

# Hydrolyzable ATP and PIP<sub>2</sub> Modulate the Small-conductance K<sup>+</sup> Channel in Apical Membranes of Rat Cortical-Collecting Duct (CCD)

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**ABSTRACT** The small-conductance K<sup>+</sup> channel (SK) in the apical membrane of the cortical-collecting duct (CCD) is regulated by adenosine triphosphate (ATP) and phosphorylation-dephosphorylation processes. When expressed in *Xenopus* oocytes, ROMK, a cloned K<sup>+</sup> channel similar to the native SK channel, can be stimulated by phosphatidylinositol biphosphate (PIP<sub>2</sub>), which is produced by phosphoinositide kinases from phosphatidylinositol. However, the effects of PIP<sub>2</sub> on SK channel activity are not known. In the present study, we investigated the mechanism by which hydrolyzable ATP prevented run-down of SK channel activity in excised apical patches of principal cells from rat CCD. Channel run-down was significantly delayed by pretreatment with hydrolyzable Mg-ATP, but ATP $\gamma$ S and AMP-PNP had no effect. Addition of alkaline phosphatase also resulted in loss of channel activity. After run-down, SK channel activity rapidly increased upon addition of PIP<sub>2</sub>. Exposure of inside-out patches to phosphoinositide kinase inhibitors (LY294002, quercetin or wortmannin) decreased channel activity by 74% in the presence of Mg-ATP. PIP<sub>2</sub> added to excised patches reactivated SK channels in the presence of these phosphoinositide kinase inhibitors. The protein kinase A inhibitor, PKI, reduced channel activity by 36% in the presence of Mg-ATP. PIP<sub>2</sub> was also shown to modulate the inhibitory effects of extracellular and cytosolic ATP. We conclude that both ATP-dependent formation of PIP<sub>2</sub> through membrane-bound phosphoinositide kinases and phosphorylation of SK by PKA play important roles in modulating SK channel activity.

**KEY WORDS:** cortical-collecting duct (CCD) • small-conductance potassium channel (SK) • adenosine triphosphate (ATP) • phosphoinositide kinase (PI kinase) • phosphatidylinositol biphosphate (PIP<sub>2</sub>)

## INTRODUCTION

The cortical-collecting duct (CCD)\* is an important site for potassium secretion (Wang et al., 1997; Giebisch, 1999). The secretory process is confined to principal cells and initiated by active uptake of potassium across the basolateral membrane through the activity of Na-K-ATPase. This is followed by passive diffusion along a favorable electrochemical gradient across the apical membrane through potassium channels. A small-conductance potassium channel (SK) in the apical membrane of CCD is the major pathway for this potassium secretion (Wang et al., 1997). SK channels in principal cells of the mammalian nephron are regulated by pH, phosphorylation and dephosphorylation processes, cell messengers, and the cytoskeleton (Wang et al., 1992, 1997; Kubokawa et al., 1995b; Palmer, 1999; Schulte and Fakler, 2000).

A distinguishing feature of the SK channel is the modulation of activity by adenosine triphosphate (ATP) (Wang and Giebisch, 1991a), typical of other

ATP-sensitive (K<sub>ATP</sub> or Kir6.x) K<sup>+</sup> channels (Ashcroft and Ashcroft, 1990; Yokoshiki et al., 1998; Ashcroft, 2000). Low concentrations of ATP in the presence of protein kinase A (PKA) often restore the activity of run-down SK channels in CCD, whereas higher concentrations of ATP inhibit channel activity (Wang and Giebisch, 1991a). The former is thought to involve several distinct phosphorylation processes, whereas the latter may be due to direct binding of ATP to the channel as in other K<sub>ATP</sub> channels (Tanabe et al., 1999, 2000; Ashcroft, 2000; MacGregor et al., 2001). ADP also affects SK channel activity by antagonizing the inhibitory effect of ATP (Wang and Giebisch, 1991b).

Phosphatidylinositol phosphates (PIPs) like PIP<sub>2</sub> provide membrane signals (Martin, 1998; Corvera et al., 1999) that stimulate both K<sub>ATP</sub> (Kir6.x) channels (Fan and Makielski, 1997; Baukowitz et al., 1998; Huang et al., 1998; Shyng and Nichols, 1998; Shyng et al., 2000b) and other nonnucleotide-gated inward rectifier K<sup>+</sup> channels (Huang et al., 1998; Zhang et al., 1999). The concentration of these PIPs in the membrane is regulated by phospholipases (like phospholipase C, PLC), phospholipid phosphatases, and phosphoinositide kinase (PI kinase). Hydrolyzable ATP and/or PIP<sub>2</sub> have been shown to reactivate run-down Kir6.2 channels and the effect of Mg-ATP, but not PIP<sub>2</sub>, is abolished by wortmannin, a PI kinase inhibitor (Xie et al., 1999b). Since protein kinase inhibitors did not block the ability of

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\*Abbreviations used in this paper: ATP, adenosine triphosphate; cAP, calf intestinal alkaline phosphatase; CCD, cortical-collecting duct; PIP, phosphatidylinositol phosphate; PKA, protein kinase A; SK, small-conductance K<sup>+</sup> channel.

Mg-ATP to reactivate these Kir6.2 channels, this suggests that the Mg-ATP-dependent channel recovery is mediated by membrane lipid kinases rather than protein kinases. However, another study has suggested that protein kinase-mediated phosphorylation of Kir6.2 maintains responsiveness to PIP<sub>2</sub> (Ribalet et al., 2000). A role for PI kinases in regulating K<sub>ATP</sub> channel activity is also supported by the modulation of ATP sensitivity of K<sub>ATP</sub> channels in COSm6 cells transfected with PI kinase (Shyng et al., 2000a). In addition, the hydrolysis of PIP<sub>2</sub> by phospholipases such as PLC leads to the formation of inositol triphosphate and diacyl glycerol and loss of the stimulatory effect of PIP<sub>2</sub> on Kir channels (Xie et al., 1999a; Kobrinsky et al., 2000).

ROMK, cloned from the outer medulla of rat kidney (Ho et al., 1993; Zhou et al., 1994), shares many properties with the native SK channel (Palmer et al., 1997; Wang et al., 1997). ROMK exists in three isoforms, ROMK1, 2, and 3 (Boim et al., 1995; Lee and Hebert, 1995). In situ hybridization has shown expression of ROMK1 and ROMK2 mRNA in principal cells (Lee and Hebert, 1995), and immunohistochemistry has identified ROMK in apical membranes of principal cells in CCD (Lee and Hebert, 1995; Kohda et al., 1998). Thus, ROMK is believed to encode the SK channel in apical membranes of principal cells. ROMK1 channels expressed in oocytes can be activated by PIP<sub>2</sub> (Huang et al., 1998) and this effect is modulated by PKA-dependent phosphorylation of ROMK (Liou et al., 1999), as well as by changes in cytosolic-side pH (Leung et al., 2000).

Although the studies of PIP<sub>2</sub> effects on ROMK1 channels suggest a potential role for PIPs in regulating the native SK channel, there is currently no information about the effects of these phospholipids or PI kinases on SK channel activity in the mammalian CCD. In the present study we investigated the possible roles of ATP, PI kinase, PIP<sub>2</sub>, and PKA in modulating SK channel activity in principal cells from rat kidney. We found that hydrolyzable ATP can restore the low SK channel activity that follows excision of patches through a mechanism that involves membrane-bound kinases. Full channel activation requires both PI kinases increasing PIPs (like PIP<sub>2</sub>) and PKA phosphorylation of the SK channels.

