Adenosine-induced activation of ATP-sensitive K⁺ channels in excised membrane patches is mediated by PKC

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Hu, Keli, Gui-Rong Li, and Stanley Nattel. Adenosine-induced activation of ATP-sensitive K⁺ channels in excised membrane patches is mediated by PKC. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H488–H495, 1999.—Both protein kinase C (PKC) and adenosine receptor activation have been shown to enhance ATP-sensitive K⁺ (KATP) channels. The present studies were designed to determine whether PKC mediates adenosine effects on the KATP channel. The dependence of KATP channel activity (nPo) on intracellular ATP concentration ([ATP]i) was determined in excised rabbit ventricular membrane patches. Externally adenosine (100 µM in the pipette solution) significantly increased KATP nPo at all [ATP]i (dissociation constant increased from 7.4 ± 0.8 to 22.2 ± 3.1 µM, P < 0.001), an effect blocked by the adenosine receptor antagonist 8-phenyltheophylline (10 µM). When the highly selective PKC blocker bisindolylmaleimide (BIM) was included in the internal (bath) solution, the KATP-stimulating action of adenosine was prevented. The addition of BIM to the superfusate rapidly inhibited KATP channels activated by adenosine. Endogenous PKC activation by phorbol 12,13-didecanoate (PDD), but not administration of the inactive congener 4α-PDD, enhanced KATP activity. Internal guanosine 5’-O-(2-thiodiphosphate) prevented KATP activation by adenosine, an effect which could be overridden by exposure to PDD. We conclude that PKC mediates adenosine activation of KATP channels in excised membrane patches in a membrane-delimited fashion.

BRIEF PERIODS of ischemia reduce the amount of myocyte necrosis produced by a subsequent sustained period of ischemia (30). This phenomenon, termed ischemic preconditioning, has been demonstrated in all species examined including dogs (19), rabbits (38), rats (25), pigs (32), and humans (3, 45). The precise mechanisms of ischemic preconditioning remain to be elucidated completely. The ATP-sensitive K⁺ (KATP) channel appears to play a crucial role in the protective effects of ischemic preconditioning (1, 6, 7, 34, 39, 40). Both adenosine and protein kinase C (PKC) can mimic ischemic preconditioning, and their effects can be eliminated by KATP channel blockers (1, 34, 39, 44), suggesting that adenosine receptors, PKC, and KATP channels may be interrelated, with the KATP channel as the end effector in preconditioning. It has been shown that adenosineA₁ receptor activation stimulates KATP channels via an inhibitory G protein (Gi)‑mediated pathway (13, 14, 16, 17, 20, 36). Recently, the stimulation of PKC has been shown to increase KATP channel activity in cardiac myocytes (11, 23, 26). In a number of systems, inhibitory G proteins are known to be coupled to the activation of PKC (10). PKC mediation of adenine-induced enhancement of KATP could play a role in the well-demonstrated ability of PKC inhibitors, adenosine antagonists, and KATP blockers to prevent ischemic preconditioning. The purpose of the present study was to determine whether adenosine activation of KATP channels in excised membrane patches is mediated by PKC, providing a potential link between adenosine receptor agonists, PKC, and KATP channel activation.

METHODS

Cell isolation. Single rabbit ventricular myocytes were isolated by enzymatic dissociation as described previously (11). In brief, hearts were excised and retrogradely perfused via the aorta with oxygenated (100% O₂) Tyrode solution containing (in mM) 126 NaCl, 5.4 KCl, 1.0 CaCl₂, 1.0 MgCl₂, 0.33 NaH₂PO₄, 10 HEPES, and 10 glucose at 37°C. The perfusate was then changed to a Tyrode solution that was nominally Ca²⁺ free but otherwise had the same composition. When cardiac contraction had ceased, the hearts were perfused with the same solution containing collagenase (100–150 U/ml, type II, Worthington Biochemical, Freehold, NJ) and bovine serum albumin (0.1%, Sigma Chemical, St. Louis, MO) for 20–30 min. Softened ventricular tissues were removed, cut into small pieces, and mechanically dissociated by trituration. The isolated cells were kept in a storage solution containing (in mM) 20 KCl, 10 KH₂PO₄, 10 glucose, 70 potassium glutamate, 10 β-hydroxybutyric acid, 10 taurine, 5 mannitol, and 5 EGTA, along with 1% albumin. All cells used for experiments were rod shaped and showed clear cross-striations.

