Regulation of an Inwardly Rectifying ATP-sensitive K^+ Channel in the Basolateral Membrane of Renal Proximal Tubule

Ulrich R. Mauerer,* Emile L. Boulpaep,* and Alan S. Segal*‡

From the *Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06520; and ‡Department of Medicine, University of Vermont, Burlington, Vermont 05401

ABSTRACT Functional coupling of Na^+,K^+-ATPase pump activity to a basolateral membrane (BLM) K^+ conductance is crucial for sustaining transport in the proximal tubule. Apical sodium entry stimulates pump activity, lowering cytosolic [ATP], which in turn disinhibits ATP-sensitive K^+ (K_{ATP}) channels. Opening of these K_{ATP} channels mediates hyperpolarization of the BLM that facilitates Na^+ reabsorption and K^+ recycling required for continued Na^+,K^+-ATPase pump turnover. Despite its physiological importance, little is known about the regulation of this channel. The present study focuses on the regulation of the BLM K_{ATP} channel by second messengers and protein kinases using membrane patches from dissociated, polarized *Ambystoma* proximal tubule cells. The channel is regulated by protein kinases A and C, but in opposing directions. The channel is activated by forskolin in cell-attached (c/a) patches, and by PKA in inside-out (i/o) membrane patches. However, phosphorylation by PKA is not sufficient to prevent channel rundown. In contrast, the channel is inhibited by phorbol ester in c/a patches, and PKC decreases channel activity (nP_o) in i/o patches. The channel is pH sensitive, and lowering cytosolic pH reduces nP_o. Increasing intracellular [Ca^{2+}] ([Ca^{2+}]$_i$) in c/a patches decreases nP_o, and this effect is direct since [Ca^{2+}]$_i$ inhibits nP_o with a K_i of ~170 nM in i/o patches. Membrane stretch and hypotonic swelling do not significantly affect channel behavior, but the channel appears to be regulated by the actin cytoskeleton. Finally, the activity of this BLM K_{ATP} channel is coupled to transcellular transport. In c/a patches, maneuvers that inhibit turnover of the Na^+,K^+-ATPase pump reduce nP_o, presumably due to a rise in intracellular [ATP], although the associated cell depolarization cannot be ruled out as the possible cause. Conversely, stimulation of transport (and thus pump turnover) leads to increases in nP_o, presumably due to a fall in intracellular [ATP]. These results show that the inwardly rectifying K_{ATP} channel in the BLM of the proximal tubule is a key element in the feedback system that links cellular metabolism with transport activity. We conclude that coupling of this K_{ATP} channel to the activity of the Na^+,K^+-ATPase pump is a mechanism by which steady state NaCl reabsorption in the proximal tubule may be maintained.

KEY WORDS: ion channel • kidney • patch-clamp • sulfonylurea • epithelia

INTRODUCTION

A consistent finding in essentially all Na^+-reabsorbing or Cl^--secreting epithelia is an increase in basolateral membrane (BLM)[1] K conductance (G_K) concurrent with the activity of the Na^+,K^+-ATPase pump, and vice-versa (Schultz, 1992; Beck et al., 1994). Using rabbit kidney cortical tubule suspension, Balaban et al. (1980) directly demonstrated that inhibition of the Na^+,K^+-ATPase pump increased the ATP/ADP ratio, and concluded that [ATP]$_i$, [ADP]$_i$, or their ratio could be the link coupling cellular metabolism to transport. The demonstration of Type I K_{ATP} channels in cardiac muscle (Noma, 1983) opened the way to study the regulatory role of ATP at the single-channel level. Since this topic has been treated in several excellent reviews (e.g., see Beck et al., 1994; Lang and Rehwald, 1992; Edwards and Weston, 1993; Ashcroft and Ashcroft, 1990), as well as in Mauerer et al. (1998), the following is limited to renal K^+ channels.

In the distal nephron, Hunter and Giebisch (1988) reported a Ca^{2+}-activated K^+ channel in the apical membrane of *Amphiuma* early distal tubule that was inhibited by ATP with a K_i of ~5 mM. This was the first ATP-sensitive K^+ channel described in epithelia, and was classified as Type 3 (Ashcroft and Ashcroft, 1990) because of its Ca^{2+} activation and lower sensitivity to ATP. Moreover, this "maxi-K" channel was depolarization activated and did not exhibit rundown. Subsequently, Wang et al. (1990) demonstrated that the K^+ channel in the apical membrane of rat cortical collecting duct, first identified by Frindt and Palmer (1989), was ATP sensitive. This 35-pS channel, which mediates K^+ secretion by principal cells, is Type 1-like (Ashcroft and Ashcroft, 1990) in that it is insensitive to Ca^{2+}, volt-

Address correspondence to Alan S. Segal, Department of Medicine, University of Vermont, 55A South Park Drive, Colchester, VT 05446. Fax: 802-656-8915; E-mail: asegal@zoo.uvm.edu

[1]*Abbreviations used in this paper:* BLM, basolateral membrane; c/a, cell-attached (patch); CD, cytochalasin D; CFTR, cystic fibrosis transmembrane regulator; FK, forskolin; i/o: inside-out (patch); NBF, nucleotide binding fold; nP_o, channel activity represented as the product of the minimum number of channels (n) times the open probability (P_o) of the channel; SUR, sulfonylurea receptor.

age independent, and exhibits rundown (Wang and Giebisch, 1991*a*; Wang et al., 1990). Thus, the single-channel findings in distal nephron segments were partially consistent with the notion that $[ATP]_i$ could link cell metabolism and K^+ conductance, although Schultz (1981) had specified that the relevant K conductance was on the BLM. Two K^+ channels have been identified in the lateral membrane of rat cortical collecting duct (Wang et al., 1994), but they do not appear to be ATP sensitive.

The situation in the proximal tubule, however, is more compatible with homocellular regulation (Schultz, 1981). In their study of *Necturus* proximal tubule, Matsumura et al. (1984) showed that inhibition of Na^+,K^+-ATPase activity using ouabain, low bath K^+, or low luminal Na^+ perfusate brought about a fall in BLM G_K. They suggested that this effect might be a metabolic consequence of pump inhibition, but emphasized $[Ca^{2+}]_i$ as the proximate signal coupling pump activity to BLM G_K. Once it became evident that Type 1 K_{ATP} channels were not Ca^{2+} activated, the focus shifted to ATP itself as the link between pump activity and BLM G_K. Beck et al. (1991*a*) quantitated the inverse relationship between Na^+ transport and $[ATP]_i$ in perfused rabbit proximal tubule and confirmed a qualitative correlation between $[ATP]_i$ and G_K. Shortly thereafter, Tsuchiya et al. (1992) observed that ATP reversibly inhibited BLM K_{ATP} channel activity in four of five inside-out patches pulled off the BLM of nonperfused rabbit tubules, but regulation on the single-channel level was not pursued in that study. Beck et al. (1993) looked at regulation of stretch-insensitive BLM K^+ channels in perfused rabbit tubules and found that the addition of luminal substrates depolarized the cell and increased channel activity. In a follow-up study, the same group (Hurst et al., 1993) showed that this channel could be activated by diazoxide and inhibited by pump inhibition or direct application of ATP. These data, combined with our findings in nonperfused rabbit tubules and dissociated *Ambystoma* proximal tubule cells (see Mauerer et al., 1998) that maintain epithelial polarity (Segal et al., 1996), clearly show that a Type 1-like K_{ATP} channel exists on the BLM of the proximal tubule.

In the present study, we have used the dissociated *Ambystoma* proximal tubule cells (Segal et al., 1996) to investigate the regulation of this BLM K_{ATP} channel by protein kinases, intracellular nucleotides, pH (pH_i), Ca^{2+}, and the cytoskeleton. We also show that regulation of the K_{ATP} channel is indirectly linked to transport dynamics in the proximal tubule through changes in intracellular [ATP], resulting from altered activity of the Na^+,K^+-ATPase pump as transport is modulated.

MATERIALS AND METHODS

Solutions and Drugs

The composition of the solutions used is summarized in Table I. After titration to pH 7.5 (710A; Orion Research, Boston, MA), sucrose was added to adjust the osmolality of the solutions (3MO; Advanced Instruments Inc., Needham Heights, MA). KCl solutions containing low levels (50, 100, 200, 500, and 1,000 nM) of free Ca^{2+} were prepared by adding the appropriate amount of $CaCl_2$ (0.407, 0.579, 0.733, 0.873, and 0.933 mM, respectively) to solution *d* (Table I). Free Mg^{2+} was maintained at ~1 mM except in solution *b* (divalent-free NaCl). In solutions containing ATP,

TABLE I
Solutions

	a NaCl Ringer	*b* Ca^{2+}/Mg^{2+} free Ringer	*c* NaCl recording solution	*d* KCl recording solution	*e* NaCl 1 μM Ca^{2+}	*f* KCl 50 nM Ca^{2+}	*g* Isotonic 3/4 NaCl	*h* Hypotonic 3/4 NaCl	*i* KCl low Cl^-
Na^+	95.9	98.5	95	2.5	95	2.5	67.5	67.5	2.5
K^+	2.5	2.5	2.5	95	2.5	95	2.5	2.5	95
Cl^-	98.1	92.5	94.5	94.5	96.3	95.3	67	67	13.5
Aspartate$^-$									81
Ca^{2+} (total, mM)	1.8				0.933	0.407			
Ca^{2+} (free, nM)					1000	50			
Mg^{2+}	1		1	1	1	1	1	1	1
HEPES	5	5	5	5	5	5	5	5	5
HEPES$^-$	5	5	5	5	5	5	5	5	5
EGTA			1	1	1	1	1	1	1
EDTA		1							
$H_2PO_4^-$	0.1	0.1							
HPO_4^{2-}	0.4	0.4							
Dextrose	2	2							
pH (titrand)	7.5 (NaOH)	7.5 (NaOH)	7.5 (NaOH)	7.5 (KOH)	7.5 (NaOH)	7.5 (KOH)	7.5 (NaOH)	7.5 (NaOH)	7.5 (KOH)
Osmolality	200	200	200	200	200	200	200	140	200

the nucleotide was added as the Mg-salt to maintain the free Mg^{2+} at ~1 mM (range 0.98–1.33 mM). Chemicals used were of the highest quality and obtained from Sigma Chemical Co. (St. Louis, MO), except ADP (Boehringer-Mannheim Biochemicals, Indianapolis, IN). Nucleotides were prepared fresh daily as 20–50-mM stocks in bath solution. Stock solutions of glibenclamide (100 mM), PMA (10 mM), and 4α-PMA (10 mM) were dissolved in DMSO. Stock solutions (10 mM) of forskolin, 1,9-dideoxyforskolin (Calbiochem Corp., La Jolla, CA), and nigericin were dissolved in ethanol. The catalytic subunit of the cAMP-dependent protein kinase A (Promega Corp., Madison, WI) or protein kinase C (0.5 U/ml; Promega Corp.) was pipetted directly into the bath.

Cell Preparation

Dissociated proximal tubule cells were isolated from amphibian kidneys as previously described (Segal et al., 1996). Briefly, kidneys from *Ambystoma tigrinum* were rapidly removed and placed in iced HEPES-buffered NaCl at pH 7.5 (solution *a*). After incubation in collagenase-dispase (0.2 U/ml collagenase; Boehringer-Mannheim Biochemicals), the enzyme reaction was stopped by washing with Ca^{2+}- and Mg^{2+}-free NaCl (solution *b*). Dissociated cells were resuspended in 2.5 ml of NaCl (solution *a*) in a 35-mm culture dish, and stored at 4°C until use. The dissociated proximal tubule cells retain their epithelial polarity for up to 14 d (Segal et al., 1996). Cells were used for experiments from 2 to 12 d after dissociation. A representative cell is shown in Fig. 1 of Mauerer et al. (1998).

Electrophysiology, Data Acquisition, and Analysis

The methods are essentially as described in Mauerer et al. (1998). These include the mounting and selection of single proximal cells for electrophysiological study. The standard tight-seal patch-clamp configurations for single-channel recording were used (Hamill et al., 1981), and channel currents were acquired and analyzed as described in the accompanying paper. Briefly, channel activity (nP_o) was calculated over periods of 60–500 s, and open and closed dwell lifetimes were determined as reported elsewhere (Mauerer et al., 1998).

The number of observations or experiments is reported in the text, whereas n in the analysis denotes either the whole data set or the subset of total experiments in which precise quantitation could be reliably applied. In some figures, a running average (using a specified window width) of current versus time is displayed. Statistical values for the n elements are given as mean ± SEM. Student's t test was applied where appropriate.

RESULTS

The regulation of the BLM K_{ATP} channel by PKA, PKC, $[Ca^{2+}]_i$, and pH was studied in cell-attached (c/a) and inside-out (i/o) patches. Channel activity in response to perturbations of cell volume was examined in c/a patches, and the effect of membrane stretch and the role of the cytoskeleton was also tested. Finally, the coupling of channel behavior to changes in cellular energy levels and transport activity was investigated.

Forskolin activates the BLM K_{ATP} channel. The cAMP second messenger system was studied in c/a patches using forskolin (FK), which increases $[cAMP]_i$ by activating adenylyl cyclase. In each experiment, a cell served as its own control. Fig. 1, *A* and *B* shows a representative experiment. Under control conditions with KCl in the pipette (solution *d*) in a NaCl bath (solution *c*), steady state channel activity was recorded for 3–5 min before the application of 10 μM FK at a command potential of −60 mV. Within 1–2 min, the single-channel current begins to increase, consistent with hyperpolarization of the cell membrane potential (V_m). This is most likely due to the opening of K^+ channels on the cell membrane that precede those under the patch pipette itself. Within 5–10 min, FK increases the nP_o of the BLM K_{ATP} channels in the patch of membrane under study.

