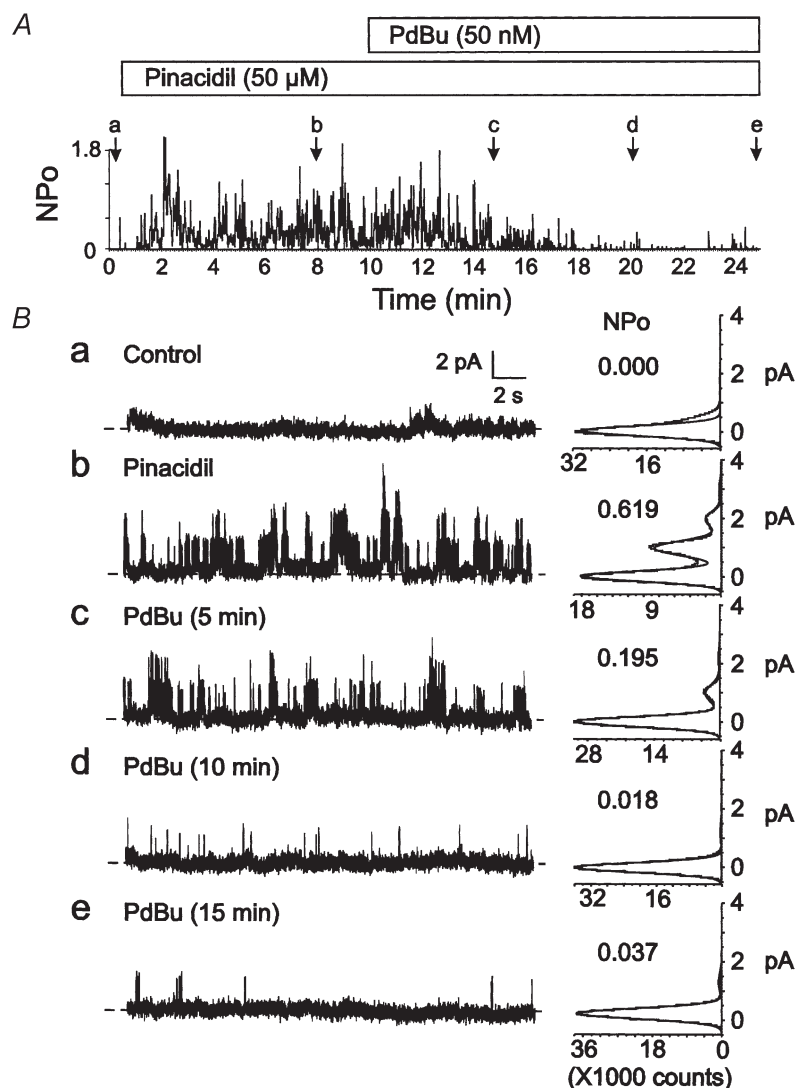


Figure 2. Inhibition of Kir6.1/SUR2B channel activity by PdBu

A, representative changes in NP_o versus time of Kir6.1/SUR2B channel activity of a C-A patch in control conditions, after treatment with pinacidil ($50 \mu\text{M}$), or the combination of pinacidil and PdBu (50 nM). Lower case letters (a–e) indicate times of representative data and amplitude histograms in B. B, representative 15 s segments and amplitude histograms (identical 1 min intervals) assessed at the times indicated by a–e in A. Respective NP_o values for each histogram are indicated.

These data support the conclusion that PKC activity was responsible for the inhibition of Kir6.1/SUR2B channels by PdBu.

Effect of PKC activation via angiotensin II on Kir6.1/SUR2B channel activity

HEK293 cells were co-transfected with cDNAs encoding the angiotensin AT₁ receptor (Burns *et al.* 1993), Kir6.1 and SUR2B. Figure 5 shows that treating these cells with

angiotensin II caused a time-dependent inhibition of Kir6.1/SUR2B channel activity in the presence of pinacidil. On average, the open probability of the channels was significantly reduced after 15 min exposure to the agonist (Table 1). The involvement of PKC in the suppression of Kir6.1/SUR2B activity by angiotensin II was confirmed using chelerythrine. Pretreatment for 10–15 min with the PKC inhibitor prior to exposure to angiotensin II abolished the inhibition produced by the agonist, confirming that