Solutions and drugs. The composition of the external solution (pipette solution for inside-out patches, bath solution for outside-out patches) was (in mM) 140 KCl, 0.5 MgCl₂, 1.0 CaCl₂, and 10 HEPES (pH adjusted to 7.4 with KOH). The internal solution (bath for inside-out patches, pipette for outside-out patches) contained (in mM) 140 KCl, 0.5 MgCl₂, 1.0 EGTA, 10 HEPES, and 0.1 GTP (pH 7.2). ATP (as K₂ATP, Sigma) was added as required from a 10 mM stock in 1 M sucrose but otherwise had the same composition. Solutions used for experiments were made fresh on the day of use. Adenosine and 8-phenyltheophylline (8-PT) were purchased from Sigma Chemical. The PKC activator phorbol 12,13-didecanoate (PDD) and its non-PKC-stimulating homolog 4α-PDD were purchased from ICN Biochemicals. Adenosine, 8-PT, PDD, and 4α-PDD were prepared as stock solutions in DMSO at concentrations of 10 mM for adenosine and 1 mM for 8-PT, PDD, and 4α-PDD. The highly selective PKC inhibitor bisindolylmaleimide hydrochloro-

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pike (BIM) (37) was obtained from Calbiochem-Novabiochem International and prepared as a stock solution (0.75 mM) in DMSO. Guanosine 5’-O-(2-thiodiphosphate) (GDP·βS) was obtained from Boehringer Mannheim and prepared as a stock solution (4.2 mM) in DMSO.

Single-channel recording and analysis. Ventricular cells were placed in a small-volume recording chamber (1 ml) on the stage of an inverted microscope. Single-channel currents were measured in the inside-out configuration using standard patch-clamp recording techniques (8). Pipettes had a resistance of 7–10 MΩ when filled with the extracellular solution. After gigaseal formation, the mouth of a multi-input perfusion pipette was brought to the cell. Solutions were changed with a rapid-switching perfusion system that changed the perfusate bathing the cell in <200 ms. The pipette and attached cell were lifted from the base of the chamber, and a rapid spurt of solution was applied to rip the cell from the pipette, leaving an excised inside-out patch. Patches were first exposed to bath solutions containing 2 mM ATP and then to ATP-free bath solution to confirm KATP channel opening. In some experiments, the outside-out configuration of the patch-clamp technique was used. The whole cell mode was first formed and then a spurt of solution was used to tear the cell from the pipette, leaving an outside-out patch.

Rundown of KATP channel activity tends to occur after patch excision. To minimize rundown, we used Ca2+-free bath solutions containing only 0.5 mM intracellular Mg2+. The rapidly switching perfusion system allowed us to obtain full curves for the relationship between KATP channel activity (nP) and intracellular ATP concentration ([ATP]i) within 5 min, minimizing the time for rundown. The patches were exposed to ATP-free bath solution at the beginning and end of each experiment to estimate the degree of rundown, and patches exhibiting >20% rundown were discarded. Only patches with less than five active channels were used to resolve discrete channel opening and closure and to minimize variability among patches. All experiments were performed at room temperature.

Single-channel currents were recorded at a holding potential of +40 mV, amplified (Axopatch 200A, Axon Instruments, Foster City, CA), digitized at 10 kHz, and stored on the hard disk of a computer for subsequent analysis. The recordings were filtered with a low-pass frequency of 1 kHz. All voltages and currents are expressed as they would be measured from the inside of the cell. Data were analyzed using pCLAMP software (version 6.0, Axon).

KATP nP0 was calculated from a multiple Gaussian fit to all-points current amplitude histograms that were constructed from data segments 20–30 s in duration using the equation

$$nP_0 = (a_1 + 2a_2 + 3a_3 + ... + na_n)/(a_0 + a_1 + a_2 + a_3 + ... + a_n),$$

where nP0 is the total number of channels in the patch (n) times open probability (P0); a2, a3, a4, ..., and an are the areas under each peak corresponding to a discrete open level; and a0 is the area under the closed state peak. The nP0 data were generally expressed in normalized form relative to the nP0 at zero intracellular ATP, to control for variations in nP0 among patches.

Statistics. Group data are presented as means ± SE. Multiple group means were compared by ANOVA with a Dunnett’s test. Differences with a two-tailed P < 0.05 were considered statistically significant. Nonlinear curve fitting of concentration-response data was performed with a Marquardt procedure for parameter estimation (Sigma Plot, Jandel Scientific).