Fig. 1 *C* summarizes the time course of channel activity (nP_o) and single-channel current (i_{sc}) of the experiment. Initially, nP_o was 0.17 and the i_{sc} was −1.3 pA (at a command potential of −60 mV) under control conditions with KCl in the pipette in a NaCl bath. After 10 μM FK, the nP_o increased nearly ninefold and i_{sc} rose to −2.2 pA. Based on an inward slope conductance of 22.2 pS under similar conditions (Mauerer et al., 1998), the change in i_{sc} of −0.9 pA in response to FK would result from a hyperpolarization of V_m by ~40 mV (0.9 pA/22.2 pS). Such a hyperpolarization is most likely due to FK-induced opening of K^+ channels over the whole cell membrane. That the rise in i_{sc} precedes the increase in nP_o is consistent with the notion that K^+ channels in the rest of the membrane are opening before those in the patch of membrane, possibly because the latter are less accessible. Similar results were obtained in 10 of 12 experiments (83%). In those experiments that allowed reliable analysis, nP_o increased by 7.5-fold (7.50 ± 1.55, $n = 6$), leading to an incremental i_{sc} of −0.52 ± 0.10 pA ($n = 6$), consistent with a concomitant membrane hyperpolarization of 23.4 ± 4.5 mV ($n = 6$). In contrast, exposure of c/a patches to 1,9-dideoxyforskolin, which does not lead to an increase in $[cAMP]_i$, has no effect ($n = 5$).

These results indicate that the BLM K_{ATP} channel is activated by a component of the cAMP second messenger system. The possibility that the channel is directly activated by cAMP is remote since the addition of cAMP to the cytoplasmic side of inside-out membrane patches does not result in channel activation ($n = 7$, data not shown).

PKA directly activates the BLM K_{ATP} channel. To test the hypothesis that the channel is activated by the cAMP-dependent protein kinase, the effect of the catalytic subunit of PKA was tested in i/o patches. To prevent channel rundown, i/o patches were excised in a bath containing 0.2 mM ATP (see Mauerer et al., 1998). After a control period in 0.2 mM ATP, 50–100 U/ml PKA was directly added to the bath in the continued presence of ATP ($n = 29$). Channel activation occurred after a delay of 0.5–3 min ($n = 26$). This delay is most likely due to the time necessary for the reagents to diffuse to the patch, and then phosphorylate the chan-

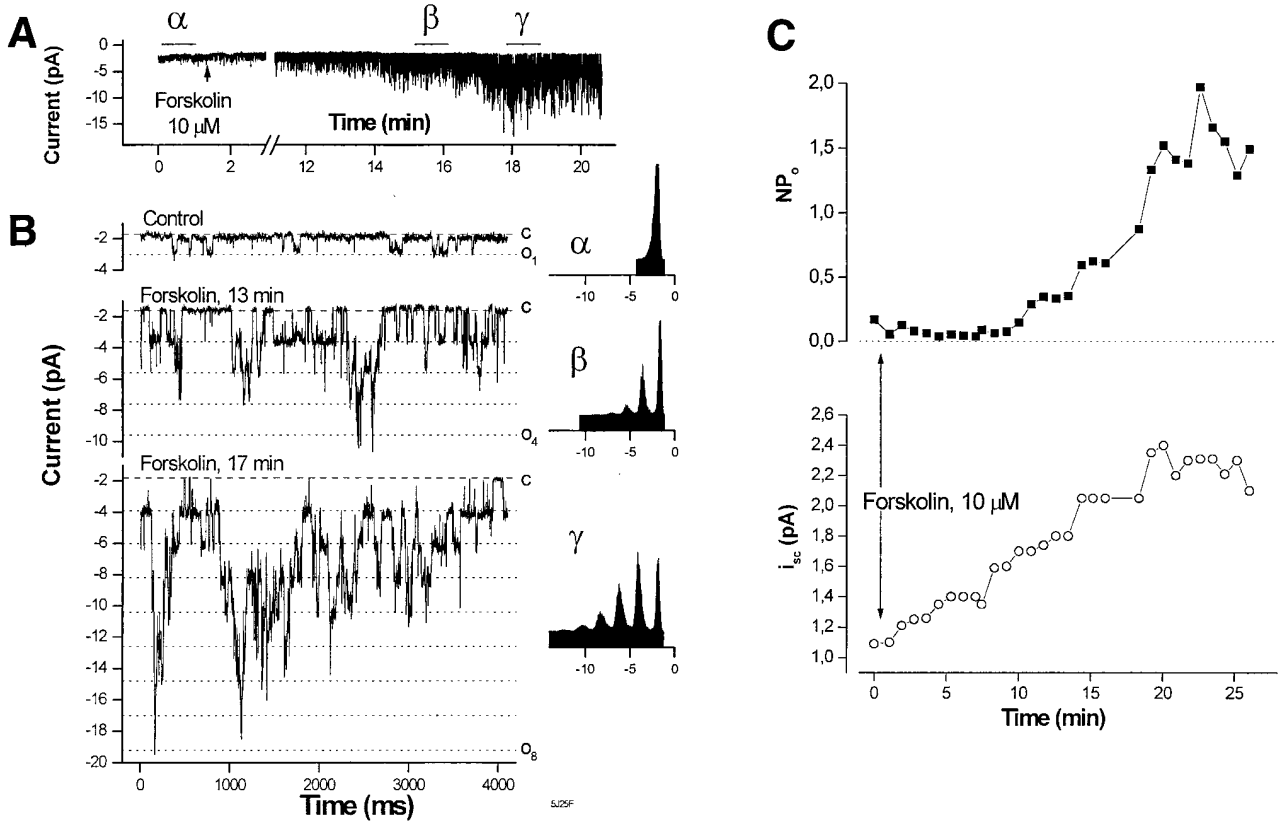

FIGURE 1. cAMP agonists activate the BLM K_{ATP} channel. (*A*) Forskolin (10 μM) activates the BLM K_{ATP} channel in cell-attached patches. In the experiment depicted, the pipette contains KCl (solution *d*), the bath contains NaCl (solution *c*), and the command potential is −60 mV. A running average of current versus time (window width 16 ms) is shown. (*B*) Sample traces taken from within the regions marked α, β, and γ in *A*. The corresponding amplitude histograms were taken for each region. After the addition of forskolin, channel activity increases ninefold (nP_o increases from 0.17 to 1.46). Note the increase in the single channel current (i_{sc}) due to the hyperpolarization of the cell membrane as K^+ channels open over the BLM. The all channels closed level (*dashed line*) and open channel levels (*dotted lines*) are indicated. (*C*) Forskolin increases both channel activity (NP_o) and single channel current (i_{sc}). nP_o (■) and i_{sc} (○) as measured in the experiment described in *A* are plotted versus time. i_{sc} increases as K^+ channels on the BLM open. From the change in i_{sc} ($\Delta i_{sc} = -0.9$ pA), the hyperpolarization (ΔV_m) is ~−40 mV. Note that the increase in i_{sc} precedes the increase in nP_o, consistent with other BLM K_{ATP} channels opening before those in the patch.

nel(s). In some experiments, a low [Cl] bath was used to isolate the effect of PKA on the K^+ channel since phosphorylation via PKA also activates a cystic fibrosis transmembrane regulator (CFTR)–like Cl^- channel present in the BLM (Segal et al., 1996). The mean nP_o increase was 5.1 ± 0.7-fold in patches containing K^+ channels exclusively ($n = 6$).

A representative experiment is shown in Fig. 2. With only 0.2 mM ATP in the NaCl bath, infrequent and brief channel openings are seen. The addition of the catalytic subunit of PKA (csPKA) results in more frequent channel opening and bursting, with a nearly fourfold increase in nP_o, from 0.032 to 0.118. These results confirm that activation of the cAMP second messenger system stimulates the channel via phosphorylation by PKA.

Phosphorylation of the channel (or an associated protein) by PKA is quite stable. The activated state is maintained after washout of the PKA, and reapplication of PKA did not result in additional activity. These observations suggest that endogenous phosphatase activity is not characteristic of the BLM K_{ATP} channel, at least in excised patches. This is in contrast to a Mg^{2+}-dependent phosphatase believed to be endogenous to patches containing the renal K channel ROMK (Kubokawa et al., 1995; McNicholas et al., 1994). PKA phosphorylation of the BLM K_{ATP} channel is also insensitive to exogenous alkaline phosphatase (100 U/ml, $n = 21$).

PKA phosphorylation is necessary but not sufficient for channel activation. ATP can serve as a substrate for (*a*) a kinase in a phosphorylation reaction, and/or (*b*) an ATPase in a hydrolysis reaction. It is possible that either or both are required for channel activation, but the use of ATP does not allow the two to be dissociated. To test the hypothesis that phosphorylated channels remain active after the removal of hydrolyzable nucleotides, the poorly hydrolyzable nucleotide ATP-γS (0.2 mM)

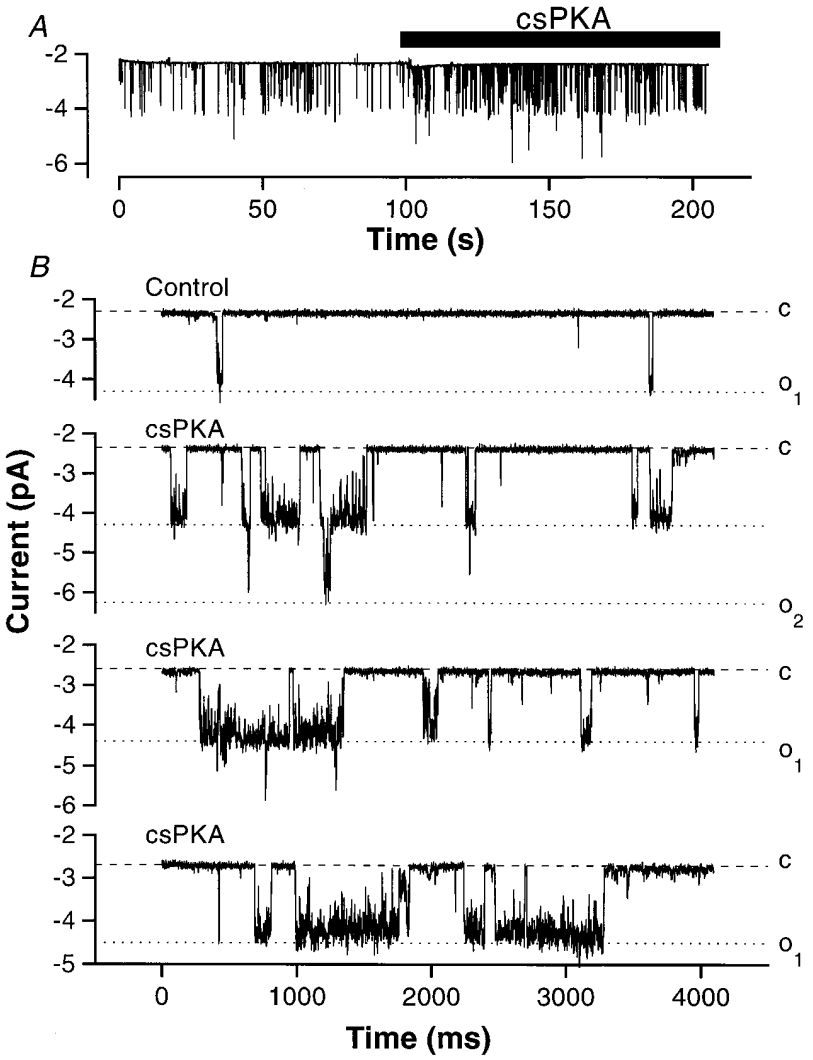

FIGURE 2. The cAMP-dependent protein kinase directly activates the BLM K_{ATP} channel. In this inside-out experiment, the pipette contains KCl (solution *d*), the bath contains NaCl (solution *c*) plus 0.2 mM ATP, and the command potential is −60 mV. (*A*) The running average of current versus time (16-ms window) is shown. The catalytic subunit of PKA (*csPKA*, 100 U/ml) was added to the bath where indicated. (*B*) Original current traces representative of channel activity during the control period (*top trace*) and after stimulation by PKA (*bottom three traces*). In this experiment, PKA led to a fourfold increase in nP_o. The all channels closed level (*dashed line*) and open channel levels (*dotted lines*) are indicated.

was substituted for 0.2 mM ATP in the presence of PKA. In this condition, the patch can be fully phosphorylated since ATP-γS is a substrate for PKA. Indeed, thiophosphorylation may be even more resistant to phosphatases than phosphorylation (Eckstein, 1985). Fig. 3 shows that, after a period of steady channel activity in 0.2 mM ATP, activity begins to decrease upon substitution of ATP with 0.2 mM ATP-γS. Subsequent addition of csPKA to thiophosphorylate the patch did not restore activity; in fact, the channels run down. Thus, because ATP-γS and PKA were not sufficient to restore or even sustain channel activity ($n = 7$), it appears that phosphorylation per se is necessary but not sufficient to maximally activate the BLM K_{ATP} channel. That channel rundown occurs despite the presence of ATP-γS and PKA confirms our previous finding that the BLM K_{ATP} channel also requires submillimolar levels of a hydrolyzable nucleotide (see Mauerer et al., 1998).