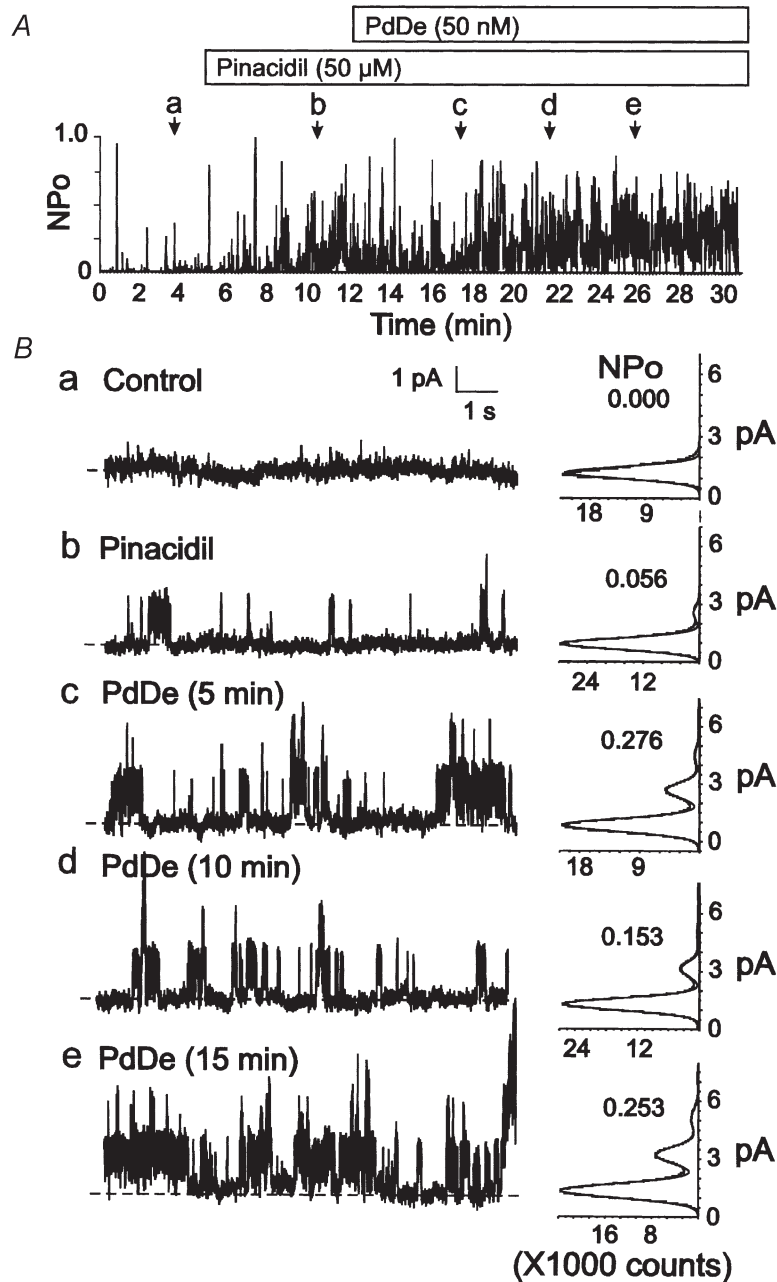


Figure 3. Lack of effect of PdDe on Kir6.1/SUR2B channel activity

A, representative changes in NP_o versus time of Kir6.1/SUR2B channel activity of a C-A patch in control conditions, after treatment with pinacidil (50 μM), or the combination of pinacidil and PdDe (50 nM). Lower case letters (a–e) indicate times of representative data and amplitude histograms in *B*. *B*, representative 10 s segments and amplitude histograms (identical 1 min intervals) assessed at the times indicated by a–e in *A*. Respective NP_o values for each histogram are indicated.

PKC activation was required for the reduction of Kir6.1/SUR2B open probability by angiotensin II treatment (Fig. 6 and Table 1).

Effect of PKC on Kir6.1/SUR2B channel activity in I-O patches

To assess directly the effect of PKC on Kir6.1/SUR2B channel activity, patches were excised into bath solution containing MgATP (0.5 mM) and MgADP (0.5 mM) and

exposed to the purified, constitutively active kinase in the absence or presence of the PKC inhibitor peptide, PKC(19–31). The activity of the PKC preparations employed in these experiments was confirmed prior to use via an *in vitro* assay (Parente *et al.* 1992; Light *et al.* 1995). In contrast to the absence of channel activity in MgATP-free solution, patches excised into solutions containing ATP and ADP displayed sustained activity that was stable

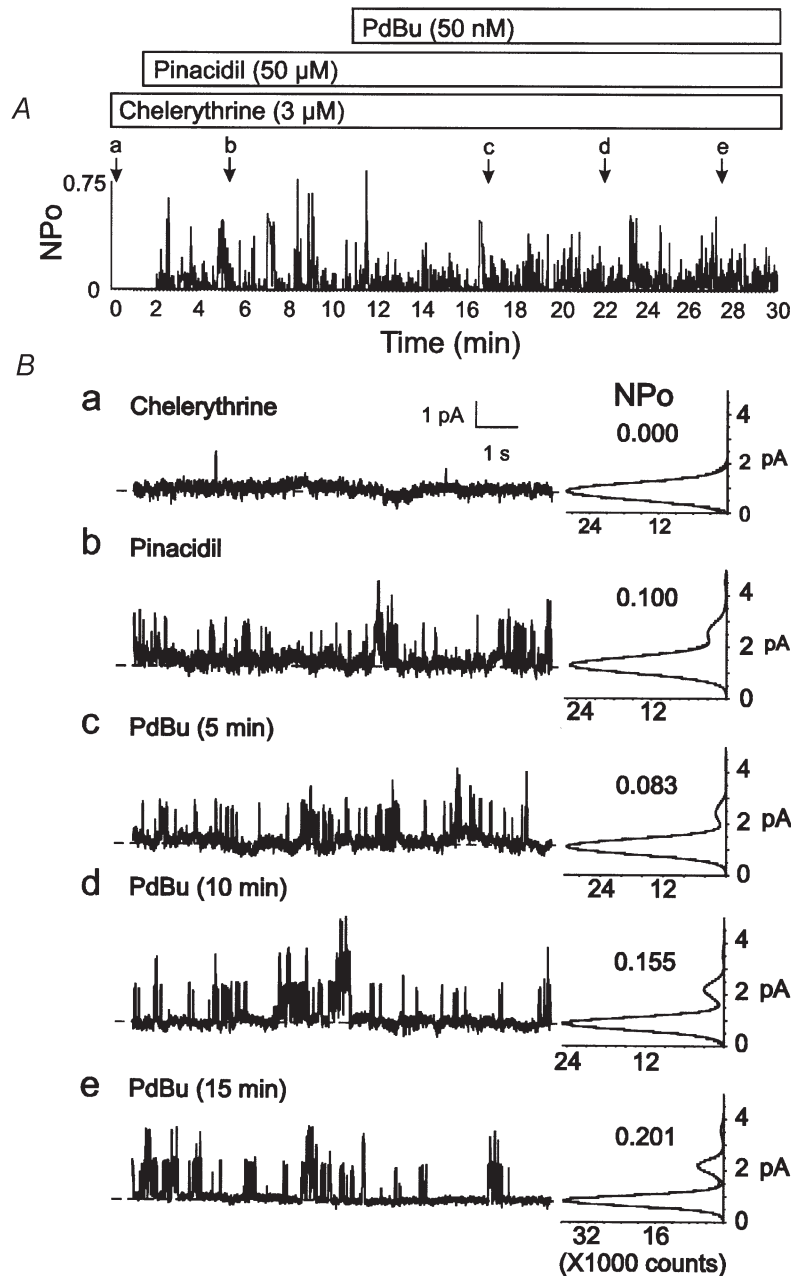


Figure 4. Lack of effect of PdBu on Kir6.1/SUR2B channel activity following pretreatment with PKC inhibitor