RESULTS

Effects of PKC inhibition on adenosine-induced KATP channel activation. We first tested the effects of adenosine on KATP channel activity in the presence and absence of BIM (50 nM) in the bath. Figure 1 shows channel activity from representative inside-out patches studied at bath ATP ([ATP]i) of 10 µM. Current recordings are on the left and corresponding histograms are at the right of each panel. Under control conditions, channel activity was relatively low (Fig. 1A). When 100 µM adenosine was included in the pipette, channel activity was greater (Fig. 1B). With BIM in the bath and either no adenosine (Fig. 1C) or adenosine (100 µM) in the pipette (Fig. 1D), KATP channel activity was similar to control without adenosine in the pipette (Fig. 1A).

Figure 2 shows mean data for the relations between nP0 and [ATP]i in cells studied under control conditions (n = 7), in the presence of adenosine in the pipette (n = 6), in the presence of BIM in the bath but no adenosine (n = 6), and with BIM in the bath and adenosine in the pipette (n = 7). Values of nP0 are expressed as a percentage of the value in each experiment under ATP-free conditions. The results were fitted by the equation given in the legend to Fig. 2, providing the curves shown in Fig. 2. Under each condition, channel activity decreased as [ATP]i increased, and all curves were roughly parallel. In the absence of BIM, the inclusion of adenosine in the pipette increased nP0 significantly at all [ATP]i between 10 and 50 µM. Results in the presence of BIM were not significantly different from control, whether or not adenosine was present in the pipette. The [ATP]i associated with 50% maximal KATP channel activation (IC50) was increased from 7.4 ± 0.8 µM in the absence of adenosine to 22.2 ± 3.1 µM in the presence of adenosine (P < 0.001), whereas the Hill coefficient was unchanged (2.19 ± 0.31, control vs. 1.76 ± 0.19, adenosine; P = NS). The [ATP]i IC50 values for BIM alone (7.6 ± 1.2 µM) or adenosine plus BIM (7.1 ± 1.5 µM) were similar to the control value. These results show that adenosine activates KATP channels by reducing their ATP sensitivity, an action that requires functional intactness of PKC.

One limitation of the kind of experiment shown in Fig. 2 is that each condition is studied in a separate set of patches, raising the possibility that different intrinsic properties of the patches used for each condition may have influenced the results. We therefore performed the experiments shown in Fig. 3 to ascertain the effects of PKC inhibition with BIM on KATP channel activity in the absence and presence of adenosine. Patches (n = 6/group) were allocated to study either with or without adenosine (100 µM) in the pipette. Each patch was then exposed to ATP-free solution and then solution containing 10 µM ATP. The solution was then rapidly altered to one containing 10 µM ATP and 50 nM BIM. Typical original recordings in the absence...
of adenosine are shown in Fig. 3A, whereas recordings in the presence of adenosine are shown in Fig. 3B. Mean data for $nP_o$ under each condition are shown in Fig. 3, insets. Absolute $nP_o$ was similar in the presence of ATP-free solution in the presence or absence of adenosine (0.61 ± 0.10 with adenosine vs. 0.57 ± 0.10 without adenosine; $P \geq NS$). In the presence of 10 µM [ATP], channel activity was significantly greater in the presence of adenosine ($nP_o$ 0.43 ± 0.08 vs. 0.19 ± 0.03 in patches studied without adenosine; $P < 0.02$). The addition of BIM inhibited $K_{ATP}$ activity within 30 s in patches exposed to adenosine, decreasing mean $nP_o$ to 0.22 ± 0.05 ($P < 0.01$). In patches not exposed to adenosine, $nP_o$ was not altered by BIM, averaging 0.19 ± 0.03 before and 0.18 ± 0.03 after BIM ($P = NS$).

Effects of adenosine receptor antagonism. To confirm that the increase in $K_{ATP}$ channel activity observed with 100 µM pipette adenosine is mediated by activation of adenosine receptors, we evaluated the response when 10 µM 8-PT was included in the extracellular (pipette) solution along with 100 µM adenosine. As shown by the mean data in Fig. 4, the $nP_o$ vs [ATP] relation in six inside-out patches exposed to adenosine in the presence

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Fig. 1. Representative recordings from patches studied with (B and D) or without (A and C) 100 µM adenosine (Ade) in pipette, in absence (A and B) or presence (C and D) of 50 nM bisindolylmaleimide (BIM) in bath. All-points histograms of current amplitude throughout recording period are shown to right of corresponding recordings. [ATP], intracellular ATP concentration; Ctrl, control.