Kinetics of activated channels. Kinetic analysis was performed on the two patches that contained only one channel both before and after stimulation with FK (c/a) or PKA (i/o with 0.2 mM ATP in the bath). This analysis reveals that the major effect of PKA phosphorylation is to activate the channel by shortening, or possibly eliminating, the dwell time of the longest closed state. Under control conditions, the mean lifetime of the longest closed state is ~397 (c/a, Fig. 4 *A, top*) and 502 (i/o, Fig. 4 *B, top*) ms (see Mauerer et al., 1998). After activation by FK or the catalytic subunit of PKA, the mean dwell time of this state decreases to 7 (c/a, Fig. 4 *A, bottom*) and 100 (i/o, Fig. 4 *B, bottom*) ms, respectively. Note that there is more pronounced shortening occurring in the c/a mode (98%) compared with the i/o mode (80%). Activation by FK or PKA did not affect the open state dwell times.

Stimulation of PKC inhibits the BLM K_{ATP} channel. To determine whether PKC is involved in the regulation of this K^+ channel, c/a patches were treated with the phorbol ester PMA. As shown in Fig. 5, the application of 10 μM PMA markedly decreases channel activity concomitant with a reduction in i_{sc}, consistent with depolarization of V_m. Similar results were obtained in eight

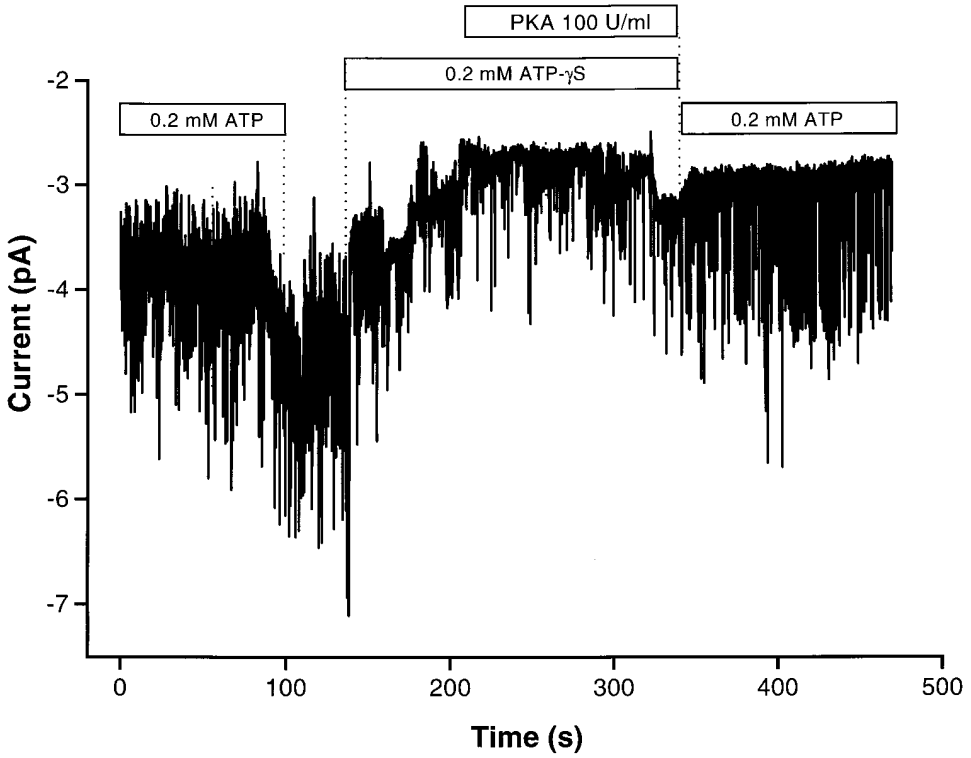

FIGURE 3. Phosphorylation itself does not prevent channel rundown. Activity of the BLM K_{ATP} channel is maintained in this representative inside-out patch excised in a bath containing 0.2 mM ATP, but rundown commences when the poorly hydrolyzable ATP-γS is substituted for ATP. Rundown continues despite subsequent addition of PKA, suggesting that phosphorylation per se does not rescue rundown of the BLM K_{ATP} channel.

cells with an average reduction in nP_o of 63 ± 11% (n = 6). Based on an inward slope conductance of 22.2 pS under similar conditions (see Mauerer et al., 1998), the mean decrease of i_{sc} of 0.28 ± 0.11 pA (n = 6) reflects an average depolarization (ΔV_m) of 12.0 ± 4.6 mV. A phorbol ester that does not activate PKC was used as a control. In five cell-attached patches, the addition of 10 μM 4α-PMA did not affect nP_o (Fig. 5).

To ensure that PKC was specifically responsible for the inhibitory effect of PMA, i/o patches were exposed to rat brain PKC. PKC activity is dependent on phospholipids (supplied by the patch of membrane) and the presence of $[Ca^{2+}]_i$. In these experiments, the bath contained only 50 nM $[Ca^{2+}]_i$ since $[Ca^{2+}]_i$ itself inhibits its channel activity with a K_i = 166 nM (see below). The addition of 50 nM Ca^{2+} itself inhibited channel activity by <25%. Subsequent addition of 0.5 U/ml rat brain PKC led to a sharp decrease in nP_o within several minutes (n = 13), as exemplified in Fig. 6. The addition of PKC results in a mean decrease in nP_o of 71.6 ± 4.8% (n = 4). The effect of PKC does not appear to be readily reversible.

In summary, the proximal tubule BLM K_{ATP} channel is regulated by both PKA and PKC, but in opposing directions. Phosphorylation by PKA activates the channel, while PKC phosphorylation has an inhibitory effect.

pH Sensitivity

The activity of the BLM K_{ATP} channel is pH sensitive. Directly lowering the pH on the cytoplasmic side of an i/o patch from 7.5 to 6.8 (a fivefold increase in $[H^+]_i$) decreases channel activity by 81 ± 2% (n = 3, Fig. 7 *A*). To assess whether this effect occurs under more physiological conditions, an intracellular acidosis was generated during cell-attached recording using the K^+/H^+ exchanger nigericin (Margolis et al., 1989) in a low K^+ bath (solution *c*, Fig. 7 *B*). We have previously shown that under these conditions, pH_i decreases by ~0.4 pH units in the presence of 10 μM nigericin (Segal et al., 1992). This protocol decreases the nP_o of the BLM K_{ATP} channel by 61 ± 3% (n = 3).

Effect of Calcium

The BLM K_{ATP} channel does not require Ca^{2+} for activity since excising membrane patches into an EGTA-buffered Ca^{2+}-free solution (solution *c*) does not alter channel current. However, increases in $[Ca^{2+}]_i$ inhibit the channel. The addition of the calcium ionophore ionomycin has no effect on cell-attached channel currents recorded from cells bathed in Ca^{2+}-free solution (Fig. 8, *A* and *B*). However, in the continued presence of ionomycin, raising bath Ca^{2+} ($[Ca^{2+}]_o$) to 1 μM (solution *e*) promptly and significantly reduces nP_o by 63 ± 9% (n = 4). This effect appears to be direct since exposing the cytoplasmic side of excised patches to varying $[Ca^{2+}]_i$ produces a dose-dependent decrease in channel activity with a K_i = 166 nM (Figs. 8, *C* and *D*). However, since the excised membrane may still contain elements of Ca^{2+}-activated signal transduction pathways (e.g., Ca^{2+}/calmodulin kinase, PKC), a possible

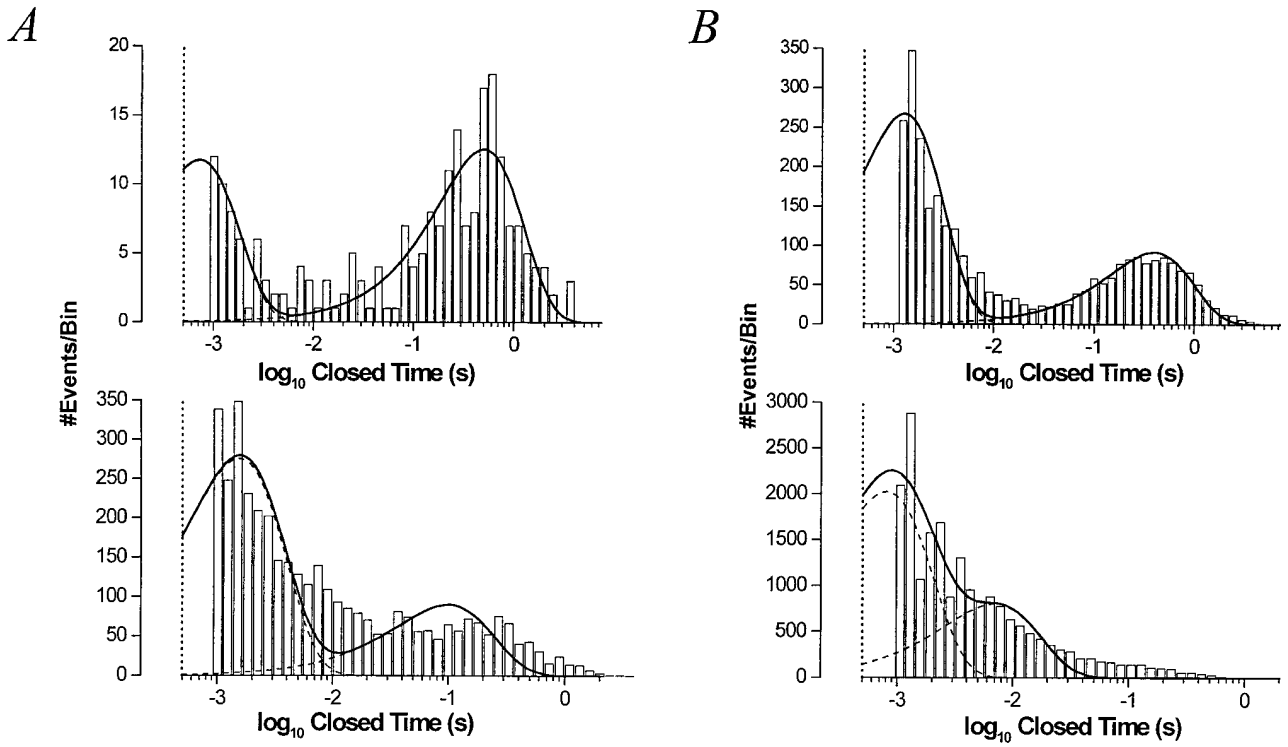

FIGURE 4. PKA phosphorylation shortens the longest closed time of the BLM K_{ATP} channel. (*A*) Closed-time histogram for a K_{ATP} channel in a cell-attached patch at −60 mV before (*top*) and after (*bottom*) forskolin. (*top*) Logarithmically binned (Sigworth and Sine, 1987) data before forskolin was fitted with two probability density functions (*dashed lines*) to give the overall fit (*solid line*), yielding time constants of τ_{c1} = 1.27 ms (74%) and τ_{c2} = 397 ms (26%). (*bottom*) Logarithmically binned data after forskolin was fitted with two probability density functions (*dashed lines*) to give the overall fit (*solid line*) yielding time constants of τ_{c1} = 0.81 ms (72%) and τ_{c2} = 7 ms (28%). Note that the main effect of forskolin is to reduce the longest closed lifetime from 397 to 7 ms. Based on the bandwidth of the recording system, the data and the fit were cutoff at 500 μs (*dotted vertical line*). (*B*) Closed-time histogram for a K_{ATP} channel in an inside-out patch at −60 mV before (*top*) and after (*bottom*) csPKA. (*top*) Logarithmically binned data before csPKA were fitted with two probability density functions (*dashed lines*) to give the overall fit (*solid line*), yielding time constants of τ_{c1} = 0.72 ms (48%) and τ_{c2} = 502 ms (52%). (*bottom*) Logarithmically binned data after csPKA was fitted with two probability density functions (*dashed lines*) to give the overall fit (*solid line*), yielding time constants of τ_{c1} = 1.5 ms (75%) and τ_{c2} = 100 ms (25%). As was the case for forskolin, the main effect of PKA phosphorylation was to reduce the longest closed lifetime from 502 to 100 ms. Based on the bandwidth of the recording system, the data and the fit were cutoff at 500 μs (*dotted vertical line*).

indirect effect cannot be fully ruled out. Indeed, the Hill coefficient of 0.73 may suggest that Ca^{2+} has both direct and indirect effects. In any case, an increase in $[Ca^{2+}]_i$ leads to a decrease in channel activity.

Membrane stretch. Several classes of cationic channels are known to be stretch-activated, including a K^+ channel on the BLM of *Necturus* proximal tubule (Sackin, 1989). Application of negative pressure up to −60 mm Hg to the patch pipette using a calibrated electronic negative pressure generator (DPM-1B; Bio-Tek Instruments, Inc., Winooski, VT) had no detectable effect on the nP_o of this BLM K_{ATP} channel in either c/a or i/o patches ($n > 100$). Thus, the BLM K_{ATP} channel of *Ambystoma* proximal tubule is not stretch activated and is distinct from the SA-K found on the BLM of *Necturus* proximal tubule (Filipovic and Sackin, 1992).

Cell swelling. To assess whether the BLM K_{ATP} channel might be involved in cell volume regulation, nP_o in c/a patches was monitored as measured bath tonicity was reduced by 30% from 200 (solution *g*) to 140 (solution *h*) mosM/kg. Despite clearly observable cell swelling due to the hypotonic shock, K_{ATP} channel activity did not increase and actually tended to decrease. This slight inhibition may be mediated either by an increase in $[Ca^{2+}]_i$ and/or activation of PKC, since cell swelling is often associated with an increase in $[Ca^{2+}]_i$ (Robson and Hunter, 1994*a*, 1994*b*; Ubl et al., 1988). Nonetheless, measurements made in zero current clamp showed that V_m hyperpolarizes (by 15–20 mV) during hypotonicity, probably due to the opening of a swelling-activated K^+ exit pathway. It does not appear that the BLM K_{ATP} channel mediates swelling-activated K^+ efflux.