A, representative changes in NP_o versus time of Kir6.1/SUR2B channel activity of a C-A patch after pretreatment with chelerythrine ($3 \mu\text{M}$), and sequential exposure to pinacidil ($50 \mu\text{M}$) followed by pinacidil and PdBu (50 nM) in the continued presence of chelerythrine. Lower case letters (a–e) indicate times of representative data and amplitude histograms in B. B, representative 10 s segments and amplitude histograms (identical 1 min intervals) assessed at the times indicated by a–e in A. Respective NP_o values for each histogram are indicated.

for > 20 min without rundown (data not shown). Patches were exposed to PKC alone (in the continued presence of ATP and ADP) followed by > 5 min washout, or the combination of PKC and PKC(19–31) followed by PKC alone and washout (Table 2). A similar inhibition of channel activity was observed in the presence of PKC in both experiments. Figure 7 shows a representative example illustrating that treatment with PKC (7.5 nM) caused an inhibition of Kir6.1/SUR2B channel activity, but not in the presence of the specific peptide inhibitor of the enzyme, PKC(19–31) (5 μ M). Average values for NP_O illustrating

the inhibition by PKC of Kir6.1/SUR2B channel activity, the ability of the peptide inhibitor to block this effect of PKC and the reversibility of the effects of PKC treatment on open probability are indicated in Table 2. Treatment of eight I-O patches of HEK293 cells expressing Kir6.1/SUR2B channels with inactive PKC (confirmed by *in vitro* assay) due to prolonged tryptic digestion, failed to alter K_{NDP} activity (data not shown). These results indicate that active PKC inhibits the activity of Kir6.1/SUR2B channels at intracellular ATP and ADP concentrations of 0.5 mM: this result is similar to that previously described for native

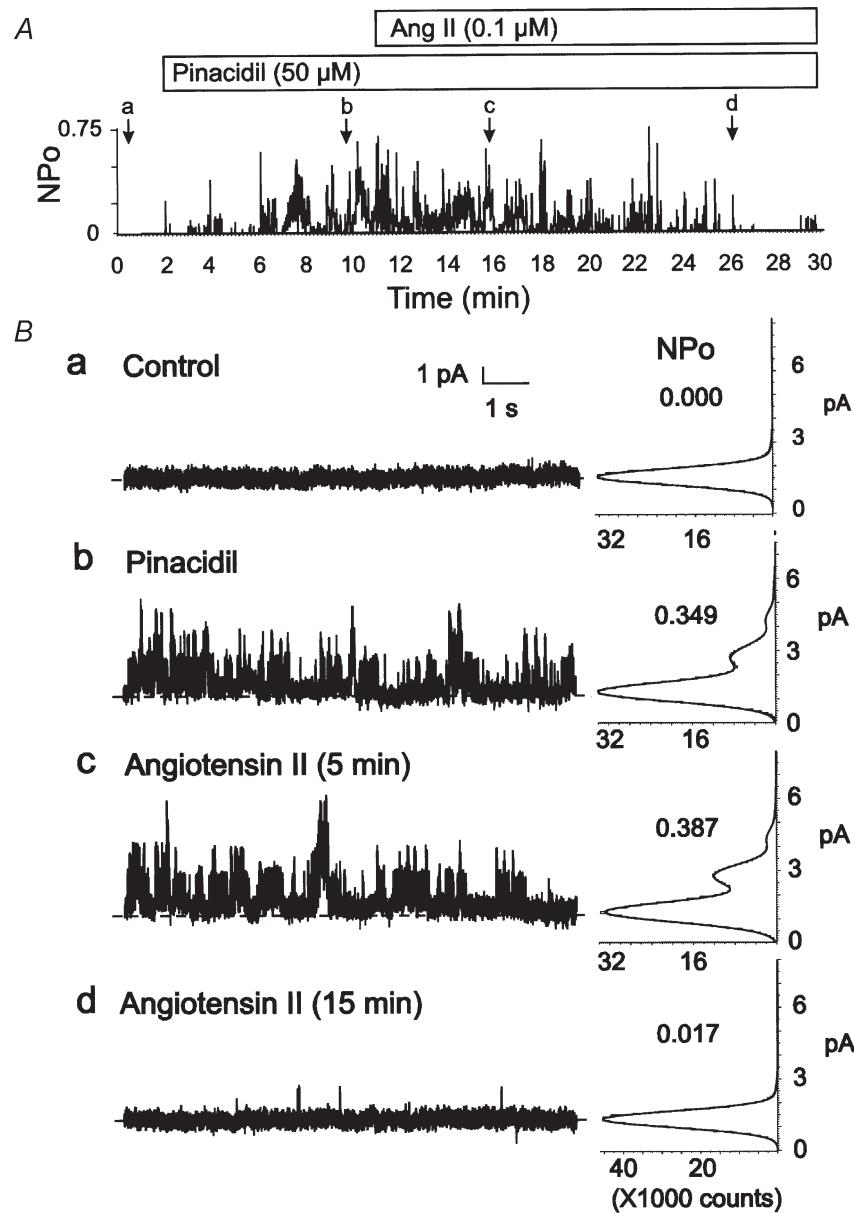


Figure 5. Inhibition of Kir6.1/SUR2B channel activity by angiotensin II

A, representative changes in NP_O versus time of Kir6.1/SUR2B channel activity of a C-A patch of a HEK293 cell co-transfected with cDNA encoding the AT_1 receptor in the presence of pinacidil (50 μ M) followed by pinacidil and angiotensin II (0.1 μ M). Lower case letters (a–d) indicate times of representative data and amplitude histograms in B. B, representative 10 s segments and amplitude histograms (identical 1 min intervals) assessed at the times indicated by a–d in A. Respective NP_O values for each histogram are indicated.