Fig. 2. Concentration-response curves for ATP inhibition of ATP-sensitive K+ channels under control (n = 7); Ade, 100 µM in pipette (n = 6); BIM, 50 nM in bath (n = 6); and 100 µM Ade in pipette plus 50 nM BIM in bath (n = 7). Data were fitted by Hill equation: normalized $nP_o = nP_o /nP_{o,max} = 1 / \left(1 + ([ATP] / K_d)^n \right)$, where $nP_o$ is open probability at a given [ATP], $nP_{o,max}$ is control open probability at zero [ATP], $K_d$ is concentration for half-maximal effect, and $n$ is Hill coefficient.

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$2 pA$

$[ATP] = 10 \mu M$

A

B

C

D

Ctrl

Ade

BIM

Ade+BIM

0

1 sec

Number of points

Number of points

Number of points

Number of points

0

10

20

30

40

50

60

70

80

90

100

Amplitude (pA)

Amplitude (pA)

Amplitude (pA)

Amplitude (pA)
of 8-PT was similar to that of control patches and significantly different from that of patches exposed to extracellular adenosine alone. Overall, the IC₅₀ averaged 7.37 ± 0.89 µM in the presence of adenosine and 8-PT, compared with 22.20 ± 3.11 µM (P < 0.01) in the presence of adenosine alone and 7.37 ± 0.81 µM (P = NS) under control conditions.

Activation of KATP channels by the stimulation of endogenous PKC activity. To determine whether stimulation of endogenous PKC results in KATP activation in excised membrane patches, we studied the effects of adding PDD to the bath (intracellular side of patches). The inactive congener 4α-PDD was used as a negative control. Figure 5 shows the ATP dependence of KATP

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**Fig. 3.** Effects of bath (intracellular side of patch) application of BIM on ATP-sensitive K⁺ (KATP) channel activity in presence of 10 µM [ATP] in absence and presence of 100 µM Ade in pipette. Results in absence of Ade (control conditions) are in A, and results in presence of 100 µM Ade are in B. Original recordings of KATP channel activity from one patch under ATP-free conditions and then in presence of 10 µM [ATP], without and with BIM in bath are shown in each panel, with mean ± SE nPₒ values under each condition (n = 6 patches/group) shown in insets.

**Fig. 4.** KATP nPₒ-[ATP] relation in absence of Ade in pipette (Ctrl) and in presence of 100 µM Ade in pipette, with or without 10 µM 8-phenyltheophylline (8-PT). Data were fitted by Hill equation given in legend to Fig. 2 (n = 6 for each group). *P < 0.05, **P < 0.01 vs. control.

**Fig. 5.** KATP nPₒ-[ATP] relation in absence of phorbol ester (Ctrl) and in presence of protein kinase C-stimulating phorbol ester phorbol 12,13-didecanoate (PDD) and its inactive congener 4α-PDD. Curves were obtained by fitting mean data with Hill equation given in legend to Fig. 2 (n = 7 for each group).
channels in the presence of 1 µM PDD or 4α-PDD in the bath. PDD shifted the concentration-response curve to the right relative to control, with the IC50 increased from 7.37 ± 0.81 to 23.97 ± 3.09 µM (P < 0.001). The inactive congener 4α-PDD did not alter the IC50 (7.83 ± 0.72 µM vs. 7.37 ± 0.81 µM, control; P = NS). PDD also altered the slope of the concentration-response relation: the Hill coefficient was changed from 1.71 ± 0.22 in the presence of 4α-PDD (2.19 ± 0.31 for control conditions) to 0.83 ± 0.09 in the presence of PDD (P < 0.01 vs. 4α-PDD, P < 0.01 vs. control).