In summary, neither direct mechanical deformation (stretch) nor cell swelling (which may involve direct and/or indirect transduction pathways) affect the BLM K_{ATP} channel. This suggests that the BLM K_{ATP} channel

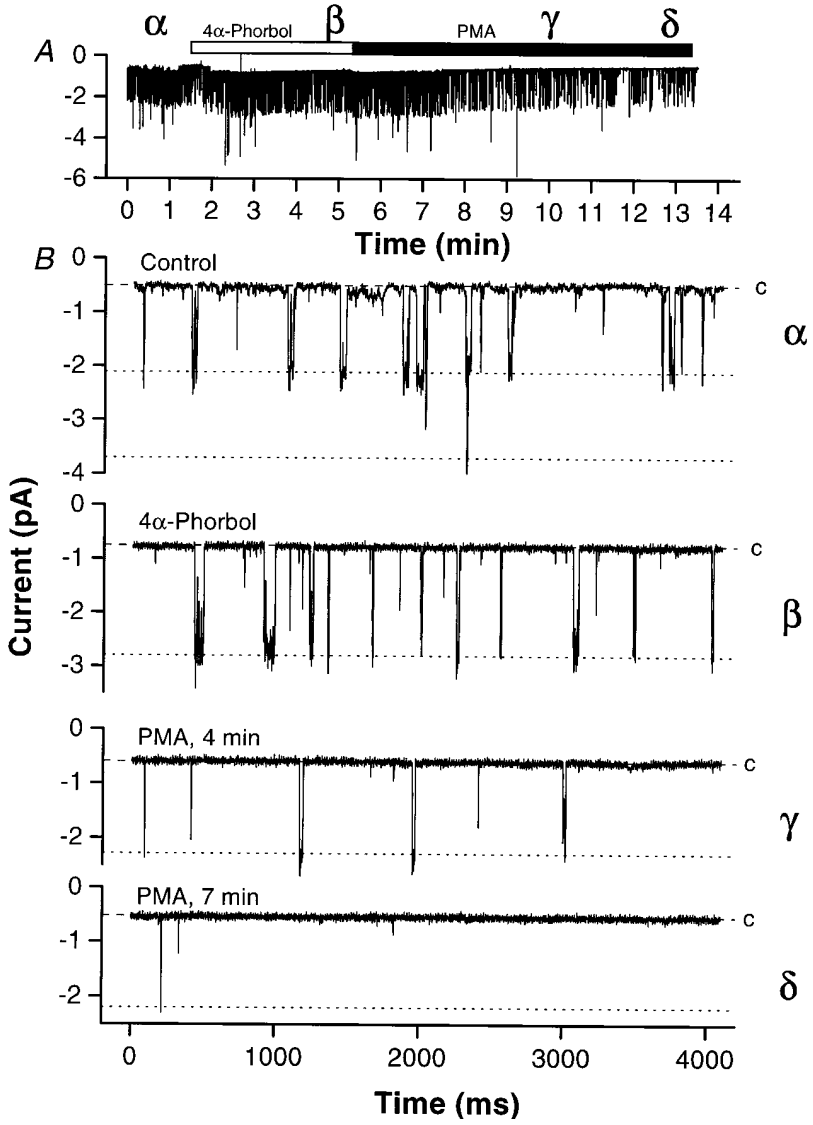

FIGURE 5. Phorbol ester inhibits the BLM K_{ATP} channel. (*A*) PMA (10 μM) inhibits the BLM K_{ATP} channel in cell-attached patches. In the experiment depicted, the pipette contains KCl (solution *d*), the bath contains NaCl (solution *c*), and the command potential is −60 mV. A running average of current versus time (16-ms window) is shown. (*B*) Sample traces taken from within the regions marked α, β, and γ, and in *A*. Addition of 4α-PMA does not alter channel activity. After the addition of PMA, however, channel activity decreases by 85% (nP_o decreases from 0.072 to 0.011). The all channels closed level (*dashed line*) and open channel levels (*dotted lines*) are indicated.

does not play a major role in subserving cell volume regulation.

Cytoskeleton. The cytoskeleton is a major factor in maintaining epithelial polarity in the dissociated cells (Segal et al., 1996). It is also well established that cytoskeletal elements are important in anchoring some proteins to their membrane (Morrow et al., 1989). More recently, it has been demonstrated that Na^+ (Prat et al., 1993), K^+ (Wang et al., 1994), and CFTR Cl^- channels (Prat et al., 1995) can be regulated by the cytoskeleton.

To begin to ask whether an intact cytoskeleton is necessary for normal function of the K_{ATP} channel, the actin cytoskeleton was disrupted with cytochalasin D (CD) during c/a patch recordings. Typically, nP_o increases during the first 60–90 s of exposure to CD, and then progressively decreases (n = 12). The mean nP_o decrease was 86.0 ± 8.4% (n = 4). Fig. 9 shows a representative experiment in which 10 μM CD was added to the bath once steady state channel activity had been recorded ($nP_o \cong 0.78$). Within 10 s, the transient increase in nP_o occurred, after which channel activity decreased to 0.14. Despite washout, the inhibition by CD is largely irreversible, as channel activity continues to decrease even 3 min after the wash. Thus, disruption of actin filaments has a biphasic and long-lasting effect on the behavior of the BLM K_{ATP} channel, suggesting that the channel is not only linked to, but also regulated by, the cytoskeleton.

Functional Coupling of the BLM K_{ATP} Channel to Na^+,K^+-ATPase Pump Activity

Sustained reabsorption of salt and water in the proximal tubule requires the action of the Na^+,K^+-ATPase pump on the BLM. As K^+ ions are pumped into the cell, a basolateral exit pathway must exist to allow K^+ to recycle, thus permitting continuous operation of the

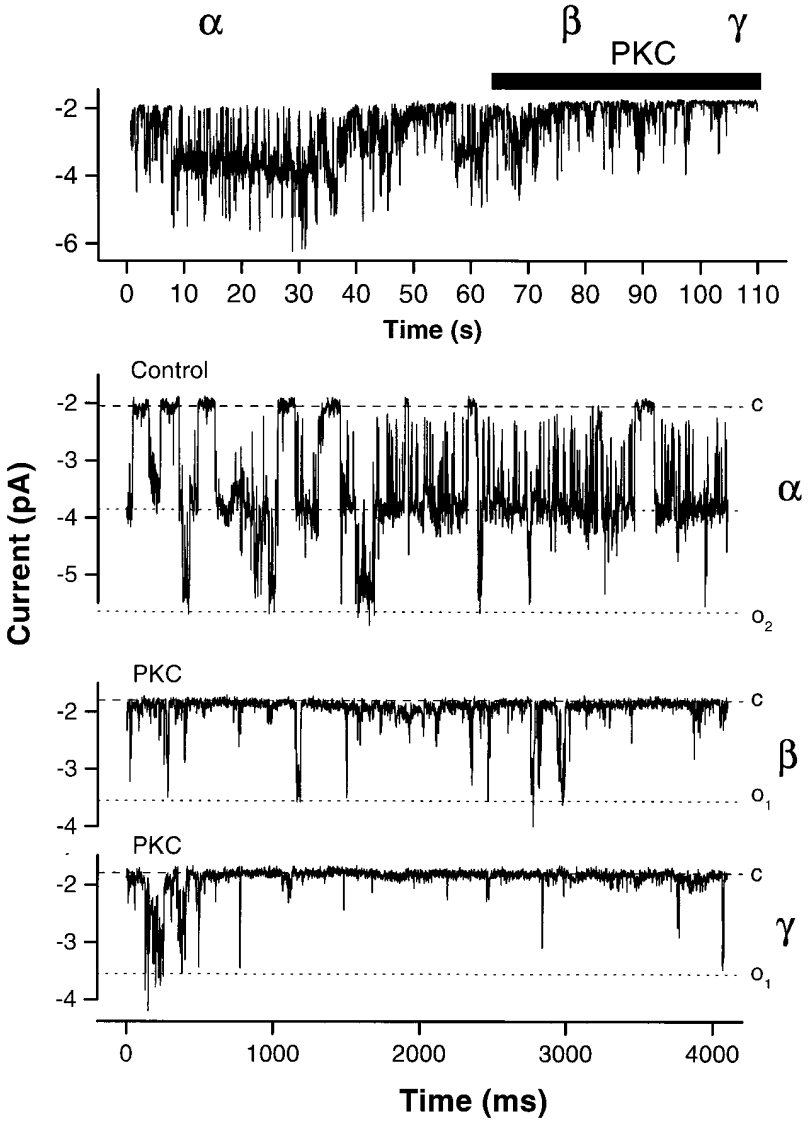

FIGURE 6. Protein kinase C inhibits the BLM K_{ATP} channel. PKC directly inhibits the BLM K_{ATP} channel. In this inside-out experiment, the pipette contains KCl (solution *d*), the bath contains NaCl (solution *c*) plus 0.2 mM ATP and 50 nM Ca^{2+}, and the command potential is −60 mV. (*A*) The running average of current versus time (16-ms window) is shown. PKC (0.5 U/ml) was added to the bath where indicated. (*B*) Original current traces representative of channel activity during the control period (*top trace*) and after inhibition by PKC (*bottom two traces*). In this experiment, PKC produced an 89% decrease in nP_o. The all channels closed level (*dashed line*) and open channel levels (*dotted lines*) are indicated.

pump. The functional coupling of these two transport elements may be linked by the intracellular [ATP] ($[ATP]_i$) level. This was examined in c/a patches.

Inhibition of transport. Retarding pump activity should result in less ATP consumption and a rise in $[ATP]_i$. This was achieved either using either (*a*) cardiac glycosides (ouabain or strophanthidin), or (*b*) removal of extracellular K^+. Both methods were used in an attempt to dissociate the rise in $[ATP]_i$ from a voltage-dependent effect, since the former depolarizes V_m while the latter (at least initially) hyperpolarizes V_m. However, it must be noted that these maneuvers result in concomitant changes in V_m, $[ATP]_i$, and even $[K]_i$. Since nP_o of the K_{ATP} channel decreases with depolarization (see Fig. 3 *C* in Mauerer et al., 1998), it is not possible to be certain how much of the fall in nP_o is due to ΔV_m versus $\Delta[ATP]_i$. Applying any of these maneuvers resulted in a gradual decrease of BLM K_{ATP} channel activity, as illustrated in Fig. 10.

Fig. 10, *A* and *B* show the effect of adding 200 μM ouabain after K_{ATP} currents reached a steady state. Channel activity begins to decline within a minute. By 5 min, only rare openings to a single open channel level are observed. Similar results were obtained in five cells exposed to 200 μM of either ouabain or strophanthidin (nP_o decreased by 50 ± 13%, $n = 4$).

Fig. 10 *C* shows the effect of changing bath K^+ from 2.5 to 0 mM. At first, nP_o increases, probably as a result of the initial hyperpolarization of V_m before a significant change in $[ATP]_i$ has occurred. The increase in i_{sc} from −1.50 pA in control to −1.90 pA after 30 s is consistent with membrane hyperpolarization. Subsequently, K_{ATP} channel activity decreases, presumably as the intracellular ATP level rises and as V_m depolarizes. Moreover, after 6 min in the $[K^+] = 0$ mM bath, i_{sc} decreases from −1.90 to −1.30 pA, consistent with membrane depolarization. Similar results were obtained in six experiments, with a decrease in channel activity by 76.8 ±