Table 2. Effect of purified PKC on NP_o of recombinant Kir6.1/SUR2B channels in I-O patches

Experiment	<i>n</i>	Treatment	NP_o
1. PKC	4	Control	0.766 ± 0.277
		PKC (7.5 nM)	$0.017 \pm 0.003^*$
		Washout	0.275 ± 0.120
2. PKC/PKC(19–31)	4	Control	0.275 ± 0.91
		PKC (7.5 nM)	0.226 ± 0.146
		+ PKC(19–31) (5 μ M)	$0.037 \pm 0.031^*$
		Washout	0.199 ± 0.101

* Significantly different from value in control conditions.

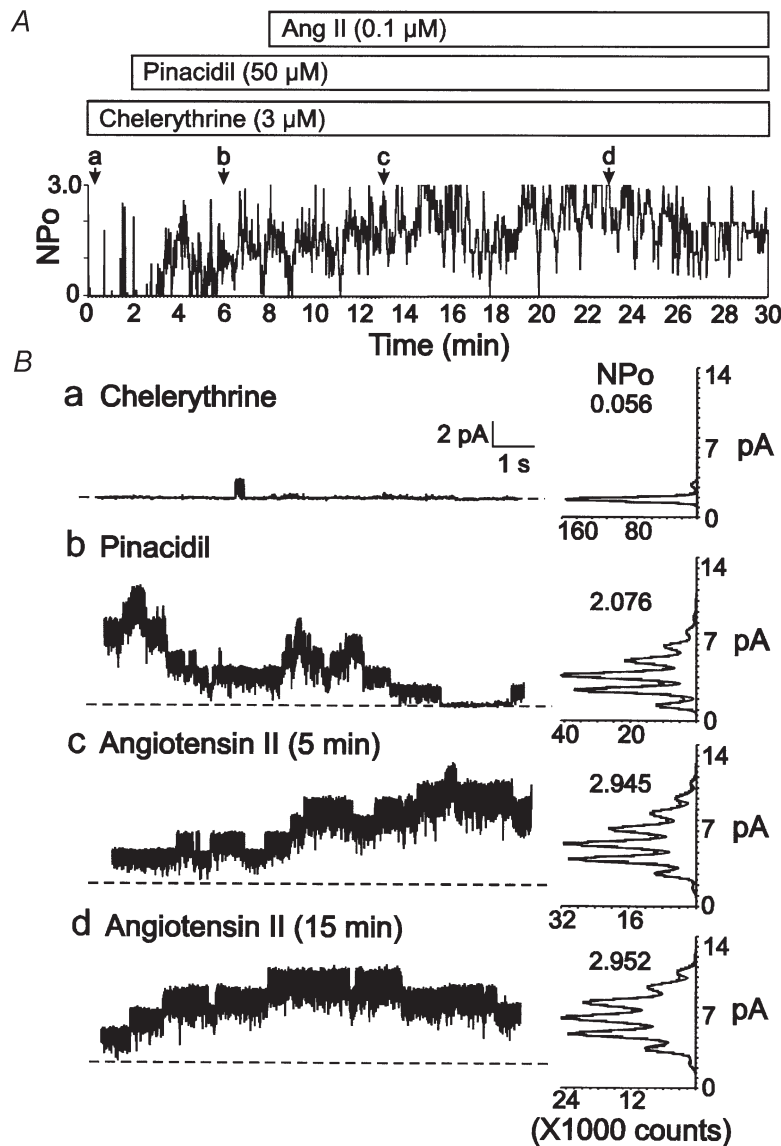


Figure 6. Lack of effect on Kir6.1/SUR2B channel activity of angiotensin II following pretreatment with PKC inhibitor

A, representative changes in NP_o versus time of Kir6.1/SUR2B channel activity of a C-A patch of a HEK293 cell co-transfected with cDNA encoding the AT_1 receptor after pretreatment with chelerythrine (3 μ M) and sequential exposure to pinacidil (50 μ M) followed by pinacidil and angiotensin II (0.1 μ M) in the continued presence of chelerythrine. Lower case letters (a–d) indicate times of representative data and amplitude histograms in *B*. *B*, representative 10 s segments and amplitude histograms (identical 1 min intervals) assessed at the times indicated by a–d in *A*. Respective NP_o values for each histogram are indicated.

vascular K_{NDP} , but is in direct contrast to the activation of cardiac K_{ATP} (Light *et al.* 1995, 1996), as well as recombinant K_{ATP} channels due to Kir6.2/SUR2A and Kir6.2/SUR1 (Light *et al.* 2000) subunits at the same intracellular concentrations of ATP.

Effect of PKC activation on Kir6.1/SUR2B channel kinetics

We previously demonstrated that the inhibition of native vascular K_{NDP} channels by PKC activation was associated with an increase in inter-burst duration but intra-burst

open and closed times, as well as burst duration, were unaffected (Cole *et al.* 2000). Multiple Kir6.1/SUR2B channels were consistently present in the patches employed in this study, so a similar analysis of inter-burst closed time was not possible. However, we were able to analyse burst duration and intra-burst kinetics for bursts of transitions to a single open level. Table 3 shows that PKC activation with PdBu failed to alter mean burst duration or mean intra-burst open and closed times, consistent with the previously reported lack of alteration in these parameters