## MATERIALS AND METHODS

### Preparation of CCD

Sprague-Dawley rats (80–100 g) were purchased from Taconic Farms, Inc. and kept on normal K-containing diet for 7–10 d before being killed. Left kidneys were removed and cut into two coronary slices for manual dissection. CCD were isolated using forceps under microscopic view in ice-cold bath solution (~4°C) as previously described (Wang and Giebisch, 1991b). One or two single CCD segments were placed on Cell-Tak (Becton Dickinson) –coated cover glass (2.5 × 2.5 mm) to immobilize them, and transferred to the experimental chamber mounted on the stage of an inverted microscope (IMT-2; Olympus). CCD tubules

were opened with a glass pipette to expose the lumen surface for patch-clamping of the apical membrane. Only principal cells, identified by their large flat surface and hexagonal shape, were chosen and all experiments were conducted at room temperature (22–24°C). The time between sacrificing the animal to opening the tubules was 5–10 min.

### Solutions and Chemicals

Control bath solutions contained (mM): 140 NaCl, 5 KCl, 1.8 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, and 10 HEPES. Ca<sup>2+</sup>-free bath solution was the same except that it also contained (mM): 0 CaCl<sub>2</sub>, 0.8 MgCl<sub>2</sub>, and 1 EGTA. Ca<sup>2+</sup>/Mg<sup>2+</sup>-free bath solution contained (mM): 140 NaCl, 5 KCl, 1 EGTA, 1 EDTA, and 10 HEPES. All solutions (pH adjusted to 7.4 with NaOH) were kept in the refrigerator and used within 3 d. ATP-containing bath solutions were freshly prepared every day. Pipette solution contained (in mM): 140 KCl, 1.8 MgCl<sub>2</sub>, and 10 HEPES (pH adjusted to 7.4 with KOH). Adenosine 5'-triphosphate (magnesium salt, Mg-ATP), adenosine 5'-[γ-thio]triphosphate (tetralithium salt, ATPγS), 5'-adenylylimido-diphosphate (AMP-PNP), wortmannin, 2-deoxy-D-glucose, antimycin A, and DL-dithiothreitol (DTT) were purchased from Sigma-Aldrich. Alkaline phosphatase from calf intestine (cAP) was purchased from New England Biolabs, Inc. LY294002, protein kinase A inhibitor (PKI), and quercetin were purchased from Calbiochem-Novabiochem. L-α-phosphatidylinositol-4, 5-bisphosphate (PIP<sub>2</sub>) was purchased from Roche Diagnostics Corp. Phosphatidylinositol 4,5-bisphosphate antiserum (anti-PIP<sub>2</sub>) was purchased from Assay Designs, Inc. Stock solutions of PIP<sub>2</sub> were sonicated in a cold bath for 30 min and kept in the freezer (–70°C). After sonicating for 1 min, PIP<sub>2</sub> was added directly to the perfusion chamber by hand using a small pipette. All chemical solutions were freshly made before each experiment. Agents were dissolved in either DMSO or ethanol with final concentrations in bath solutions of <0.1%. These solvents had no effects on SK channel activity.

### Patch-clamp Technique

Microelectrode pipettes were pulled from borosilicate glass capillaries (Dagan Corp.) using a two-step Narishige PP83 puller (Narishige Scientific Instrument Laboratory) and polished using a Narishige microforge (MF-83). The pipette resistances were 6–8 MΩ after filling with pipette solution. Single channel recordings were obtained using an EPC-7 amplifier (List Electronics) and filtered at 1 kHz by a Bessel filter (902 LPF; Frequency Devices, Inc.). Currents were digitized at a sampling rate of 4 kHz using a Digidata 1200 interface (Axon Instruments, Inc.) and stored in a PC (E-3100, Gateway 2000) for further statistical analysis and printing. NP<sub>o</sub> represents the channel activity, in which N is the number of channels in the patch and P<sub>o</sub> is the single channel open probability, calculated at a modified filter frequency of 500 Hz by using pCLAMP software (version 6.0.4 of Fetchan and pSTAT; Axon Instruments, Inc.). The changes of channel activity were determined when a steady-state was reached and calculated over a 30s interval before and after changing bath solutions or additions of agents. Statistical data are shown as mean ± SEM and P < 0.05 was considered significant. Mean paired differences were calculated by Student's *t* test.

## RESULTS

### SK Channel Activity Rapidly Decreases When Patches Are Excised into Bath Solutions Lacking ATP

Fig. 1 shows a representative single channel recording upon changing from a cell-attached to an inside-out

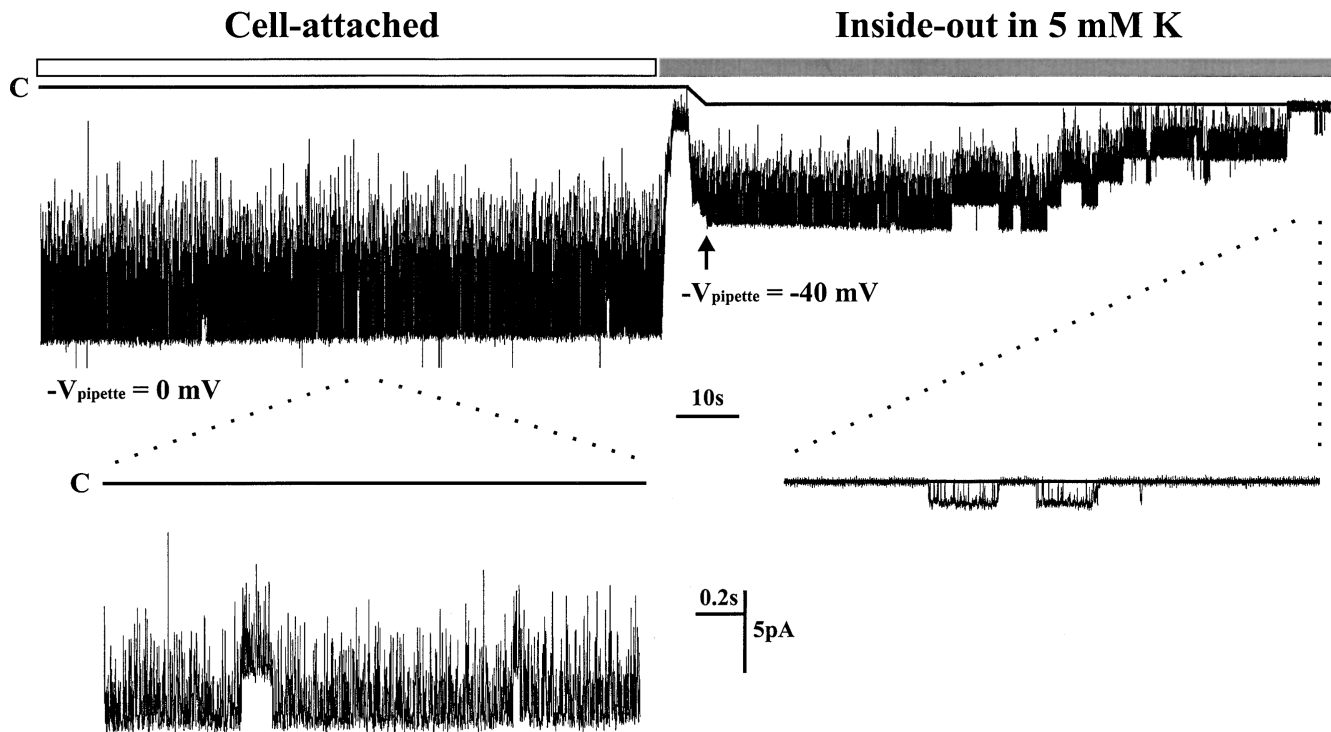


FIGURE 1. Small-conductance  $K^+$  channel run-down in a representative excised patch. In the cell-attached patch, inward  $K^+$  current was recorded showing five active SK channels. Since the pipette solution contained 140 mM KCl, the channel shows inward current displaying typical SK channel kinetics with high open probability. When the patch was excised into a 5 mM  $K^+$  bath solution, the current amplitude immediately decreased due to loss of the membrane potential. Then pipette holding potential ( $-V_{\text{pipette}}$ ) was elevated from 0 to  $-40$  mV, current amplitude was increased. SK channels completely closed in 1.7 min in the Ringer's solution after excision. The downward deflection of the traces shows inward current and the line "C" indicates the zero current level (channel completely closed state).