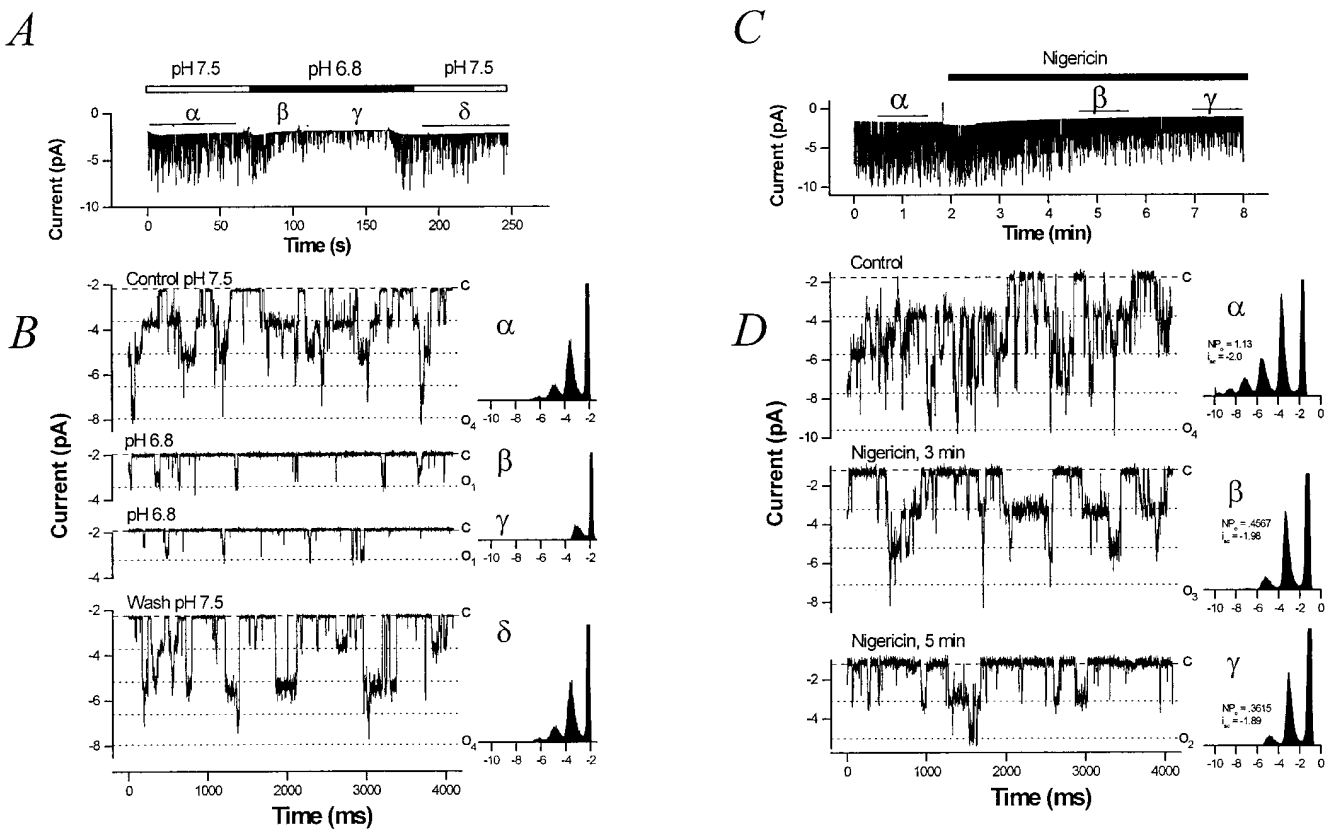

FIGURE 7. Intracellular acidosis inhibits the BLM K_{ATP} channel. (*A* and *B*) Lowering pH_i decreases channel activity in inside-out patches. In this inside-out experiment, the pipette contains KCl (solution *d*), the bath contains NaCl (solution *c*) plus 0.2 mM ATP, and the command potential is −60 mV. (*A*) The running average of current versus time (16-ms window) is shown. When the pH of the bath was lowered from 7.5 to 6.8, channel activity was inhibited by 79% (nP_o changes from 0.67 to 0.14). This effect is reversible upon returning the bath pH to 7.5. (*B*) Original current traces representative of channel activity during the control period (pH_i = 7.5, *top*), during acidosis (pH_i = 6.8, *middle*), and after returning to pH_i = 7.5 (*bottom*). The all channels closed level (*dashed line*) and open channel levels (*dotted lines*) are indicated. (*C* and *D*) Lowering pH_i with nigericin decreases channel activity in cell-attached patches. In this cell-attached experiment, the pipette contains KCl (solution *d*), the bath contains NaCl with 2.5 mM KCl (solution *c*) plus 0.2 mM ATP, and the command potential is −80 mV. (*C*) The running average of current versus time (16-ms window) is shown. When nigericin (10 μM) was added to the bath, channel activity was progressively inhibited. nP_o was reduced by 68% after 8 min in nigericin. (*D*) Original current traces and amplitude histograms representative of channel activity during the control period (*top trace*) and during nigericin (*bottom two traces*). The all channels closed level (*dashed line*) and open channel levels (*dotted lines*) are indicated.

5.2% (n = 4) and a membrane depolarization of 7.5 ± 1.6 mV (Δi_{sc} = −0.17 ± 0.04 with respect to control $[K^+]$ = 2.5 mM bath, n = 4).

Stimulation of transport. A rise in intracellular $[Na^+]$ is the primary physiological stimulus for the Na^+,K^+-ATPase pump. Na^+ entry via apical membrane Na-coupled cotransporters was enhanced by the addition of glucose (5 mM), alanine (5 mM), or both (2.5 mM each) to a NaCl bath while recording K_{ATP} channel activity from the BLM. Fig. 11 depicts a representative experiment in which 5 mM alanine was added to the bath. Within a minute of substrate addition, BLM K_{ATP} channel activity markedly increased despite an initial depolarization due to Na^+ entry. Comparable effects were seen in 17 of 19 (89%) cells exposed to glucose and/or alanine, with an approximately twofold increase in nP_o (1.95 ± 0.20, n = 5).

To try to clamp $[ATP]_i$ and still maintain a driving force for Na^+ entry, we used the following protocol (Fig. 12): it has been demonstrated that proximal tubule cells can be loaded with ATP from the bath (Tsuchiya et al., 1992). Using cell-attached patches, ATP (2 mM) was added to the bath in an effort to maintain $[ATP]_i$ constant. After reaching steady state, alanine was added to the bath to assess whether substrate was still able to stimulate K_{ATP} channel activity. This experiment shows that, with a (presumably) constant level of ATP_i, the response of K_{ATP} channels to alanine uptake is curtailed, as nP_o only slightly increases (11.3 ± 6.7%, n = 3). A possible explanation for the biphasic response of nP_o to extracellular ATP may be as follows: as ATP is taken up by the cell, K_{ATP} channels that have been quiescent due to a lack of ATP are initially activated; that is, n (the number of active chan-

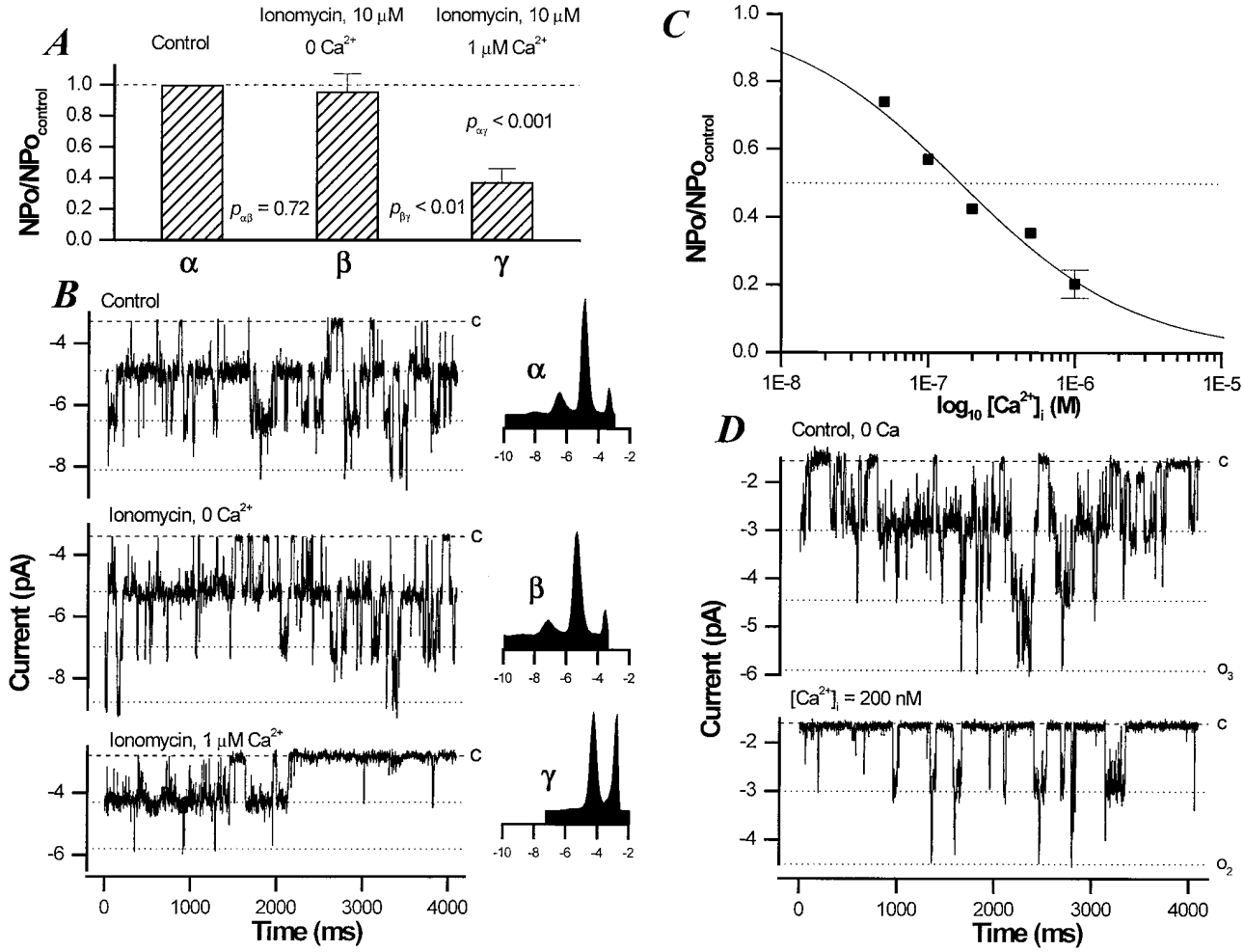

FIGURE 8. Increases in $[Ca^{2+}]_i$ inhibit the BLM K_{ATP} channel. (*A* and *B*) Elevation of $[Ca^{2+}]_i$ decreases channel activity. (*A*) Summary of normalized channel activity from cell-attached patches in which bath $[Ca^{2+}]$ was increased in the presence of a calcium ionophore (ionomycin). The addition of 1 μM ionomycin in a Ca^{2+}-free bath (solution *c*) does not significantly affect nP_o ($P = 0.72$), but subsequent addition of 1 μM Ca^{2+} (solution *e*) leads to a 63 ± 9% ($n = 4$) reduction in nP_o compared with control ($P < 0.001$). The inhibition tends to persist despite the removal of Ca^{2+} from a bath containing EGTA. (*B*) Representative single-channel traces and corresponding amplitude histograms for Ca^{2+}-free bath (*top*), Ca^{2+}-free bath plus ionomycin (*middle*), and 1 μM Ca^{2+} bath plus ionomycin (*bottom*). The all channels closed level (*dashed line*) and open channel levels (*dotted lines*) are indicated. (*C* and *D*) Increasing $[Ca^{2+}]_i$ in inside-out patches directly inhibits channel activity. (*C*) Dose–response curve of channel activity versus $[Ca^{2+}]_i$. Fitting the data with the Hill equation (*solid line*) yields a $K_i = 166$ nM and $n_H = 0.73$. (*D*) Representative single channel traces for $[Ca^{2+}]_i = 0$ (*top*) and 200 (*bottom*) nM at a command potential of −60 mV.

nels) increases. However, as ATP concentrations rise, P_o of the channels in the patch is diminished. As a consequence, the net response of channel activity, expressed by $n \cdot P_o$, depends on the balance of the increase in n and the decrease of P_o. This result is in line with the notion of an "ATP window," in which K_{ATP} channels first open (presumably as channels become phosphorylated) at low $[ATP]_i$, and then close at high $[ATP]_i$ (Priebe et al., 1996). Overall, these data are consistent with the hypothesis that $[ATP]_i$ may be a crucial coupling mediator between Na^+-K^+ pump activity and basolateral K_{ATP} channel activity.

ATP:ADP ratio. Stimulation of transport leads to activation of the Na^+,K^+-ATPase pump while the resultant fall in $[ATP]_i$ disinhibits the BLM K_{ATP} channel. However, the increased turnover of the Na^+,K^+-ATPase pump produces a rise in $[ADP]_i$ and/or a fall in the $[ATP]_i$:$[ADP]_i$ ratio. It is not clear which of these signals is the primary determinant of BLM K_{ATP} channel activity in intact cells. This issue was examined in i/o patches in two ways: (*a*) varying the ratio of [ATP]:[ADP] while keeping the [ATP] constant, and (*b*) varying the ratio of [ATP]:[ADP] while keeping the sum of [ATP] + [ADP] constant. The latter was performed for sums of 0.5, 2.5, and 5 mM.