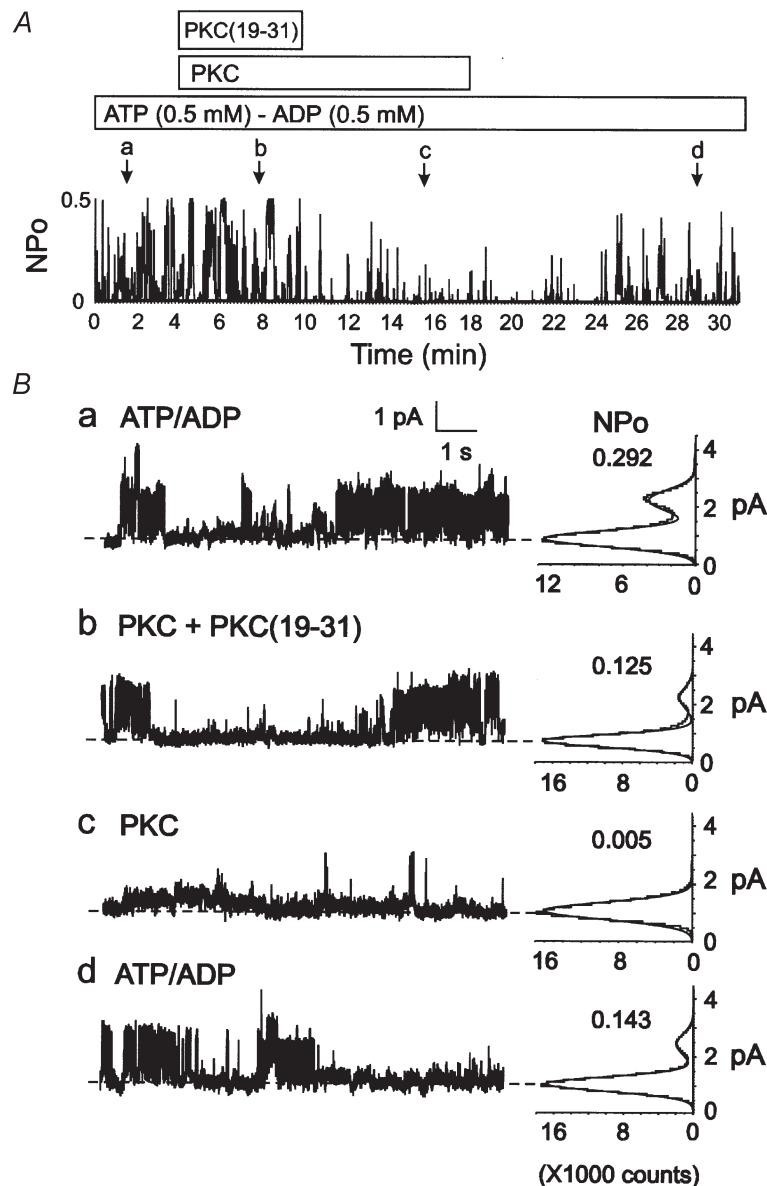


Figure 7. Inhibition of Kir6.1/SUR2B channel activity by PKC is suppressed by the peptide inhibitor PKC(19–31)

A, representative changes in NP_o versus time of Kir6.1/SUR2B channel activity of an I-O patch in the presence of ATP (0.5 mM) and ADP (0.5 mM), PKC (7.5 nM) in the presence of the peptide inhibitor PKC(19–31) (5 μ M), PKC alone and washout with ATP/ADP-containing bath solution. Lower case letters (a–d) indicate times of representative data and amplitude histograms in B. B, representative 10 s segments and amplitude histograms (identical 1 min intervals) assessed at the times indicated by a–d in A. Respective NP_o values for each histogram are indicated.

for native K_{NDP} channels in the presence of PdBu (Cole *et al.* 2000). This indicates that a lengthening of the interburst interval was probably responsible for the decreased open time of Kir6.1/SUR2B channels following PKC activation.

Effect of PdBu on recombinant K_{ATP} channels due to Kir6.2 and Kir6.1–Kir6.2 expressed with SUR2B

The effect of PKC activation with PdBu on Kir6.2/SUR2B channels was assessed using identical conditions as described above for the experiments on Kir6.1/SUR2B channels. The open probability of Kir6.2/SUR2B channels in the presence of pinacidil (Table 4) was consistently lower than that observed for homotetrameric Kir6.1 channels (Table 1),

and it was stable for more than 20 min of treatment (data not shown). In the presence of PdBu, an increase in NP_O was observed in five of five patches studied. Representative data are shown in Fig. 8 and mean values for the changes in NP_O due to PdBu are given in Table 4. The increase in Kir6.2/SUR2B channel activity following activation of PKC in this experiment is similar to that previously reported for channels due to expression of Kir6.2 with either SUR2A or SUR1 (Light *et al.* 2000).

The effect of PKC activation on the activity of recombinant K_{ATP} channels due to the co-expression of Kir6.1 and Kir6.2 with SUR2B was also determined. Figure 8B and C show representative data from two experiments. The