patch configuration on the apical membrane of a principal cell. With the cell-attached configuration and Ringer's solution in the bath, channel conductance was  $38.3 \pm 1.2$  pS (calculated at pipette holding potential [ $-V_{\text{pipette}}$ ] between 0 and  $-80$  mV). The I-V curve showed slight inward rectification ( $35.4 \pm 0.8$  pS,  $-V_{\text{pipette}}$  between 20 and  $-20$  mV;  $31.2 \pm 1.5$  pS,  $-V_{\text{pipette}}$  between 40 and 0 mV,  $n = 13$ ). In the inside-out patch configuration with symmetrical 140 mM KCl solutions, the channel conductance was  $31.3 \pm 0.6$  pS ( $-V_{\text{pipette}}$  between 20 and  $-80$  mV,  $n = 4$ ). With bath solution containing 140 mM NaCl and 5 mM KCl, the channel showed inward rectification with a conductance of  $26.0 \pm 0.3$  pS,  $-V_{\text{pipette}}$  between 0 and  $-80$  mV ( $n = 6$ ). The latter conductance is typical of the SK conductance under these conditions (Frindt and Palmer, 1989; Wang et al., 1990). In all remaining experiments, inside-out patches were exposed to 140 mM NaCl and 5 mM KCl bath solutions. Channel kinetics calculated at a pipette holding potential ( $-V_{\text{pipette}}$ ) of  $-40$  mV were typical of the SK channels in mouse (Lu et al., 2000) and rat (Wang and Giebisch, 1991a,b): one open time ( $T_o = 37.6 \pm 0.2$  ms) and one closed time ( $T_c = 1.4 \pm 0.3$  ms); open probability ( $P_o$ ) was  $0.89 \pm$

$0.17$ . Consistent with previous observations (Wang and Giebisch, 1991b; Kubokawa et al., 1995a), channel activity progressively decreased in inside-out patches and channel activity disappeared completely within  $1.4 \pm 0.4$  min ( $n = 21$ ; Fig. 1).

#### *ATP-dependent Phosphorylation Processes Are Required in Intact CCD Principal Cells for SK Channel Activity*

The concentration of ATP on the cytosolic face of principal cells plays an important role in modulating SK channel activity: in excised patches sub-mM concentrations of ATP can reactivate run-down SK channels whereas mM concentrations of Mg-ATP reduce channel activity (Wang and Giebisch, 1991a). Antimycin A, an inhibitor of mitochondrial electron transfer, and 2-deoxy-D-glucose, a glycolysis inhibitor, deplete cellular ATP stores in cultured renal epithelial cells (Mandel et al., 1994). We used these metabolic inhibitors to assess effects of depletion of intracellular ATP on SK channel activity. Fig. 2 is a representative recording from a cell-attached patch showing that SK channel activity decreased following exposure to  $20 \mu\text{M}$  antimycin A and  $20$  mM 2-deoxy-D-glucose.  $NP_o$  decreased from a control value of  $4.7 \pm 0.5$  to  $0.9 \pm 0.3$  ( $n = 3$ ) within 7 min.

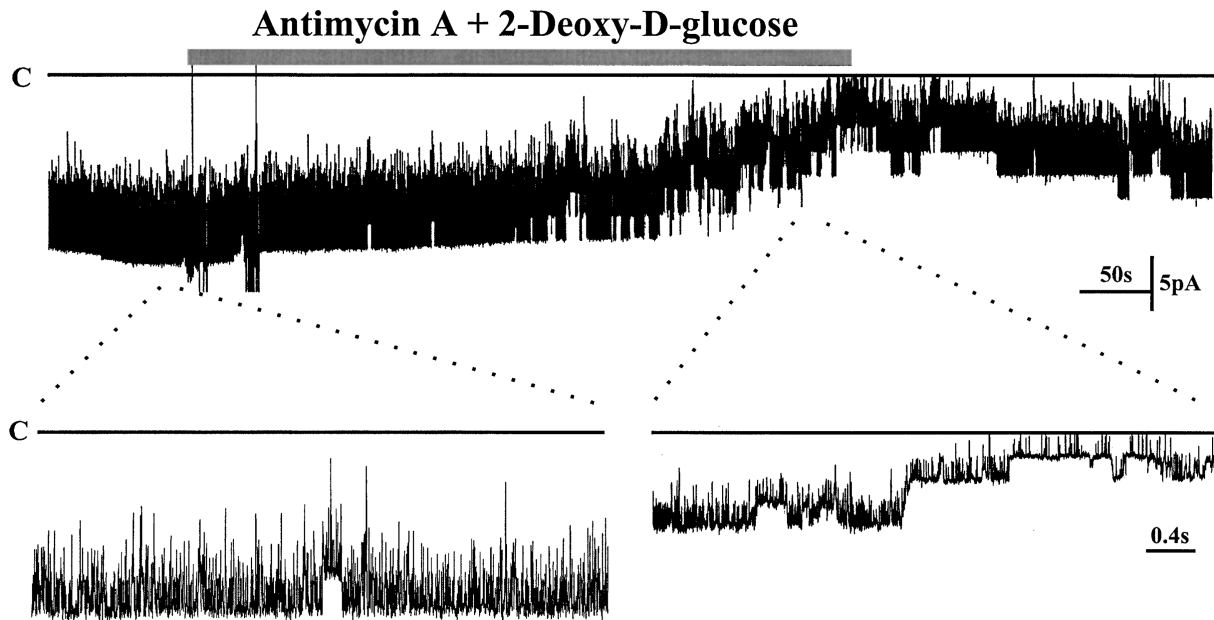


FIGURE 2. Depletion of intracellular ATP concentration decreased SK channel activity. In a representative cell-attached patch, the recording showed seven open channels before addition of 20  $\mu$ M antimycin A and 20 mM 2-deoxy-D-glucose to the bath solution. In 7.4 min after application of the metabolic inhibitors, the SK channel activity was reversibly inhibited.  $NP_o$  decreased from  $6.4 \pm 0.6$  to  $1.9 \pm 0.3$ . Pipette holding potential ( $-V_{\text{pipette}}$ ) = 0 mV. C = channel-closed state.

The inhibitory effect of antimycin A + 2-deoxy-D-glucose was partially reversible. Thus, ATP depletion in principal cells reduces SK channel activity.

To investigate further the effects of ATP on SK channel activity, we assessed the ability of Mg-ATP or ATP analogs (ATP $\gamma$ S or ANP-PNP) on reactivation of run-down SK channels. ATP $\gamma$ S has been reported to be a substrate for some protein kinases (Eckstein, 1985; Jeong and Nikiforov, 1999) but not for lipid kinases in oocytes (Liou et al., 1999) while AMP-PNP is a nonhydrolyzable ATP analogue. Fig. 3 shows the effects of Mg-ATP on SK channel run-down. Fig. 3 A shows the decline of channel activity following exposure to  $Ca^{2+}/Mg^{2+}$ -free and Mg-ATP-free bath solutions. Additions of 0.5 mM Mg-ATP ( $n = 6$ ) reactivated SK channels following run-down. Fig. 3 B shows that ATP $\gamma$ S ( $n = 11$ ) and AMP-PNP (unpublished data;  $n = 3$ ) failed to maintain channel activity, whereas addition of 0.5 mM Mg-ATP reactivated channels after run-down in each condition. To provide additional evidence that the activating effect of Mg-ATP was due to phosphorylation processes, we exposed excised patches to calf intestinal alkaline phosphatase (cAP, a broad-spectrum phosphatase) in the presence of 0.5 mM Mg-ATP. Fig. 4 shows a representative recording demonstrating that cAP abolished SK channel activity even in the presence of Mg-ATP (from an  $NP_o = 3.3 \pm 0.3$  to an  $NP_o = 0.2 \pm 0.1$ ;  $n = 16$ ). This effect of cAP was reversible ( $NP_o = 2.9 \pm 0.6$ ;  $88 \pm 11\%$  of control after 5 min of cAP wash-

out), likely due to both reestablishment of PKA-dependent phosphorylation of proteins (including the SK channel) and lipid kinase production of PIP $_2$ .