To test whether ADP is able to relieve the block by ATP on the BLM K_{ATP} channel, increasing [ADP] were added to the cytoplasmic side of i/o patches in the

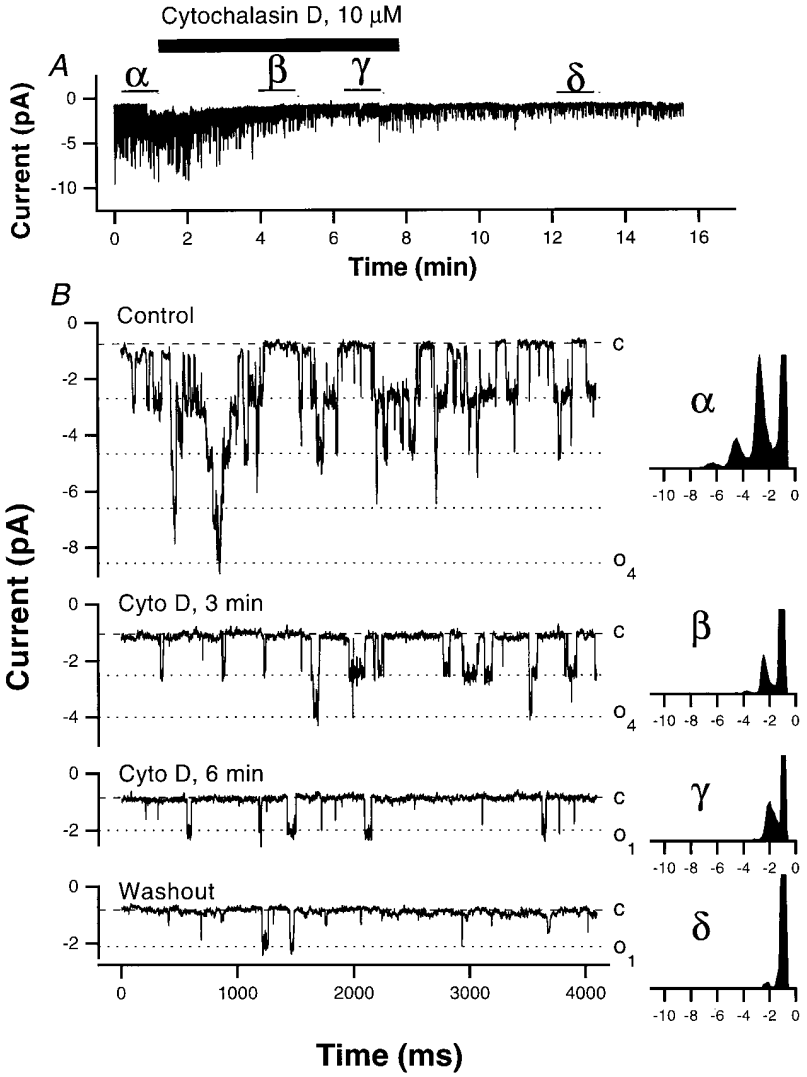

FIGURE 9. Disrupting the actin cytoskeleton has a biphasic effect on the BLM K_{ATP} channel. Cytochalasin D (10 μM) has a biphasic effect on the BLM K_{ATP} channel. In this cell-attached patch, the pipette contains KCl (solution *d*), the bath contains NaCl (solution *c*), and the command potential is −60 mV. (*A*) A running average of current versus time (16-ms window) shows that channel activity increases within the first minute of treatment, but gradually declines thereafter. Irreversible inhibition of channel activity persists after washout of cytochalasin D from the bath. (*B*) Sample traces taken from within the regions marked α, β, γ, and δ in *A*. Corresponding amplitude histograms are given for each region. After the addition of cytochalasin D, channel activity initially increases, but subsequently declines to 18% of control activity (region marked γ: nP_o = 0.14, compared with 0.78 during region α). The inhibition persists after washout of cytochalasin D (region marked δ: nP_o = 0.01, 1% of control). Note the decrease in the single channel current (i_{sc}) due to the depolarization of the cell membrane as K^+ channels close over the BLM. The all channels closed level (*dashed line*) and open channel levels (*dotted lines*) are indicated.

presence of 5 mM ATP (Fig. 13 *A*). Channel activity was almost completely and reversibly blocked by 5 mM ATP alone (Fig. 13 *A*, *middle*), and addition of 5 (*left*) or 10 (*right*) mM ADP did not relieve the block. Indeed, the block became more pronounced as the total concentration of nucleotide was raised.

Turning to the possibility that the channel is sensitive to the ratio of [ATP]:[ADP], i/o patches were exposed to ratios varying from 12.5:1 to 1:12.5 while keeping the sum [ATP] + [ADP] constant. The case for a sum near the K_i for ATP (~2.5 mM nucleotide, Fig. 13 *B*) shows that varying the ratio of [ATP]:[ADP] did not affect the degree of block. Similar results were obtained for a sum of 5 mM (the sum of 0.5 mM did not exert sufficient block to assess the ratio effect). Thus, neither protocol demonstrated any antagonism by ADP on the ATP block (n = 16).

DISCUSSION

The properties of the BLM K^+ (K_{ATP}) channel were the subject of the companion paper (Mauerer et al., 1998). The regulation of basolateral K^+ channels was not examined in most previous studies (Hunter, 1991; Sackin and Palmer, 1987; Kawahara et al., 1987; Parent et al., 1988; Gögelein and Greger, 1987). In the present study, we have investigated the regulation of this Type 1-like K_{ATP} channel in the BLM of dissociated *Ambystoma* proximal tubule cells that maintain epithelial polarity (Segal et al., 1996).

The BLM K_{ATP} Channel Is Regulated by PKA and PKC, but in Opposing Directions

The proximal tubule BLM K_{ATP} channel is activated by PKA, both in cell-attached patches via stimulation of adenylyl cyclase–elevating cAMP levels, or directly by PKA itself in inside-out patches. In contrast, this channel is inhibited by PKC, either by treating cell-attached patches with phorbol ester, or directly by PKC in excised patches. Opposite regulation by PKA and PKC has been reported for other K channels (Chen and Yu, 1994; Fakler et al., 1994) including K_{ATP} channels (Bonev and

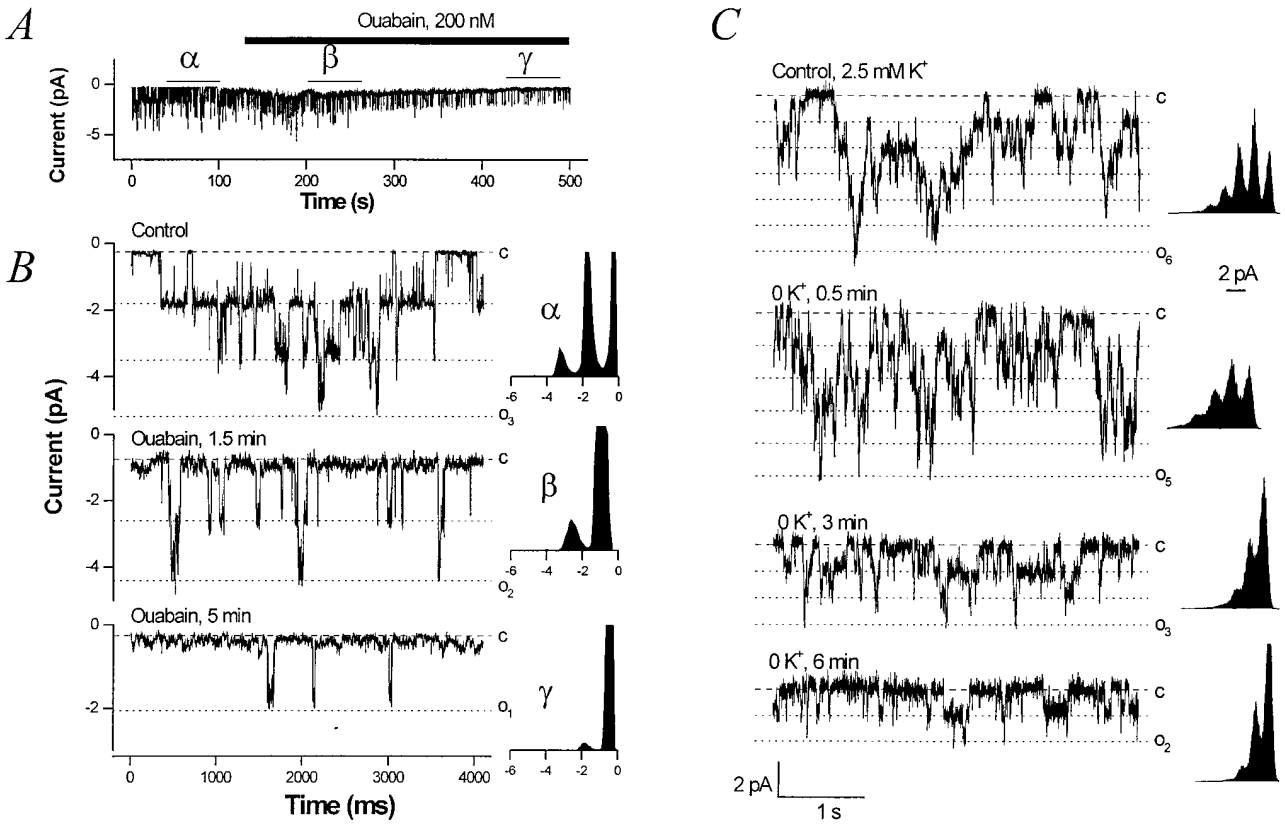

FIGURE 10. Inhibition of transport decreases BLM K_{ATP} channel activity. (*A*) Inhibition of the Na^+,K^+-ATPase pump with ouabain (200 μM) results in a decrease in activity of the BLM K_{ATP} channel in cell-attached patches. A running average of current versus time (16-ms window) is shown. The application of ouabain caused nP_o to decrease by 89% (region marked α: $nP_o = 0.46$, region marked γ: $nP_o = 0.05$) presumably due to an increase in $[ATP]_i$. In this experiment, the pipette contains KCl (solution *d*), the bath contains NaCl (solution *c*), and the command potential is −60 mV. (*B*) Sample traces taken from within the regions marked α, β, and γ in *A*. The corresponding amplitude histograms were taken for each region. Note the decrease in the single channel current due to the depolarization of the cell membrane as K^+ channels close over the BLM. The small channel appearing in the lower two traces is a CFTR-like Cl^- channel on the BLM previously described by us. An increase in $[ATP]_i$ may have led to the opening of the Cl^- channel. The all channels closed level (*dashed line*) and open channel levels (*dotted lines*) are indicated. (*C*) Inhibition of the Na^+,K^+-ATPase pump with a zero-K^+ bath (solution *c* without KCl) also results in a decrease in activity of the BLM K_{ATP} channel in cell-attached patches. Sample traces taken at the times indicated show a progressive decrease in nP_o (68%) and single channel current (control: $nP_o = 1.6$, $i_{sc} = -1.6$. Zero K^+, 6 min: $nP_o = 0.51$, $i_{sc} = -1.39$) corresponding to duration in the zero-K^+ bath. In this experiment, the pipette contains KCl (solution *d*) and the command potential is −80 mV. The all channels closed level (*dashed line*) and open channel levels (*dotted lines*) are indicated.

Nelson, 1993; Honore and Lazdunski, 1993; Zhang et al., 1994) in the kidney (Wang and Giebisch, 1991*b*).

Activation by PKA. In cell-attached experiments, stimulation of endogenous cAMP-dependent protein kinase (PKA) increases channel activity and single-channel current, the latter most likely due to hyperpolarization of the cell secondary to K^+ channel opening. Activation of channel by direct exposure of cell-free membrane patches to the catalytic subunit of PKA strongly suggests that phosphorylation of a PKA site on the channel itself (or a closely associated protein) increases the mean open time. PKA increased channel activity by 5.1 ± 0.7-fold, compared with 7.50 ± 1.55-fold in the case of forskolin. This difference is at least in part due to the additional stimulatory effect of membrane hyperpolarization (see Mauerer et al., 1998) in the cell-attached experiments. Kinetic analysis limited to patches containing one channel reveals that PKA phosphorylation does not significantly affect the open states, rather the major effect appears to be shortening of the longest closed state.

In contrast to the regulation of ROMK1 (McNicholas et al., 1994) and the secretory K^+ channel found in principal cells (Wang and Giebisch, 1991*a*), our experiments indicate that, at least in cell-free patches, an endogenous phosphatase does not appear to be closely associated with the BLM K_{ATP} channel. Indeed, after activation by PKA, neither removal of PKA nor exogenous alkaline phosphatase affected channel activity. The PKA phosphorylation site also appears to be distinct from the site(s) important in channel rundown, since thiophosphorylation of the patch by PKA and ATP-γS does not support channel activity.

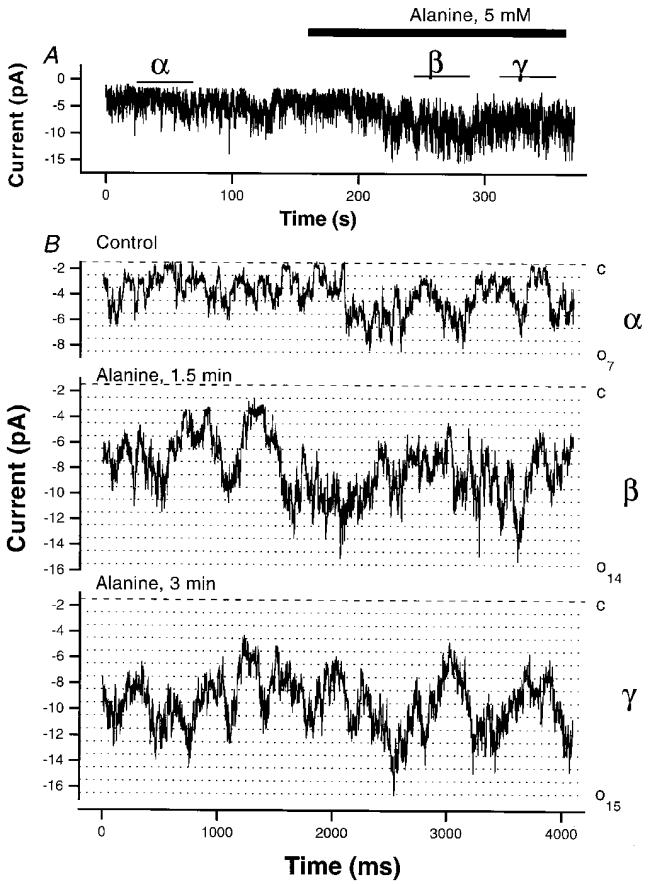

FIGURE 11. Stimulation of transport increases BLM K_{ATP} channel activity. (*A*) Activation of the Na^+,K^+-ATPase pump with substrates, in this case 5 mM alanine, results in an increase in activity of the BLM K_{ATP} channel in cell-attached patches. A running average of current versus time (16-ms window) is shown. The addition of alanine caused nP_o to increase nearly twofold (region marked α: $nP_o = 2.53$, region marked β: $nP_o = 4.44$). In this experiment, the pipette contains KCl (solution *d*), the bath contains NaCl (solution *c*), and the command potential is −60 mV. Sample traces taken from within the regions marked α, β, and γ in *A*. Note that despite the opening of BLM K_{ATP} channels, the single channel current remains essentially constant due to a net balance of hyperpolarizing (K^+ channel activation) and depolarizing (Na^+ entry) forces. The all channels closed level (*dashed line*) and open channel levels (*dotted lines*) are indicated.