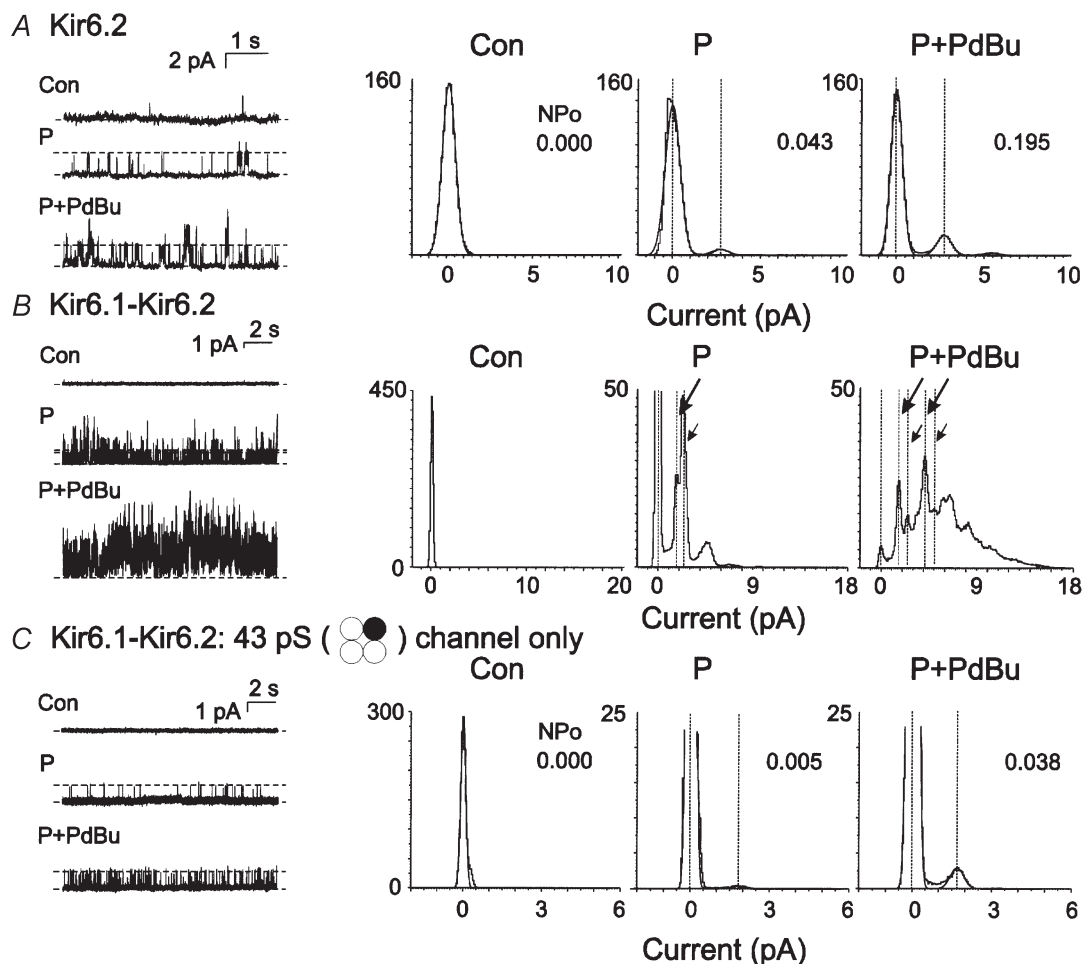


Figure 8. Stimulation of Kir6.2/SUR2B and Kir6.1–Kir6.2/SUR2B channel activity by PdBu

A, representative recordings (left) and amplitude histograms (right) of Kir6.2/SUR2B channel activity in control conditions (Con) and after sequential exposure to pinacidil (P; $50 \mu\text{M}$) and pinacidil + PdBu (P + PdBu; 50 nM). Data for P + PdBu were obtained after 15 min of treatment. The NP_O value for each condition is indicated in the histogram. B, representative recordings (left) and amplitude histograms (right) of Kir6.1–6.2/SUR2B channel activity in control conditions and after sequential exposure to pinacidil ($50 \mu\text{M}$) and pinacidil + PdBu (50 nM). Data for P + PdBu were obtained after 15 min of treatment. The large and small arrows indicate the levels of openings of the 43 and 55 pS channels, respectively, before and after PdBu treatment. Note the increase in double openings of both channels after PdBu addition. C, representative recordings (left) and amplitude histograms (right) for a patch containing only the 43 pS subtype of Kir6.1–Kir6.2/SUR2B channel in control conditions and after sequential exposure to pinacidil ($50 \mu\text{M}$) and pinacidil + PdBu (50 nM). Data for P + PdBu were obtained after 15 min of treatment. The NP_O value for each condition is indicated in the histogram.

Table 3. Effect of PKC activation on recombinant Kir6.1/SUR2B channel burst kinetics

Treatment	Burst duration (ms)*	Intra-burst dwell times (ms) †	
		Open	Closed
1. Pinacidil	57.8 ± 4.4	1.71 ± 0.27	0.46 ± 0.12
2. PdBu/Pinacidil	57.9 ± 4.6	1.81 ± 0.24	0.44 ± 0.14

* Analysis based on burst duration for 155 and 157 individual bursts in three patches in the absence (pinacidil alone) and presence of PdBu + pinacidil, respectively. † Analysis based on 37 972 and 42 023 transitions in > 1000 bursts in four patches in the absence (pinacidil alone) and presence of PdBu + pinacidil, respectively.

activity of the Kir6.1–Kir6.2/SUR2B channels was stable for more than 20 min in the presence of pinacidil (data not shown). The activation of PKC with PdBu caused an increase in heterotetrameric Kir6.1–Kir6.2/SUR2B channel activity in 11 of 11 patches; however, in nine of 11 experiments, multiple channels of different conductance, as shown in Fig. 1, were present in the recordings. The presence of multiple channels resulted in amplitude histograms with a very complex appearance and precluded a quantitative evaluation of the magnitude of increase in activity of the different channel sub-types. However, it was apparent that the open probability of the heterotetrameric channels due to all possible mixtures of Kir6.1 and Kir6.2 was increased by PdBu treatment, in contrast to the decline in activity observed for Kir6.1/SUR2B channels. This is demonstrated by the representative data shown in Fig. 8B. In this case, PdBu treatment resulted in an increase compared to control conditions in the amplitude of the peaks corresponding to the open levels of the 43 and 55 pS channels (large and small arrows, respectively), composed of Kir6.1 and Kir6.2 with respective stoichiometries of 3:1 and 2:2. A similar increase was apparent in nine of nine patches containing multiple channels. In two patches, transitions of only a single subtype were apparent in more than 20 min of recording; Fig. 8C shows data for a patch containing only the 43 pS channel subtype (Kir6.1–Kir6.2 at 3:1). In this case, the open probability of the channel was increased by 8.5-fold in the presence of PdBu. A similar 10-fold increase in open probability was observed for a patch containing only 53 pS channels (Kir6.1–Kir6.2 at 2:2; NP_o increased from 0.010 to 0.100). These data indicate that the presence of Kir6.2 in the Kir6.1–Kir6.2/SUR2B channels resulted in a stimulatory response to PKC that was identical to that observed for Kir6.2/SUR2B, but opposite to the inhibition of Kir6.1/SUR2B channels.