In rat principal cells, phosphorylation processes have been associated with both channel activation (PKA) and inhibition (PKC or  $Ca^{2+}$ -dependent calmodulin-activated kinase II [CaMK II]). Increasing cytosolic  $Ca^{2+}$  reduces SK channel activity in cell-attached patches by activating PKC (Wang and Giebisch, 1991b; Wang et al., 1993) and/or CaMK II (Kubokawa et al., 1995a,b). However, addition of bath  $Ca^{2+}$  in excised patches did not reduce channel activity in the absence of exogenous PKC (Wang et al., 1990). Moreover,  $Mg^{2+}$  can activate membrane-bound phosphatases and reduce SK channel activity in excised patches (Kubokawa et al., 1995a). To test whether  $Ca^{2+}$  and/or  $Mg^{2+}$  affect channel run-down after patch excision, experiments were performed in bath solutions lacking  $Ca^{2+}$  or both  $Ca^{2+}$  and  $Mg^{2+}$ . There was no difference in time to run-down between experiments using  $Ca^{2+}$ -free ( $Mg^{2+}$ -containing) solutions or  $Ca^{2+}/Mg^{2+}$ -free solutions ( $1.8 \pm 0.3$  min [ $n = 6$ ] vs.  $1.8 \pm 0.6$  min [ $n = 5$ ], respectively). These times to run-down were also not different from the time of run-down in control  $Ca^{2+}/Mg^{2+}$ -containing solutions (Figs. 1 and 3 A). Thus, these kinase inhibitors appear to play no significant role in our present experiments with rats fed a normal K diet. Although in a previous study in rats fed a high K diet, the time to run-down was significantly prolonged in the absence of



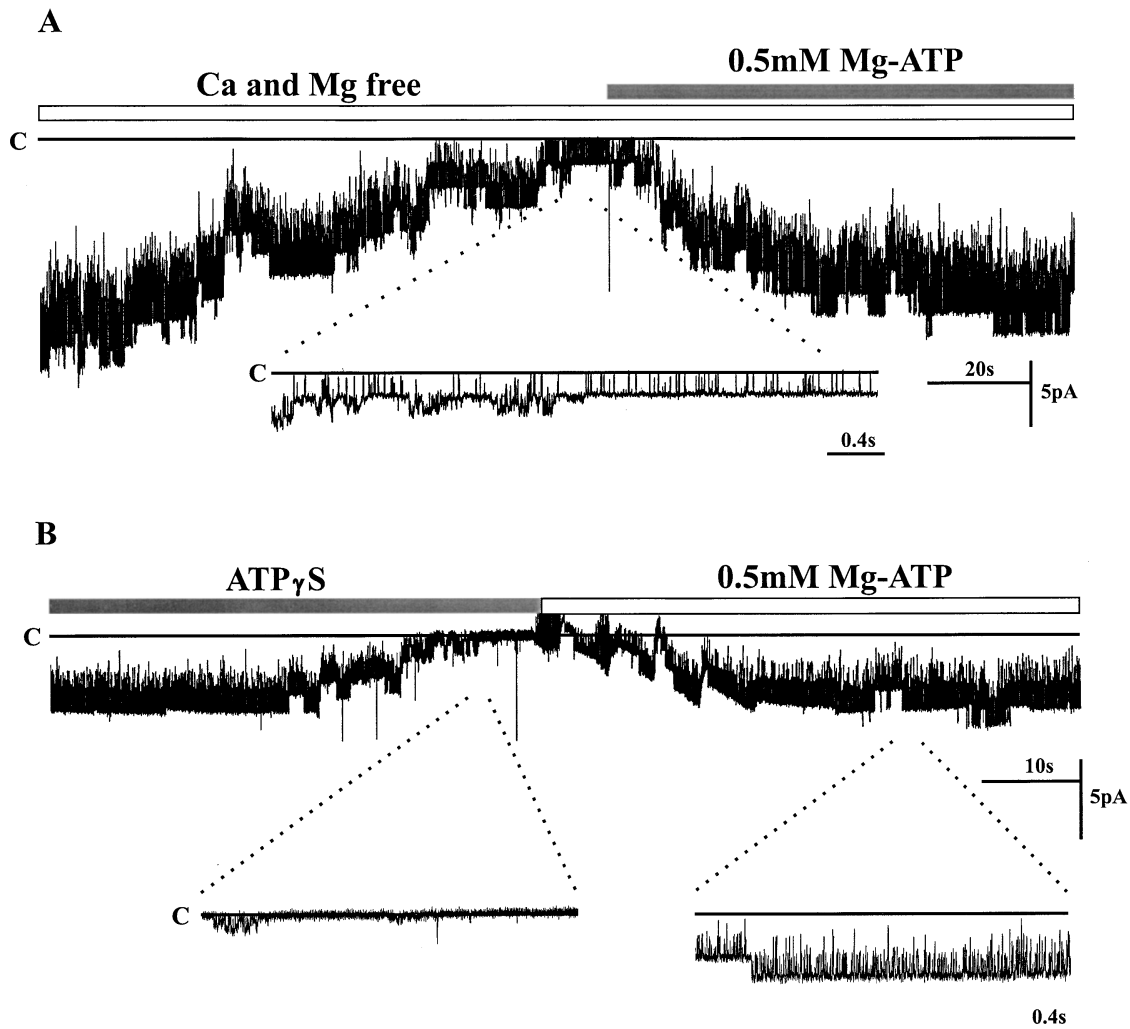


FIGURE 3. Hydrolyzable ATP prevented SK channel run-down in inside-out patches. (A) In the absence of Mg-ATP, channel activity rapidly decreased in the  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Ringer's solution. 0.5 mM Mg-ATP reactivated the run-down channel. (B) SK channel activity recorded from an inside-out patch shows run-down in the presence of ATP $\gamma$ S (0.5 mM)-containing,  $\text{Ca}^{2+}$ -free bath solution. The SK channel activity could be reactivated by 0.5 mM Mg-ATP in a  $\text{Ca}^{2+}$ -free Ringer solution. The time to run-down was similar to those shown in Figs. 1 and 3 A. C = channel-closed state.  $-V_{\text{pipette}} = -40$  mV.

$\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (Kubokawa et al., 1995a), it is not clear whether this difference was due to K in the diet or to other undetermined factors.

#### Phosphatidylinositol-4, 5-bisphosphate ( $\text{PIP}_2$ ) Modulates SK Channel Activity

In further experiments, we examined the role of PI kinases in mediating the Mg-ATP-dependent phosphorylation effects on maintaining SK channel activity (Figs. 5–7). Two experimental approaches were used to test whether  $\text{PIP}_2$  affects SK channel activity. First, we investigated the effects of  $\text{PIP}_2$  applied to the cytosolic face of excised membrane patches in which activity had declined after exposure to solutions free of Mg-ATP and  $\text{Ca}^{2+}$ . As shown in Fig. 5 A, a significant increase of  $\text{NP}_o$

was produced by addition of 50  $\mu\text{M}$   $\text{PIP}_2$  ( $\text{NP}_o$  from  $0.9 \pm 0.3$  to  $3.7 \pm 0.7$ ;  $n = 8$ ). This result is similar to the increase in the activity of ROMK1 produced by  $\text{PIP}_2$  in oocytes (Huang et al., 1998).