Inhibition by PKC. In contrast to the effect of FK, treatment of cell-attached patches with PMA, a phorbol ester known to stimulate protein kinase C, results in a decrease in channel activity and depolarization as K^+ channels close. Channel activity did not change in control experiments with 4α-PMA, a phorbol ester that does not stimulate PKC. Subsequent removal of PMA did not restore channel activity, suggesting that phosphorylation by PKC is long lasting or that adequate specific phosphatase activity was not present. In inside-out patches from rabbit ventricular myocytes, Light et al. (1995) found PKC reversibly inhibited the K_{ATP} channel, and that a phosphatase inhibitor (okadaic acid) prevented channel recovery. These observations strongly suggested that the membrane patch contained an endogenous protein phosphatase. For the BLM K_{ATP} channel, the irreversible inhibitory effect of PKC in inside-out patches strongly suggests that such a phosphatase was not present in our membrane patches. Future experiments will be aimed at determining the role of phosphatases in the regulation of the BLM K_{ATP} channel.

Metabolic Regulation of the BLM K_{ATP} Channel

Schultz (1981) emphasized the implications of the Koefoed-Johnsen and Ussing model originally developed for frog skin (Koefoed-Johnsen and Ussing, 1958) as applied to Na-transporting epithelia operating over a wide range of transport rates, and reviewed the data then emerging that the K conductance of the basolateral membrane was correlated to pump activity, an example of what Schultz termed a "homocellular regulatory mechanism." Accordingly, Na^+ entry across the apical membrane should be matched by basolateral Na^+ efflux, thus maintaining a low intracellular [Na^+] as Na^+ is transported across the epithelium. This process is mediated by cross talk between opposing membranes. Schultz's group reported cross talk operating in leaky epithelia when apical Na^+ entry was increased in amphibian small intestine via rheogenic Na-coupled cotransport (Grasset et al., 1983; Gunter-Smith et al., 1982). They found that addition of alanine to the mucosal solution promptly increased the Na conductance of the apical membrane (with depolarization), and secondarily brought about an increase in K conductance of the BLM (with hyperpolarization). In the Koefoed-Johnsen and Ussing model, maintenance of unidirectional Na transport requires that K moves in a closed circuit (recycling) across the BLM. Coordination of pump activity and basolateral K conductance is necessary to avoid large fluctuations in intracellular [K^+] and volume, and to maintain a driving force for Na entry across the apical membrane.

It is important to recognize that pump activity and BLM K conductance can be coordinated in parallel or in series. For instance, if the initial perturbation is considered to be the incremental change in cell Na^+ content due to increased apical Na^+ entry, pump activity and BLM K conductance could be coupled via: (*a*) an increase in cell volume (Sackin, 1987, 1989; Cemerikic and Sackin, 1993; Kawahara, 1990), (*b*) increased $[Ca^{2+}]_i$ secondary to either a decrease in Na/Ca exchange (Yang et al., 1988) or swelling-induced opening of Ca^{2+} channels (McCarty and O'Neil, 1992), (*c*) an increase in pH_i dependent on the relative fractions of Na entering via the Na/H exchanger and Na-coupled cotransporters (Ohno-Shosaku et al., 1990; Beck et al., 1993), (*d*) increased $[K^+]_i$ due to pump activation (Messner et al., 1985*b*), (*e*) hyperpolarization of V_m due to pump ac-

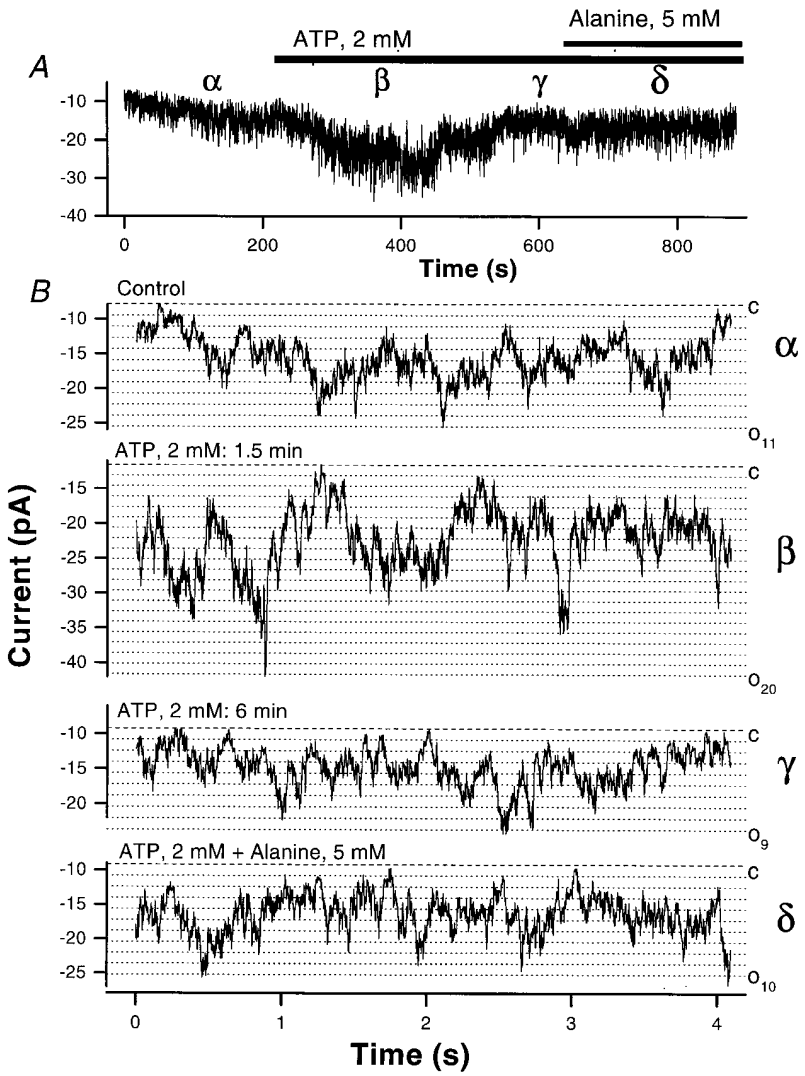

FIGURE 12. Stimulation of transport does not increase BLM K_{ATP} channel activity in ATP-loaded cells. Preloading the cell with ATP from the bath prevents the increase of BLM K_{ATP} channel activity due to alanine in cell-attached patches. A running average of current versus time (16-ms window) is shown in *A*, and sample traces are shown in *B*. Once inward K^+ channel currents reached a steady state (region α, up to 11 open channels), 2 mM ATP added to the bath is taken up by the cell, which maintains a constant $[ATP]_i$ (see text for details). The initial activation of current (region β, up to 20 open channels) may represent the opening of K_{ATP} channels that had been quiescent due to a lack of ATP. Presumably, as $[ATP]_i$ continues to rise, K_{ATP} currents are inhibited (region γ, up to 9 open channels). In the presence of steady state currents and $[ATP]_i$, the ability of alanine to increase channel activity is significantly diminished (region δ, up to 10 channels open). Similar results were observed in two other cells. In this experiment, the pipette contains KCl (solution *d*), the bath contains NaCl (solution *c*), and the command potential is −60 mV. The all channels closed level (*dashed line*) and open channel levels (*dotted lines*) are indicated.

tivation (Lapointe and Duplain, 1991), and/or (*f*) decreased $[ATP]_i$ due to pump activation. Note that the first set of three signals simultaneously mediate changes in both pump activity and BLM K conductance in parallel; the latter three signals are already the result of activation of the pump and mediate, in series, the increase in BLM K conductance (G_K). The behavior of the BLM K_{ATP} channel in regards to the first set of three will be considered separately, while the last set of three will be discussed together in the context of pump activity.

Cell volume, stretch, and the cytoskeleton. An increase in apical Na^+ entry will increase steady state cell volume (Beck et al., 1991*a*), which has been shown to lead to a rise in BLM G_K (Beck et al., 1991*b*; Macri et al., 1993). It is likely that multiple factors are involved in this response, including signal transduction via the cytoskeleton and effects of membrane deformation (e.g., membrane stretch; for review, see Sackin, 1995). Swelling- (Sackin, 1989) and stretch-activated (Kawahara, 1990) K channels on the BLM of *Xenopus*, *Rana* (Cemerikic and Sackin, 1993), and *Necturus* (Sackin, 1989) proximal tubule cells exist, but they are not ATP sensitive. Although we have detected stretch-activated channels (Segal and Boulpaep, 1994) on the BLM of the *Ambystoma* cells, they are not K selective. We and others (Parent et al., 1988; Beck et al., 1993) have shown that rabbit BLM K^+ channels are not stretch activated. The *Ambystoma* BLM K_{ATP} channel reported in the present study was not affected by stretch or hypotonic swelling and thus is analogous to the K_{ATP} channels described in mammalian preparations, which are also insensitive to membrane stretch (Beck et al., 1993; Tsuchiya et al., 1992).

A possible role of the BLM K_{ATP} channel in cell volume regulation was examined by exposing the cells to hypotonic shock (30% reduction of osmolality) during cell-attached recordings. This maneuver did not increase nP_o; indeed, activity tended to decrease. However, zero current-clamp measurements show that the cell membrane hyperpolarizes during hypotonicity, sug-

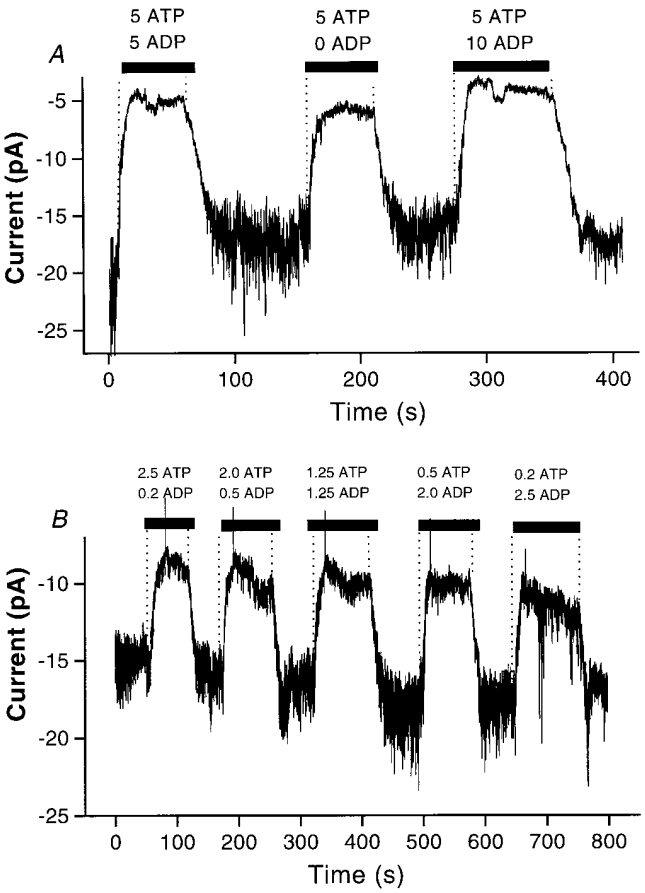

FIGURE 13. ADP does not relieve the block by ATP in excised patches. (*A*) Neither ADP nor a decrease in the ATP:ADP ratio antagonize the block by 5 mM ATP. In this inside-out patch, the pipette contains KCl (solution *d*), the bath contains K-aspartate (solution *i*) to isolate K^+ current, and the command potential is −60 mV, and a running average of current versus time (16-ms window) is shown. Adding 5 mM ATP and 5 mM ADP blocked 96% of the inward current. After washout (with 0.2 ATP), 5 mM ATP alone produced nearly the same inhibition (92%), indicating that ADP enhances rather than attenuates the ATP block. This notion is confirmed after washout, when 10 mM ADP in the presence of 5 mM ATP again enhances the block (98%), despite an ATP:ADP ratio of 0.5. (*B*) Varying the ATP:ADP ratio while keeping the [ATP + ADP] constant does not antagonize the block by ATP. In this inside-out patch, the pipette contains KCl (solution *d*), the bath contains K-aspartate (solution *i*) to isolate K^+ current, and the command potential is −60 mV, and a running average of current versus time (16-ms window) is shown. The extent of block exerted by [ATP + ADP] = 2.5 mM was not significantly affected, while the ATP:ADP ratio varied from 12.5 to 0.08.

gesting that under hypotonic conditions another population of swelling-induced K^+ channels may open (Sackin, 1989; Cemerikic and Sackin, 1993).

The Ca^{2+}/PKC second messenger system may play a major role in regulating regulatory volume decrease of proximal tubule cells (Robson and Hunter, 1994*a*, 1994*b*; Ubl et al., 1988). This might explain the slight decrease of BLM K_{ATP} channel activity observed despite hyperpolarization of the cell membrane, which by itself would increase nP_o (see Mauerer et al., 1998). Furthermore, it has been shown that in the absence of bicarbonate, the response of the BLM K^+ conductance to hypotonic shock is blunted (Volkl and Lang, 1988; Beck et al., 1992). Nevertheless, BLM K_{ATP} channel activity is not increased by cell swelling nor by direct membrane stretch, rendering these phenomena unlikely mediators of pump-K_{ATP} coupling, as was recently proposed (Cemerikic and Sackin, 1993).