DISCUSSION

This study makes the novel observation that recombinant K_{ATP} channels composed of Kir6.1 and SUR2B subunits are inhibited by PKC activation. The depression in Kir6.1/SUR2B channel activity by PKC activation observed

Table 4. Effect of PKC activation on NP_o of recombinant Kir6.2/SUR2B channels

Experiment	Treatment	NP_o
1. PdBu $n = 5$	Control	0.000 ± 0.000
	Pinacidil (50 μM)	0.026 ± 0.013
	PdBu (50 nM) + Pinacidil	
	5 min	0.037 ± 0.018
	10 min	0.065 ± 0.033 *
	15 min	0.086 ± 0.043 *
	20 min	0.137 ± 0.069 *

* Significantly different from NP_o value in pinacidil alone.

in this study mimics that which we previously reported for the small conductance, K_{NDP} subtype of native smooth muscle K_{ATP} channel (Cole *et al.* 2000). This inhibitory effect contrasts with the stimulation by PKC of native cardiac K_{ATP} (Light *et al.* 1995, 1996) and recombinant K_{ATP} channels due to the expression of Kir6.2/SUR2A and Kir6.2/SUR1 (Light *et al.* 2000), which are postulated to be the molecular basis of cardiac and pancreatic K_{ATP} channels, respectively (Seino, 1999; Fujita & Kurachi, 2000). Moreover, this study shows that expressing Kir6.2, or the combination of Kir6.1 and Kir6.2, with SUR2B produces channels that are stimulated by PKC. The findings of this study have important implications, therefore, concerning the molecular identity of the specific K_{ATP} subtype of vascular smooth muscle cells affected by vasoconstrictor agonists that act via PKC, as well as the molecular basis for the divergent effect of PKC activation on K_{ATP} currents of cardiac myocytes, pancreatic β cells and vascular smooth muscle cells.

The inhibition of Kir6.1/SUR2B channels by PKC activation observed in this study provides functional evidence consistent with the view that these subunits contribute to the K_{NDP} subtype of vascular K_{ATP} channel. Three different approaches were employed to determine the modulation by PKC of Kir6.1/SUR2B channels: (1) PKC was activated directly with a phorbol ester, PdBu; (2) cells were co-transfected with cDNA encoding the angiotensin AT_1 receptor and subsequently treated with angiotensin II; and (3) Kir6.1/SUR2B channels of excised, I-O membrane patches were exposed to purified, constitutively active PKC. The possibility that non-specific effects of phorbol ester treatment were involved was ruled out by using the inactive phorbol ester, PdDe. The involvement of PKC was confirmed by pretreating cells with chelerythrine prior to exposure to PdBu or angiotensin II, and by treating I-O patches with purified PKC in the presence of the specific peptide inhibitor PKC(19–31). In all three sets of experiments, a decrease in Kir6.1/SUR2B channel activity was associated with PKC activation. K_{ATP} channels of cardiac myocytes were previously shown to be activated by PKC at a normal intracellular concentration of ATP (~1 mM), but the modulation was inhibitory when ATP

was reduced to $< 100 \mu\text{M}$ (Light *et al.* 1996). In this study, the C-A patches were employed in most experiments to allow for activation of endogenous PKC with PdBu or angiotensin II. It is unlikely that the intracellular ATP concentration was depressed in these cells, but the possibility of microenvironments of low ATP concentration adjacent to the intracellular surface of the channels cannot be ruled out. The I-O membrane patch experiments were therefore performed using a bath solution with ATP at $500 \mu\text{M}$, identical to the concentration used by Light and co-workers (1996, 2000) to show stimulation of Kir6.2/SUR2A and Kir6.2/SUR1 channels by PKC. Our results therefore provide strong evidence that Kir6.1/SUR2B channels are inhibited by PKC activation.

Our findings provide novel information concerning the molecular basis of the K_{ATP} channel subtype involved in the modulation of vascular smooth muscle membrane potential by vasoconstrictors that activate PKC. The molecular identity of smooth muscle K_{ATP} channels is not known with certainty (Clapp & Tinker, 1998), but it is unlikely that SUR1 or SUR2A are involved, since neither is expressed in smooth muscle cells (Clapp & Tinker, 1998; Koh *et al.* 1998; Cui *et al.* 2002; Sim *et al.* 2002) and recombinant channels containing these subunits have a pharmacology that is distinct from that of vascular K_{NDP} channels (Seino, 1999; Fujita & Kurachi, 2000). Yamada *et al.* (1997) first proposed that Kir6.1/SUR2B represent the molecular basis of the vascular K_{NDP} channel subtype. Native K_{NDP} and Kir6.1/SUR2B channels have similar sensitivity to ATP and nucleoside diphosphates, similar pharmacology with respect to the K^+ channel opening drugs, diazoxide and pinacidil, and similar unitary conductance (Yamada *et al.* 1997; Satoh *et al.* 1998). The present experiments show, for the first time, that Kir6.1/SUR2B channels also mimic the modulation by PKC previously described for native vascular K_{NDP} channels (Cole *et al.* 2000). This observation adds the novel feature of an identical regulation by a protein kinase to the existing list of shared biophysical and pharmacological properties (Yamada *et al.* 1997; Satoh *et al.* 1998).

Our results also suggest that Kir6.2/SUR2B and/or Kir6.1–Kir6.2/SUR2B channels do not contribute to modulation of whole-cell vascular K_{ATP} currents by PKC (Nelson & Quayle, 1995; Quayle *et al.* 1997). K_{NDP} channels are exclusively expressed in some smooth muscle tissues, such as rabbit portal vein (Kajioka *et al.* 1991; Cole *et al.* 2000), rat mesenteric artery (Zhang & Bolton, 1995) and guinea pig coronary artery (Dart & Standen, 1993, 1995). However, glibenclamide-sensitive K_{ATP} channels of larger conductance are also present (e.g. the 70 pS cardiac-like K_{ATP} channel of rat portal vein (Zhang and Bolton, 1996)), perhaps as a result of Kir6.2/SUR2B expression (Isomoto *et al.* 1996; Fujita & Kurachi, 2000) as identified for mouse colonic smooth muscle (Koh *et al.* 1998) and guinea pig