Second, we also investigated the effect of  $\text{PIP}_2$  antibodies (anti- $\text{PIP}_2$ ). As shown in Fig. 5 B, the addition of anti- $\text{PIP}_2$  to the cytosolic face of excised membrane patches led to a prompt decline of SK channel activity in the presence of Mg-ATP-containing bath solution.  $\text{NP}_o$  decreased from  $5.6 \pm 1.2$  to  $0.1 \pm 0.1$  ( $n = 10$ ). Fig. 5 C shows that the inhibition of channels could not be reversed by repeated wash-out with anti- $\text{PIP}_2$ -free and Mg-ATP-containing/ $\text{Ca}^{2+}$ -free bath solutions. However, adding DL-dithiothreitol (DTT, 100  $\mu\text{M}$ ) to the bath restored channel activity to  $88 \pm 9\%$  of control values ( $\text{NP}_o = 4.9 \pm 0.9$ ,  $n = 5$ ). These results are consistent

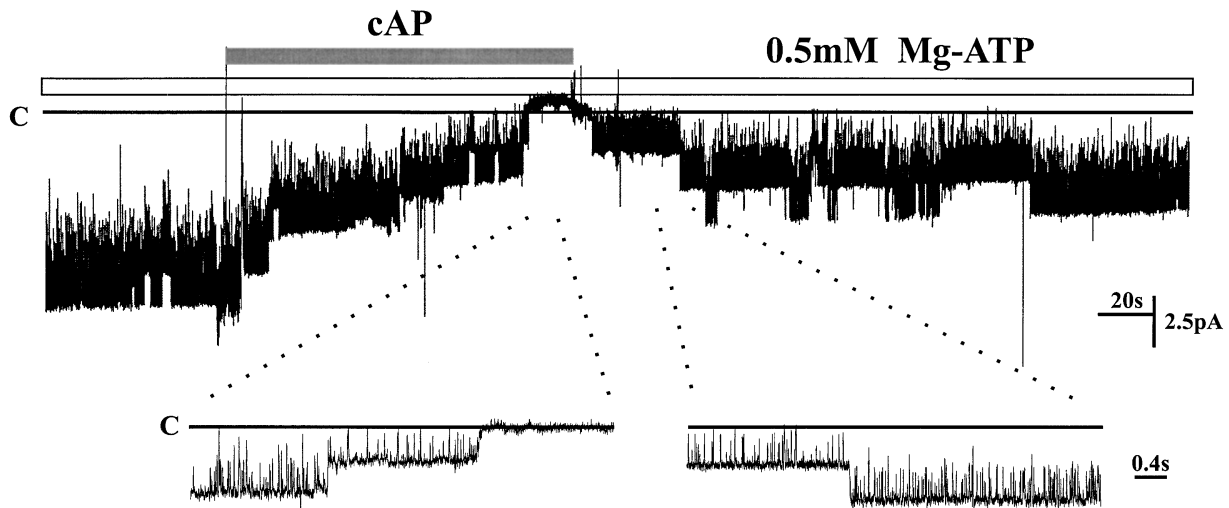


FIGURE 4. A representative inside-out patch recording showing that alkaline phosphatase (cAP, 20 U/ml) reversibly inhibited SK channel activity in the presence of 0.5 mM Mg-ATP. After removal of cAP, SK channel activity was rapidly restored. C = channel-closed state.  $-V_{\text{pipette}} = -40$  mV.

with those of Huang and coworkers in ROMK1 expressed in oocytes showing that the reversal of the PIP<sub>2</sub> antibody effect also required exposure to reducing agents like DTT (Liou et al., 1999). This effect of DTT may be due reduction of bonds in the anti-PIP<sub>2</sub> immunoglobulin molecule (Liou et al., 1999). In another study, the low pH-inhibited ROMK1 channels expressed in oocytes required reducing agents for reactivation after returning pH to control (Schulte et al., 1998).

In further experiments we investigated the relationship between hydrolyzable ATP and PI kinases. The effect of LY294002 on SK channel activity is shown in Fig. 6. Although Fig. 6 A shows that LY294002 abolished SK channel activity in this patch, this was not always the case (Fig. 6 B). On average, SK channel activity was reversibly diminished to  $17 \pm 3\%$  of control by 20  $\mu\text{M}$  LY294002, a selective inhibitor of PI kinase (NP<sub>o</sub> decreased from  $4.3 \pm 0.6$  to  $0.7 \pm 0.3$  and recovered to  $3.2 \pm 0.6$ ;  $n = 14$ ; Figs. 6 A and 7). This inhibition was also rapidly reversed in the continued presence of LY294002 by addition of 50  $\mu\text{M}$  PIP<sub>2</sub> (Fig. 6 B; NP<sub>o</sub> decreased from  $3.1 \pm 0.1$  to  $0.4 \pm 0.1$  and then increased to  $2.6 \pm 0.5$  after addition of PIP<sub>2</sub>;  $n = 7$ ).

Experiments were also performed using two additional PI kinases inhibitors: quercetin (60  $\mu\text{M}$ ), a non-selective inhibitor of PI kinase, and wortmannin (20  $\mu\text{M}$ ), a specific covalent inhibitor of PI kinase. Results of experiments using these two PI kinase inhibitors are summarized in Fig. 7. NP<sub>o</sub> was reduced to  $37 \pm 4\%$  of control by quercetin ( $n = 5$ ) or  $25 \pm 7\%$  of control by wortmannin ( $n = 6$ ). The average reduction by all PI kinase inhibitors was to  $26 \pm 6\%$  of control, a value significantly different from zero. Thus, none of the PI ki-

nase inhibitors completely abolished channel activity. PIP<sub>2</sub> reversed channel activity in the presence of quercetin and wortmannin (Fig. 7). Similar reactivation was also observed by addition of PIP<sub>3</sub> (20  $\mu\text{M}$ ,  $n = 3$ ; unpublished data).

#### Full Activation of SK Channels Requires PKA-dependent Phosphorylation

In *Xenopus* oocytes, PKA phosphorylation of ROMK has several effects that enhance channel activity. Phosphorylation of specific sites on ROMK modulates either P<sub>o</sub> or channel density, NP<sub>o</sub> (MacGregor et al., 1998). In addition, the stimulation of channel activity by PIP<sub>2</sub> is enhanced by PKA phosphorylation of ROMK1 (Liou et al., 1999). To investigate the interaction of PKA and PIP<sub>2</sub> in the native SK channel, two approaches were used.

Fig. 8 A summarizes the effect of blocking PKA activity by protein kinase A inhibitor (PKI) in the presence of 0.5 mM Mg-ATP. In control experiments NP<sub>o</sub> was maintained over 10 min after patch excision in the absence of PKI (Fig. 8 A, solid circles). SK channel activity declined to  $64 \pm 13\%$  of control (Fig. 8 A, open circles) with 20  $\mu\text{M}$  PKI in the presence of Mg-ATP. This 36% reduction in SK channel activity is consistent with our previous observations of the activating effect of PKA phosphorylation on SK channel activity (Wang and Giebisch, 1991a,b). In the presence of PKI (Fig. 8 A), Mg-ATP would be expected to maintain the concentration of PIPs in the membrane. Thus, PKA phosphorylation appears to be necessary for full activation of SK channels even in the presence of PIPs.

To further investigate the interplay of PKA phosphorylation processes and PIPs, we took advantage of the