The cytoskeleton is a major factor in maintaining epithelial polarity in the dissociated cells of this preparation (Segal et al., 1996). It is well established that cytoskeletal elements are important in anchoring some proteins to their membrane (Smith et al., 1991; Morrow et al., 1989), and recently it has been demonstrated that Na^+ (Prat et al., 1993), K^+ (Wang et al., 1994), and CFTR Cl^- channels (Prat et al., 1995) can be regulated by the cytoskeleton. Disruption of the actin cytoskeleton by cytochalasin D has a biphasic effect on BLM K_{ATP} channel activity. nP_o initially increases, but then greatly decreases and continues to decline even after washout of cytochalasin D. Thus, we propose that, like some epithelial ion channels, the BLM K_{ATP} channel is not only linked to, but also regulated by, the actin cytoskeleton.

$[Ca^{2+}]_i$. Single-channel data on the calcium sensitivity of proximal tubule BLM K^+ channels is limited to a few studies (Parent et al., 1988; Gögelein and Greger, 1987), and, as discussed in Mauerer et al. (1998), it is not likely that these were the K_{ATP} channels we studied. The *Ambystoma* BLM K_{ATP} channel was sensitive to increases in $[Ca^{2+}]_i$ in both cell-attached and excised patches. In a Ca^{2+}-free bath, ionomycin itself had no effect on K_{ATP} channel in cell-attached patches, but raising bath Ca^{2+} to 1 μM reduced channel activity by 63%. Experiments using inside-out patches showed that Ca^{2+} inhibited channel activity with a K_i of 166 nM, within the physiologic range. This effect in excised patches suggests that Ca^{2+} is acting directly on the channel, but does not exclude the involvement of Ca^{2+}-activated kinases such as PKC or Ca^{2+}/calmodulin-dependent protein kinase.

pH_i. Extracellular acidosis is known to depolarize proximal tubule cells, a finding attributed to a decrease in G_K (Steels and Boulpaep, 1987; Kuwahara et al., 1989; Bello-Reuss, 1982; Biagi and Sohtell, 1986), probably mediated by a corresponding decrease in pH_i (Kuwahara et al., 1989). Cell-attached recordings from the BLM of perfused rabbit proximal tubules showed that lowering pH from 7.4 to 6.5 in either the lumen or bath decreased K channel activity (Beck et al., 1993). In the present study, nigericin was used to produce an intracellular acidosis in the dissociated *Ambystoma* cells without altering external pH (Segal et al., 1992) during cell-attached recordings, and BLM K_{ATP} channel activ-

ity fell by 61%. Since acidosis may reduce intracellular K^+ activity (Cemerikic et al., 1982), the effect of pH_i was studied in excised patches where K^+ activity remains constant. These experiments showed that lowering pH_i from 7.5 to 6.8 reduced BLM K_{ATP} channel activity by 81%, confirming a direct effect of acidosis on the BLM K_{ATP} channel.

We observed that the inhibitory effect of acidosis is almost fully reversible in inside-out patches, but is not readily reversible in the cell-attached mode. This finding is in accordance with recent findings in mouse proximal tubule, in which extracellular metabolic acidosis led to intracellular acidosis and depolarization of the BLM due to closing of K^+ channels (Volkl et al., 1994). Despite a rise in pH_i upon reversal of the extracellular acidosis, recovery of V_{bl}, t_k, and R_{bl}/R_a was incomplete, suggesting a chronic decrease in BLM G_K after acidosis. Although the exact basis for this phenomenon in our cells is unclear, other inhibitory effectors (e.g., absence of bicarbonate, Ca^{2+}) may contribute to the long-lasting decrease of nP_o.

Modulation of BLM K_{ATP} Channel Activity by Signals Dependent on Pump Activation

In many tissues, K_{ATP} channels serve to couple cell metabolism to electrical activity. In nerve and muscle, the opening of K_{ATP} channels hyperpolarizes the cell and thus reduces electrical excitability. In the pancreatic cell, inhibition of glycolysis during hypoglycemia leads to a fall in $[ATP]_i$ and a rise in $[ADP]_i$. These metabolic changes activate K_{ATP} channels and the resultant hyperpolarization leads to a decrease in insulin secretion (Ashcroft and Ashcroft, 1990). Recently, a close coupling between Na^+,K^+-ATPase pump activity and K_{ATP} channel current was demonstrated in cardiac myocytes (Priebe et al., 1996).

Likewise in epithelial cells, transport-induced changes in cell metabolism as a result of pump activity may regulate K_{ATP} channels to optimize efficiency of transport, as discussed in Mauerer et al. (1998) and elsewhere (Schultz, 1992; Tsuchiya et al., 1992; Beck et al., 1994). BLM K^+ conductance responds to changes in apical sodium entry (Messner et al., 1985*a*; Matsumura et al., 1984) as well as alterations in basolateral sodium exit (Beck et al., 1991*a*; Messner et al., 1985*b*; Matsumura et al., 1984). There is evidence from studies on rabbit proximal tubule that $[ATP]_i$ is the signal that couples pump activity to channel activity (Beck et al., 1993; Tsuchiya et al., 1992). In the present study, modulation of transport in dissociated *Ambystoma* cells yielded results consistent with this proposal.

Retarding pump activity should lead to less ATP consumption and a rise in $[ATP]_i$. Experimentally, this was attempted by using either cardiac glycosides or by re-

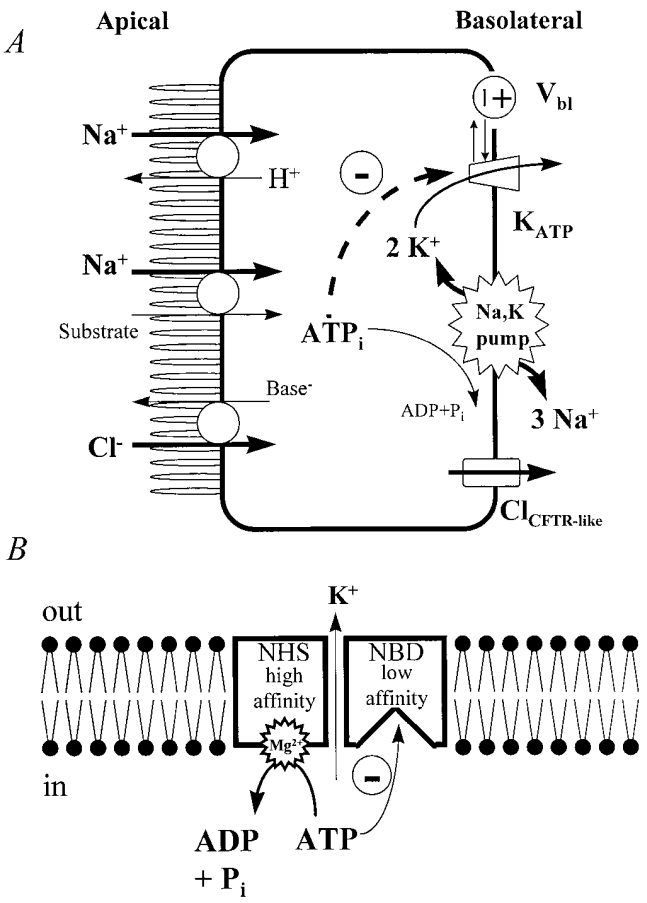

FIGURE 14. Summary of the regulation of BLM K_{ATP} channel activity. (*A*) A simplified model of transcellular transport in the proximal tubule is shown. Driven by the electrochemical gradient, Na^+ enters the cell across the apical membrane in exchange for protons or together with low molecular substrates like amino acids or glucose. Cl^- is taken up across the apical membrane by Cl^--base exchange and leaves the cell across the BLM through a CFTR-like Cl^- channel. Na^+ is pumped out of the cell across the BLM by means of the Na^+,K^+,ATPase pump, which breaks down ATP and brings K^+ ions into the cell. For steady state transport to continue, K^+ has to recycle across the BLM. Recycling is mediated by basolateral K_{ATP} channels, which is activated by PKA and by the fall in $[ATP]_i$ induced by the action of the pump when transport is stimulated. The BLM K_{ATP} channel is inhibited by decreased pH_i, increased $[Ca^{2+}]_i$, PKC, and increases in $[ATP]_i$ when transport is inhibited. This model links apical uptake of Na^+ to cellular metabolism (ATP), which in turn is linked to the basolateral K^+ conductance. (*B*) The dual effect of ATP: in the presence of Mg^{2+}, a low concentration (100–200 μM) of ATP (or another hydrolyzable nucleotide triphosphate) is required to maintain K_{ATP} channel activity by acting at a high affinity nucleotide hydrolysis site (*NHS*). However, millimolar levels of ATP_i (or another NTP, NDP, or NMP) inhibit the channel, presumably by binding to a low affinity nucleotide binding domain (*NBD*). Nucleotide hydrolysis does not appear to be necessary for the inhibitory action of nucleotides at the NBD. K channel openers such as diazoxide may act by interfering with nucleotide binding to the NBD.

moval of extracellular K^+. A possible downside of the former is the introduction of nonspecific glycoside effects, while the latter has the disadvantage of altering V_m, which by itself changes channel activity. In both cases, inhibition of pump activity resulted in a decrease of BLM K_{ATP} channel activity. In the case of extracellular K^+ removal, the initial increase of channel current (i_{sc}) and nP_o suggests that hyperpolarization of the cell membrane precedes the eventual decrease in nP_o. A similar transient increase in K_{ATP} channel current also occurs in cardiac myocytes (Priebe et al., 1996). However, since inhibition of the pump also tends to depolarize V_m, a fall in BLM K_{ATP} channel activity due to a voltage-dependent effect cannot be excluded. Conversely, stimulation of transport leads to an increase of BLM K_{ATP} channel activity. Although V_m was not monitored in this study, the addition of a substrate to the bath is known to increase Na-coupled cotransport across the apical membrane in the proximal tubule, thus stimulating Na^+,K^+-ATPase pump activity (Beck et al., 1993).

In an attempt to examine the effect of changes in V_m alone, we made efforts to clamp $[ATP]_i$ since proximal tubule cells are known to take up ATP from the bath (Tsuchiya et al., 1992; see Fig. 12). This maneuver significantly curtailed the increase in BLM K_{ATP} channel activity when alanine was subsequently added, suggesting that changes in $[ATP]_i$ are coupled to BLM K_{ATP} channel activity. Taken together, the BLM K_{ATP} channel responds to alterations in transport activity in both directions, most likely mediated by changes in V_m and $[ATP]_i$ (Beck et al., 1993; Tsuchiya et al., 1992).

Direct evidence for the functional coupling of K_{ATP} channels to changes in cytosolic ATP directly relating to Na^+,K^+-ATPase pump activity was recently demonstrated in guinea pig heart myocytes (Priebe et al., 1996). Under conditions in which the pump was running forward, addition of strophanthidin halted ATP consumption, leading to closure of K_{ATP} channels. Conversely, under conditions in which the pump was running backwards (by removal of extracellular K), addition of strophanthidin halted ATP production, leading to opening of K_{ATP} channels. These authors proposed that K_{ATP} current is modulated by cytosolic ATP within an "ATP window" that can be approached from both sides. They concluded that in cardiac myocytes (*a*) Na^+,K^+-ATPase pump activity and K_{ATP} channels are closely coupled, and (*b*) pump activity is the major determinant of cytosolic ATP. Our findings in renal proximal tubule cells are consistent with these conclusions.

Channel Regulation by ADP. Activity of the Na^+,K^+-ATPase pump results in a fall of $[ATP]_i$, while $[ADP]_i$ rises, and there is substantial evidence that the ATP:ADP ratio is an important regulator of K_{ATP} activity (Wang and Giebisch, 1991*a*; Ashcroft and Ashcroft, 1990). Nucleotide diphosphates have several modulatory actions on K_{ATP} channels, often dependent on whether Mg^{2+} is present. In the absence of Mg^{2+}, ADP inhibits K_{ATP} channels (Ashcroft and Ashcroft, 1990; Allard and Lazdunski, 1992), including the BLM K_{ATP} channel (Mauerer et al., 1998). Mg-ADP can restore activity to some K_{ATP} channels that are running down (Tung and Kurachi, 1991). Mg-ADP (but not ADP^{3-}) can also relieve channel inhibition by ATP (Nichols et al., 1996; Allard and Lazdunski, 1992) by shifting the K_i for ATP to the right.

In the present study, we varied the ratio of ATP:ADP over a wide range but ADP failed to relieve inhibition by ATP on the BLM K_{ATP} channel. This unexpected result may imply that if ADP does play a role in relieving the block by ATP on the BLM K_{ATP} channel in the intact cell, key intracellular components may not be present once the membrane patch has been excised. Recently, Nichols et al. (1996) have shown that Mg-ADP antagonizes ATP inhibition of the pancreatic cell K_{ATP} channel (made up of Kir6.2 and the sulfonylurea receptor [SUR], Inagaki et al., 1995) by specifically binding to the second nucleotide binding fold (NBF2) of SUR. Mutations around this site in NBF2, but not equivalent mutations in NBF1, eliminate stimulation by Mg-ADP. The failure of ADP to relieve the ATP inhibition of the BLM K_{ATP} channel may indicate that the renal K_{ATP} channel (at least in excised membrane patches) is not associated with a SUR-like molecule, or that a SUR-like molecule may be present but a specific NBF2 binding site for Mg-ADP is lacking. Similar reasoning may in part explain the relatively low sensitivity of renal K_{ATP} channels to sulfonylureas.

A simplified model incorporating the major regulatory features of the BLM K_{ATP} channel in association with other transport elements on the apical and basolateral membranes is given in Fig. 14. This model of transcellular NaCl transport links the apical uptake of Na^+ to cellular metabolism (ATP), which in turn is linked to the basolateral K^+ conductance. Our study has explored some of the mechanisms by which the predominant basolateral K^+ conductance, namely the BLM K_{ATP} channel, is regulated.

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