urinary bladder (Gopalakrishnan *et al.* 1999), or the presence of heterotetramers of Kir6.2 and Kir6.1 expressed with SUR2B (Cui *et al.* 2001). We found that Kir6.2/SUR2B channel activity did not decrease during PdBu treatment, rather a significant, sixfold increase in open probability was observed. This is similar to the activation of Kir6.2/SUR2A or Kir6.2/SUR1 channels previously reported (Light *et al.* 2000). Hence, in addition to differences in unitary conductance at ~ 70 and ~ 40 pS, respectively, Kir6.2/SUR2B and vascular K_{NDP} channels also do not share functional identity with respect to their modulation by PKC. Interestingly, Kir6.2 and SUR2B were concluded to be the molecular basis of small conductance (27 pS) murine colonic smooth muscle K_{ATP} channels. These channels also exhibit inhibition by PKC activation with PdBu or acetylcholine and activation by the nucleoside diphosphate, ADP (Koh *et al.* 1998; Jun *et al.* 2001). The conductance of the murine Kir6.2/SUR2B channels in symmetrical 140/140 mM KCl solutions in the present study was ~ 71 pS and their activity was increased by PKC. The reason for these differences remains to be determined.

It is also unlikely that Kir6.1–Kir6.2/SUR2B channels contribute to vascular K_{ATP} current inhibited by PKC. Cui *et al.* (2001) postulated that co-assembly of Kir6.1 and Kir6.2 may account for reports of K_{ATP} channels with multiple levels of conductance in smooth muscle. Expression of Kir6.1 and Kir6.2 with SUR2B in the present study yielded an identical series of channels with distinct levels of unitary conductance ranging from ~ 35 to ~ 70 pS as described by Cui *et al.* (2001). The values at the extremes of this range are consistent with those of Kir6.1/SUR2B and Kir6.2/SUR2B channels at ~ 35 and ~ 70 pS, respectively. Cui *et al.* (2001) attributed the three intermediate levels to channels containing Kir6.1 and Kir6.2 at stoichiometries of 3:1, 2:2 and 1:3. This view is supported by expression of tandem Kir6.1–Kir6.2 constructs (to constrain the subunit stoichiometry to 2:2) with SUR2B (Cui *et al.* 2001) or SUR2A (Kono *et al.* 2000) which yields channels with a single conductance of ~ 48 pS, similar to the mid-conductance level observed following co-expression. In this study, PdBu caused an increase in activity of the intermediate conductance channels, but we could not determine average NP_{OS} due to the complexity of the amplitude histograms; accurate analysis will require cDNA constructs engineered to obtain single channel populations with known stoichiometries of Kir6.1 and Kir6.2. Regardless of this limitation, however, the data clearly show that activity of Kir6.1–Kir6.2/SUR2B channels was increased rather than decreased by PdBu, suggesting that channels composed of both pore-forming subunits do not contribute to the vascular K_{ATP} currents inhibited by PKC.

Kir6.2/SUR2B channels, or channels composed of SUR2B and a combination of Kir6.1 and Kir6.2 subunits, do not share functional identity with native vascular K_{NDP}

channels, but they are similar to the Kir6.2-containing channels of cardiac myocytes and pancreatic β cells, respectively, which are also stimulated by PKC (Light *et al.* 2000). The PKC phosphorylation site responsible for increased Kir6.2/SUR2A and Kir6.2/SUR1 activity was localized to the intracellular C-terminal region of the Kir6.2 subunit (Light *et al.* 2000). Site-directed mutation analysis showed that the modulation of these channels by PKC was prevented when a threonine residue (T180) within this PKC consensus site was replaced with an alanine or glutamate (Light *et al.* 2000). Light and co-workers (2000) postulated that PKC-dependent phosphorylation at this site may alter the modulation of gating by ATP and/or phospholipids (Tucker *et al.* 1997; Shyng & Nichols, 1998). Interestingly, the consensus site identified in Kir6.2 is conserved in Kir6.1, but we were unable to determine its role because mutant Kir6.1 subunits in which the equivalent threonine residue (T190) was substituted with an alanine or cysteine residue expressed with SUR2B had an extremely low open probability (< 0.0001 , data not shown). The results of our analysis of Kir6.1/SUR2B kinetics following PdBu treatment, however, imply that a site(s) on the pore-forming subunit may be involved. Kir6.1/SUR2B channels exhibit bursts of transitions between the open and closed states that are separated by extended intervals in a relatively long-lived closed state. Since neither burst duration nor intra-burst open and closed dwell times were affected by PKC activation, it is likely that the decline in P_o was due to an increase in inter-burst interval. Intra-burst kinetics and burst duration of native vascular K_{NDP} channels were similarly unaffected by PKC activation, but a significant increase in the inter-burst dwell time was identified (Cole *et al.* 2000). This inter-burst interval has been suggested to reflect transition into a long-lived closed state(s) in which ATP is bound to the channel (Takano & Noma, 1993). ATP regulation of deletion mutant Kir6.2 channels is maintained in the absence of SUR subunit indicating that the effect on channel gating occurs via an interaction with the Kir subunit (Tucker *et al.* 1997). The lack of effect of PKC activation on burst duration and intra-burst kinetics of vascular K_{NDP} and Kir6.1/SUR2B channels suggests that phosphorylation by PKC may reduce the dissociation of ATP from its binding site on the Kir6.1 subunit and stabilize the channels in this inter-burst closed state. An alternate biochemical approach will be required to identify the phosphorylation site, but the present data are consistent with the view that the presence of Kir6.1 may underlie the unique regulation by PKC of vascular smooth muscle compared to cardiac and pancreatic β cell K_{ATP} channels.

In summary, our findings provide the first evidence that Kir6.1/SUR2B channel activity is inhibited by PKC activation. The expression of SUR2B with Kir6.2 alone, or the combination of Kir6.1–Kir6.2, to form K_{ATP} channels with unique properties may occur in some vascular tissues,

but only the combination of Kir6.1 and SUR2B produces K_{ATP} channels with a modulation by PKC that is consistent with the inhibition of whole-cell vascular K_{ATP} currents by vasoactive agonists.

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