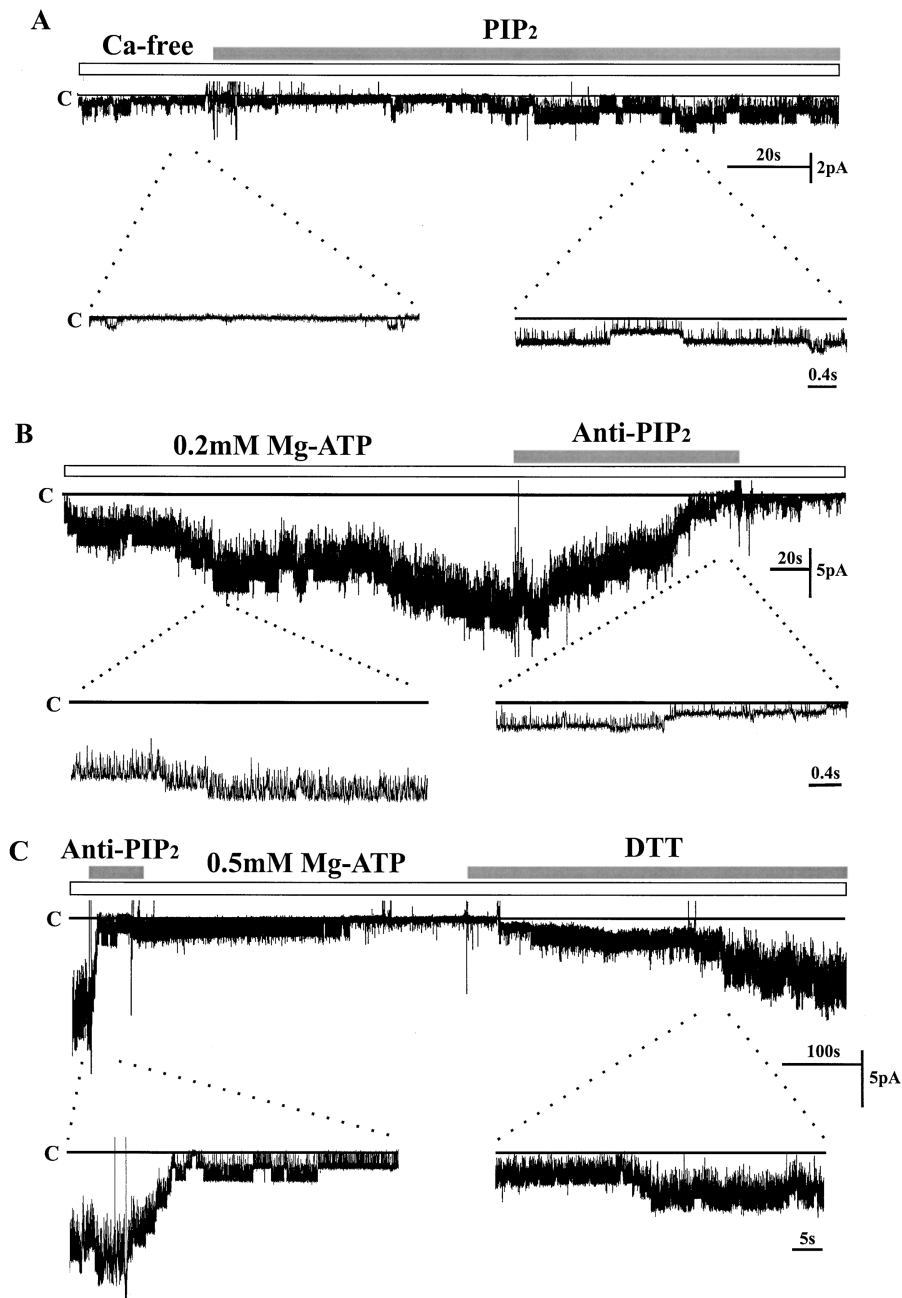


FIGURE 5. PIP<sub>2</sub> reactivated run-down SK channels in a representative inside-out patch. (A) After run-down in the Ca<sup>2+</sup>-free, ATP-free bath solution, SK channel activity was restored by application of 50  $\mu$ M PIP<sub>2</sub>. (B) PIP<sub>2</sub> antibodies (anti-PIP<sub>2</sub>, 20  $\mu$ M) caused SK channel run-down in the presence of Mg-ATP. (C) After removal of PIP<sub>2</sub> antibodies, SK channel activity was not restored by Mg-ATP. However, SK channel activity was observed following addition of reducing agent, 100  $\mu$ M DL-dithiothreitol (DTT). C = channel-closed state.  $-V_{\text{pipette}} = -40$  mV.

ability of cAP to abolish channel activity in the presence of Mg-ATP, as shown in Fig. 4. Fig. 8 B shows the run-down of channel activity by cAP in the presence of both 0.5 mM Mg-ATP and 20  $\mu$ M PKI. Following removal of cAP, but in the continued presence of Mg-ATP and PKI, channel activity was only partially restored by endogenous production of PIP<sub>2</sub> even after prolonged observation ( $\sim 10$  min;  $68 \pm 7\%$  recovery;  $n = 7$ ). These observations support that PKA phosphorylation is necessary for full PIP<sub>2</sub> responsiveness, and are consistent with observations in oocytes that PKA phosphorylation reduced the concentration of PIP<sub>2</sub> necessary for activation of ROMK1 channels (Liou et al., 1999). A similar

conclusion regarding the necessity for protein phosphorylation of Kir6.2 in maintaining responsiveness to PIP<sub>2</sub> had also been reached (Ribalet et al., 2000).

#### ATP-PIP<sub>2</sub> Interactions in Regulating SK Channel Activity

To begin to understand the relevance of ATP-PIP<sub>2</sub> interactions in regulating SK channel activity, we examined the effects of PIP<sub>2</sub> in modulating both extracellular and intracellular ATP-mediated channel inhibition. We showed previously that extracellular ATP could reduce SK channel activity in mouse principal cells by stimulated purinergic (P<sub>2</sub>Y<sub>2</sub>) receptors (Lu et al., 2000). This effect of extracellular ATP on SK channel

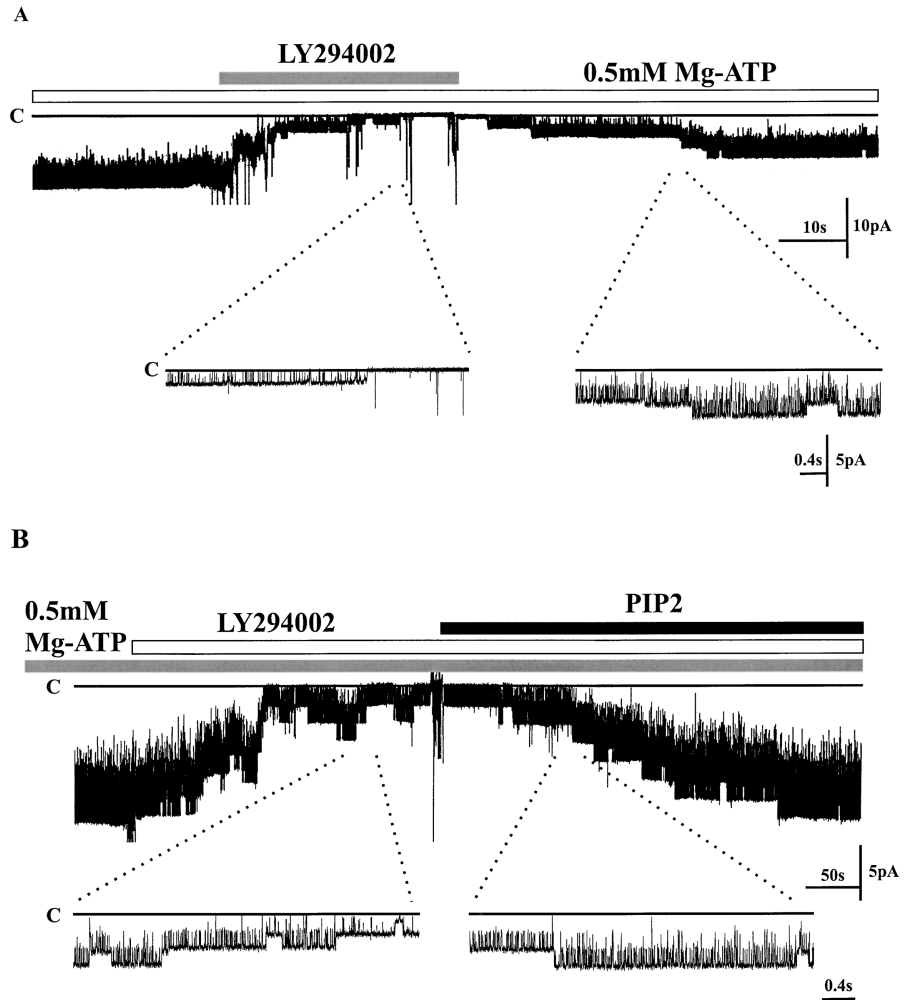


FIGURE 6. (A) The PI kinase inhibitor, LY294002 (20  $\mu$ M), reversibly reduced SK channel activity in the presence of 0.5 mM Mg-ATP/ $\text{Ca}^{2+}$ -free bath solution in an inside-out patch. (B)  $\text{PIP}_2$  (50  $\mu$ M) reactivated SK channel activity inhibited by the PI kinase inhibitor, LY294002 (20  $\mu$ M). Channel current was recorded in an inside-out patch bathed in 0.5 mM Mg-ATP/ $\text{Ca}^{2+}$ -free solution. C = channel-closed state.  $-V_{\text{pipette}} = -40$  mV.

activity was due to enhanced phosphatase activity as it could be prevented by inhibiting phosphatases by okadaic acid, activating PKA with cyclic AMP, or blocking protein kinase G with KT5823.