Role of G proteins in adenosine response. To evaluate the role of guanine nucleotide-binding proteins in the response to adenosine, we used the GDP analog GDPβS, which was applied to the intracellular side of patches at a concentration of 200 µM. Figure 6A shows the effect of extracellular application of adenosine to outside-out patches (10 µM ATP in the pipette solution). Adenosine substantially increased channel opening in a reversible fashion. Figure 6B shows recordings obtained from another outside-out patch with 200 µM GDPβS and 10 µM ATP in the pipette (internal) solution. Under these conditions, adenosine had no perceptible effect on channel activity. In five outside-out patches studied without GDPβS in the pipette, adenosine increased nPo from 0.19 ± 0.05 to 0.49 ± 0.07 (P < 0.01), and nPo returned to 0.20 ± 0.04 (P = NS vs. control) after adenosine washout. In five other outside-out patches studied with GDPβS in the pipette, nPo averaged 0.18 ± 0.04, 0.17 ± 0.03, and 0.17 ± 0.02 under control, adenosine, and washout conditions, respectively.

Figure 6C shows the effect of adding GDPβS to the bath on KATP current (I_KATP) activity in inside-out patches in the presence of adenosine in the pipette. The addition of GDPβS reduced channel opening. The ability of PKC activation to restore I_KATP activity is shown by the result of adding PDD (1 µM) to the bath. The increased activity caused by PDD despite the continued presence of GDPβS indicates that PKC activates I_KATP at a step distal to those involving G proteins. In six patches studied in this fashion, nPo averaged 0.78 ± 0.10 under ATP-free conditions, 0.45 ± 0.05 in the presence of 10 µM ATP, 0.18 ± 0.04 in the presence of 10 µM ATP and GDPβS (P < 0.01 vs. 10 µM ATP alone), and 0.50 ± 0.10 in the presence of PDD, GDPβS, and 10 µM ATP (P < 0.05 vs. GDPβS and 10 µM ATP without PDD). The importance of G protein-coupled receptors in the inhibitory action of GDPβS is shown by the experiment in Fig. 6D, which was performed in the same fashion as the experiment in Fig. 6C, but without including adenosine in the pipette. In this case, single-channel activity was not altered when GDPβS was added to the bath (although it was increased by PDD). In six patches studied with the same protocol, nPo averaged 0.75 ± 0.09 in ATP-free conditions, 0.17 ± 0.14 in the presence of 10 µM ATP alone, 0.18 ± 0.04 in the presence of 10 µM ATP and GDPβS (P = NS vs. 10 µM ATP alone), and 0.48 ± 0.08 in the presence of 10 µM ATP, GDPβS, and PDD (P < 0.01 vs. 10 µM ATP alone).

DISCUSSION

The present experiments demonstrate that adenosine applied to the extracellular side of rabbit ventricular membrane patches enhances K_ATP channel activity via G protein-mediated activation of PKC. Because the results were obtained in excised membrane patches, this PKC-mediated effect of adenosine does not require a diffusible cytoplasmic second messenger, and all the
elements of the signal transduction system (adenosine receptor, G proteins, phospholipases, membrane phospholipid, PKC, and K_{ATP} channel(s)) must be available in the membrane and able to interact effectively.

Comparison with results of previous studies of adenosine-mediated activation of K_{ATP} channels. Kirsch et al. (16) showed that adenosine activates K_{ATP} channels at reduced [ATP], via the α-subunit of inhibitory G protein (16), in contrast to the G_{iβγ}-mediated regulation of acetylcholine-sensitive K⁺ current (13). G protein regulates K_{ATP} by removing [ATP]-induced inhibition (14, 15, 36). We observed similar modulation of I_{KATP} sensitivity to [ATP], by extracellular adenosine.

Potential role of PKC-mediated phosphorylation in modulating K_{ATP} channel function. In noncardiac systems, PKC modulates K_{ATP} channel activity (4, 29, 43), with an inhibition (43), activation (4, 29), or inhibition followed by activation (5) having been reported. The present findings are in general agreement with previous findings regarding cardiac ATP activation by PKC. PKC reduced channel sensitivity to ATP, and as in the work of Light and co-workers (22, 23), the Hill coefficient was reduced by ~50%. In contrast to our present and previous observations (11), Light and co-workers (22, 23) did not observe a change in the ATP concentration for 50% activation. This discrepancy may be due to their use of a purified, constitutively activated PKC from rat brain, containing a mixture of α-, β₁-, γ-, and ε-isoforms (22, 23). Endogenous PKC exists as a broad range of isoforms, with differing tissue distribution, Ca²⁺ sensitivity, and possibly substrate specificity (33). It is therefore possible that different isoforms interact differently with the K_{ATP} channel and that the response to PKC activation depends on the specific isoform(s) involved.