In the representative experiments shown in Fig. 9, cell-attached apical membrane patches were obtained, extracellular 0.2 mM Mg-ATP added to the bath, and then the patch was excised in the inside-out configuration. Extracellular Mg-ATP inhibited SK channel activity in 1–3 min in cell-attached patches, confirming our previous observations in the mouse (Lu et al., 2000). As expected, no SK channel activity was restored after the patch was excised into  $\text{Ca}^{2+}$ -free and Mg-ATP-free bath solution (Fig. 9 A). However, patch excision in a bath containing 0.5 mM Mg-ATP (Fig. 9 B) led to  $48 \pm 7\%$  recovery of SK channel activity in 10 of 12 patches:  $\text{NP}_o$  changed from  $6.68 \pm 0.92$  (cell-attached) to  $3.17 \pm 0.68$  (inside-out). If  $\text{PIP}_2$  (50  $\mu$ M) alone was present in the bath upon patch excision, SK channel activity could be restored to  $56 \pm 9\%$  of control:  $\text{NP}_o$  changed from  $9.79 \pm 1.25$  (cell-attached) to  $5.45 \pm 0.82$  (inside-out);  $n = 3$  (unpublished data). Further addition of 50 U of

PKA to the 0.5 mM Mg-ATP-containing bath restored channel activity to  $81 \pm 10\%$  of that in the cell-attached configuration:  $\text{NP}_o$  changed from  $6.53 \pm 2.14$  (cell-attached) to  $5.28 \pm 1.30$  (inside-out);  $n = 4$ ; Fig. 9 B. Thus, channel recovery requires both  $\text{PIP}_2$  and PKA-dependent phosphorylation processes.

Since the intracellular ATP concentration is in the range of 2–4 mM in rat principal cells and 1 mM Mg-ATP in the bath inhibits SK channel activity in inside-out patches (Wang and Giebisch, 1991a), mechanisms must exist in intact cells to maintain  $\text{K}^+$  secretion through SK channels. One possibility is  $\text{PIP}_2$  competition of Mg-ATP binding to the SK channel.  $\text{PIP}_2$  has been shown to modulate the ATP sensitivity of the pancreatic  $\beta$ -cell  $\text{K}_{\text{ATP}}$  channel, Kir6.2 (Shyng and Nichols, 1998; Enkvetchakul et al., 2000; Loussouarn et al., 2001) and to directly compete with ATP for binding to the COOH-termini of Kir1.1 (ROMK) and Kir6.1 channels (MacGregor et al., 2001).

To examine the interactions of Mg-ATP and  $\text{PIP}_2$  in rat principal cells, we assessed the Mg-ATP concentration dependence of SK channel activity in the presence



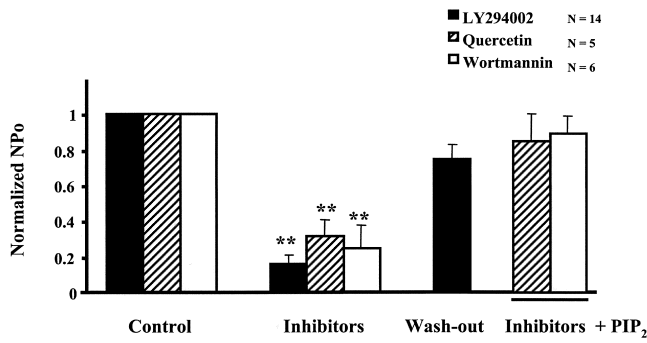


FIGURE 7. Summary of SK channel activity inhibited by the PI kinase inhibitors, LY294002 (20  $\mu$ M), quercetin (60  $\mu$ M), and wortmannin (20  $\mu$ M). Channel activity ( $NP_o$ ) was normalized to the control value.  $Ca^{2+}$ -free bath solution contained 0.5 mM Mg-ATP.  $PIP_2$  concentrations was 50  $\mu$ M. \*\* indicates  $P < 0.01$  versus control.

of 50  $\mu$ M  $PIP_2$  in excised patches (Fig. 10). SK channel activity was completely inhibited by 1 mM Mg-ATP in the absence of added  $PIP_2$  (Fig. 10 B, solid circle), confirming our previous observation in rat SK channels in principal cells (Wang and Giebisch, 1991a). Mg-ATP

sensitivity is considerably lower in ROMK channels expressed in *X. laevis* oocytes ( $EC_{50} \sim 2-3$  mM; McNicholas et al., 1996), but similar to native SK channels when ROMK is coexpressed with CFTR (Ruknudin et al., 1998). A representative experiment showing the concentration dependence of Mg-ATP inhibition in the presence of  $PIP_2$  is shown in Fig. 10 A. The patch was excised into a  $Ca^{2+}$ -free and Mg-ATP-free bath solution containing 50  $\mu$ M  $PIP_2$  that maintained SK channel activity for 2 min. Addition of 1 mM Mg-ATP had almost no effect on SK channel activity: control  $NP_o$   $3.12 \pm 0.66$  versus  $2.90 \pm 0.62$  ( $n = 9$ ). Increasing Mg-ATP led to a concentration-dependent inhibition of SK channel activity with 3 mM Mg-ATP abolishing activity (Fig. 10 B).  $PIP_2$  reduced the sensitivity of SK channels to Mg-ATP:  $EC_{50}$  for Mg-ATP was  $1.80 \pm 0.02$  mM. Thus,  $PIP_2$  reduced the Mg-ATP affinity of SK channels to a value close to that of the normal cell ATP concentration.

#### DISCUSSION

The present study provides evidence that both PI kinase and PKA play important roles in the regulation of the native SK channels in principal cells from rat kidney.