We found that K_{ATP} channel activation by adenosine requires intact endogenous PKC; however, unlike phosphoryl esters, adenosine did not alter the Hill coefficient of the K_{ATP}-[ATP] relation. This may be due to the activation of different PKC isoforms by adenosine compared with phosphoryl ester. Alternatively, adenosine may have other actions that modulate the PKC effect on the slope of the ATP-sensitivity curve.

Previous studies have pointed to PKC translocation to the cell membrane as a potentially important event during ischemic preconditioning (27) and suggested that adenosine causes PKC translocation (9). In the dog, PKC translocation does not accompany ischemic preconditioning (31). Because we studied adenosine effects on excised membrane patches, it appears that membrane-bound PKC is able to mediate adenosine-induced K_{ATP} activation and that PKC translocation is not essential for adenosine-induced K_{ATP} activation.

Novel aspects and potential significance. The major novel finding of the present study is that intact endogenous PKC function appears to be essential to the coupling between the adenosine receptor and the K_{ATP} channel in excised membrane patches from rabbit ventricular myocytes. Whereas adenosine is well known to couple to K_{ATP} channels by inhibitory G proteins (13, 14, 16, 20, 36), and inhibitory G proteins often couple to their effectors via PKC, the present study is the first of which we are aware to show directly a role for membrane PKC in coupling adenosine to the K_{ATP} channel. Wang and Lipsius (41) showed that a second exposure of cat atrial myocytes to acetylcholine, which like adenosine acts via inhibitory G proteins, activates I_{KATP} by a PKC-dependent mechanism. Lester et al. (18) recently postulated that PKC mediates the positive inotropic effects of adenosine on rat cardiac tissue.

Direct coupling to G proteins is an important regulator of ion channel function (2). G protein-mediated actions in excised, cell-free patches are often taken as evidence of a mechanism independent of second messengers. Our observation that PKC inhibition can prevent and reverse adenosine activation of K_{ATP} channels in excised patches suggests that membrane-bound PKC can mediate receptor coupling to the channel and that second messengers can couple receptors to ion channels in cell-free excised patches.

Both adenosine (1, 7, 24, 38, 39) and PKC (12, 28, 34, 46) participate in short-term ischemic preconditioning. The present findings may help to explain, at least in part, their interrelated effects. Our results do not exclude additional actions of adenosine and/or PKC that require cytoplasmic components, other elements of intact cellular function, and/or membrane-cytosol interactions. Liu et al. (26) suggested that both PKC activation and adenosine receptor stimulation may be needed for ischemic preconditioning. Liang (21) recently presented results consistent with PKC coupling of adenosine with the K_{ATP} channel, along with the possibility that preconditioning requires additional actions of adenosine receptor activation.

Potential limitations. In addition to acting as a ligand for the K_{ATP} channel, ATP is a substrate for enzymes that phosphorylate the channel and maintain its activity, a process that requires hydrolyzable forms of ATP (35). The latter process could complicate analyses of ligand-gated concentration dependence as performed in the present study. The phosphorylation-dependent process is manifested by gradual channel rundown in the absence of hydrolyzable ATP (35). To avoid contamination by this process, we exposed patches to 2 mM ATP before studying the ATP concentration-response curve, minimized the time for measuring the latter (<5 min), and verified that channel activity under ATP-free conditions was the same before and after the concentration-response measurement.

ATP is a substrate for PKC-dependent phosphorylation. It is therefore conceivable that our ATP concentration-response curves for I_{KATP} are contaminated by differing degrees of phosphorylation at different ATP concentrations. The Michaelis constant for cardiac PKC hydrolysis of ATP is 4.4 μM (42). Patches were superfused with 2 mM ATP for several minutes before ATP concentration-response analysis, so the level of PKC-induced phosphorylation should have been near maximal during the relatively rapid ATP concentration-response determination. We cannot, however, exclude the possibility that (particularly at ATP concentrations of 1 and 5 μM) some reversal of channel phosphoryla-
tion may have occurred due to a lack of substrate. There is extensive evidence for PKC activation of cardiac $I_{\text{KATP}}$ in the literature (11, 23, 26), and channel phosphorylation has been presumed to be involved; however, channel phosphorylation has not been directly demonstrated. Thus mechanisms other than phosphorylation could be involved, as suggested for the regulation of $I_{\text{KATP}}$ activity by Mg-ATP (35).

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