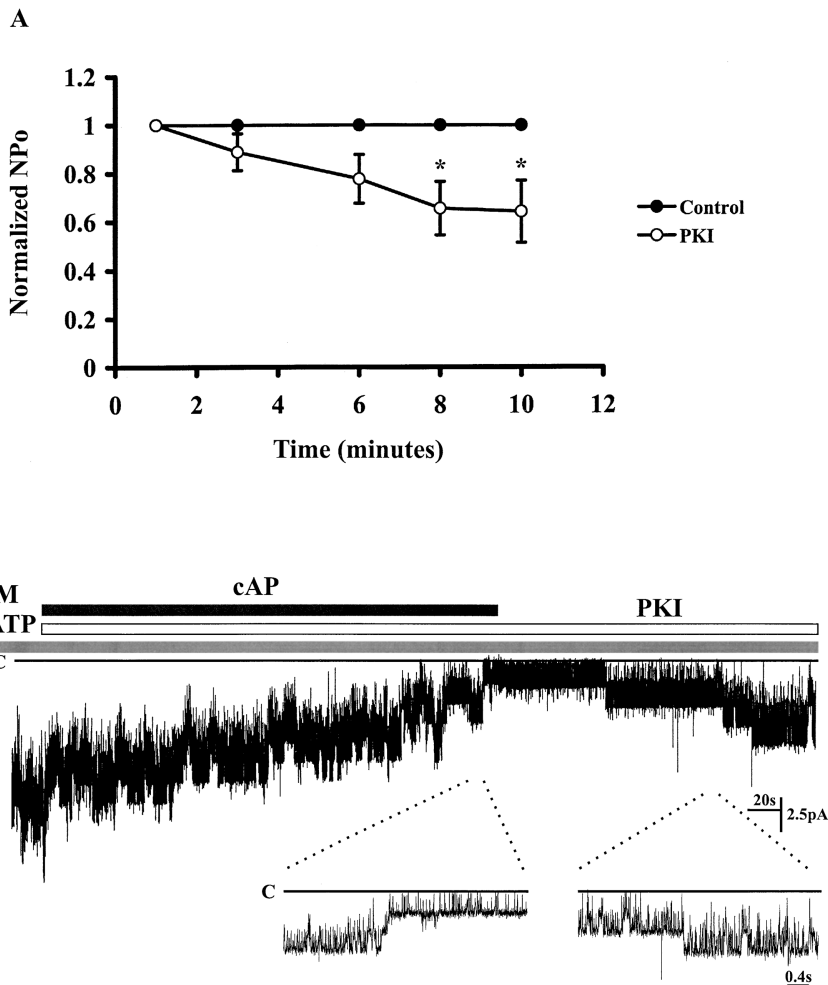
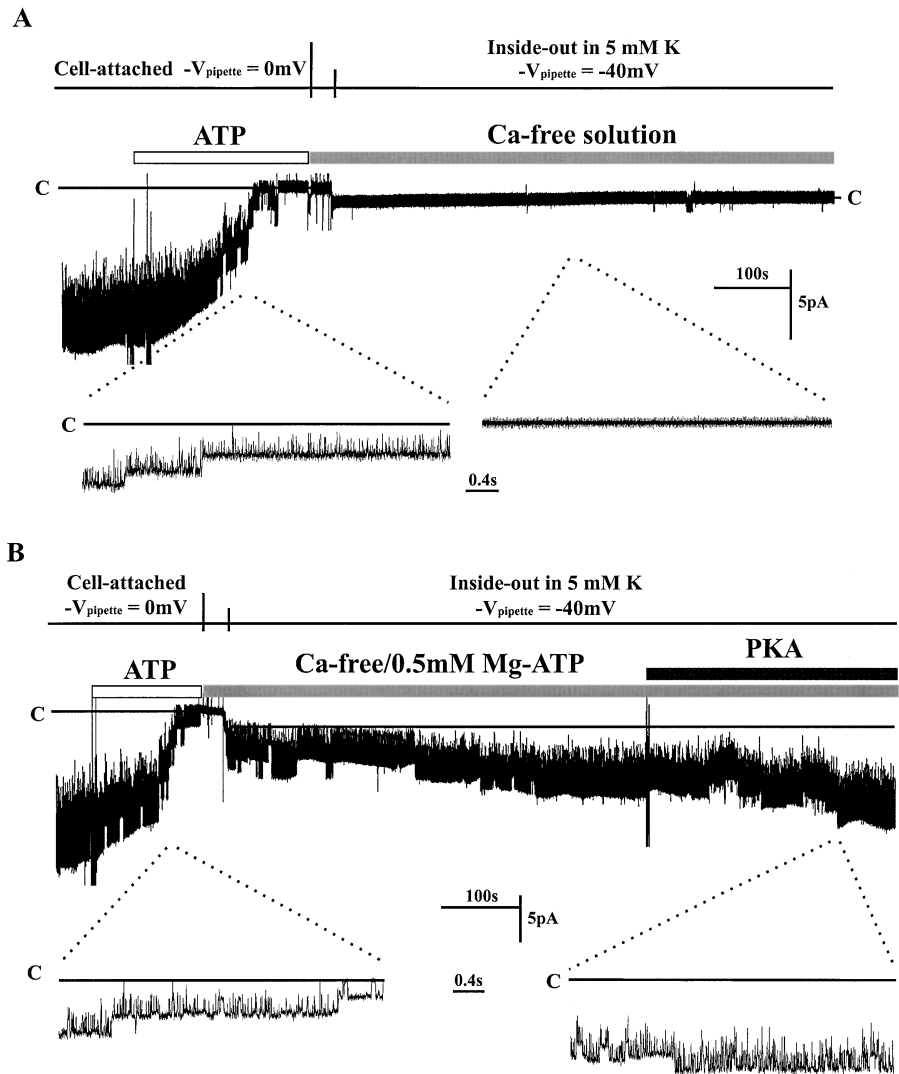


FIGURE 8. (A) PKI reduced SK channel activity in the presence of 0.5 mM Mg-ATP by 36% in 10 min. Data were from inside-out patch configuration. \* indicates  $P < 0.05$  compared with control (without PKI). (B) A representative trace showing that alkaline phosphatase (cAP) + PKI reduced SK channel activity in the presence of 0.5 mM Mg-ATP. After cAP wash-out,  $\sim 65\%$  of channel activity was restored in the presence of PKI + 0.5 mM Mg-ATP. C = channel-closed state.  $-V_{pipette} = -40$  mV.



**FIGURE 9.**  $\text{PIP}_2$  and PKA are required for restoration of SK channel activity after purinergic receptors mediated inhibition by extracellular Mg-ATP. Cell-attached apical membrane patches were obtained, extracellular Mg-ATP (0.2 mM) was added to the bath, and then patches were excised in the inside-out configuration. Representative SK channel traces are shown demonstrating inhibition of SK activity by extracellular Mg-ATP in the cell-attached configuration. In the inside-out configuration, no channel activity was observed in the absence of Mg-ATP or  $\text{PIP}_2$  (A). However, with addition of 0.5 mM Mg-ATP followed by 50 U of the catalytic subunit of PKA (B) SK channels were nearly completely reactivated. C = channel-closed state.  $-V_{\text{pipette}}$  = pipette-holding potential.

Several lines of evidence support this conclusion. First, hydrolyzable ATP (Mg-ATP) is required to maintain channel activity or to reactivate channels after run-down through the endogenous PKA and  $\text{PIP}_2$  (Figs. 3 and 4). This effect of Mg-ATP depends on phosphorylation reactions since exposure of SK channels to alkaline phosphatase in the presence of Mg-ATP nearly abolished channel activity. Second, stimulation of channel activity by Mg-ATP is mediated, at least in part, by generation of  $\text{PIP}_2$  through endogenous PI kinases. This is supported by our observations that declining channel activity after excision of membrane patches is mitigated by directly applied  $\text{PIP}_2$ , that  $\text{PIP}_2$  antibodies inhibit channel activity in the presence of Mg-ATP (Fig. 5, A and B), and that Mg-ATP or  $\text{PIP}_2$  partially reactivated SK channels inhibited by extracellular Mg-ATP (Fig. 9). It should be noted in this regard that  $\text{ATP}\gamma\text{S}$  does not interact with PI kinases to generate  $\text{PIP}_2$  (Liou et al., 1999). This explains our observation that  $\text{ATP}\gamma\text{S}$  does not sustain channel activity (Fig. 3 B). Moreover, inhibition of PI kinases re-

duced SK channel activity and attenuated Mg-ATP effects on maintenance of channel activity (Figs. 6 and 7). Third, processes involving PKA phosphorylation provide an additional mechanism for modulating SK channel activity by Mg-ATP. Maximal  $\text{PIP}_2$  effects require PKA phosphorylation (Figs. 8 and 9 B).

PI kinases are involved in the synthesis of  $\text{PIP}_2$  and several isoforms have been identified (Anderson et al., 1999). Together with membrane phosphatases and phospholipases, PI kinases are regulated by several hormones and growth factors (Martin, 1998; Leever et al., 1999).  $\text{PIP}_2$  has been shown to be an important regulator of a number of ion channels and transporters (Hilgemann et al., 2001). Our present results add the SK channel in principal cells to this growing list of  $\text{PIP}_2$ -regulated ion channels. However, the cellular distribution of PI kinases between membranes and the cytosol, and their regulation in principal cells has not been explored.

PIPs have thus emerged as important regulators of inward rectifier  $\text{K}^+$  channels (Hilgemann, 1997; Huang

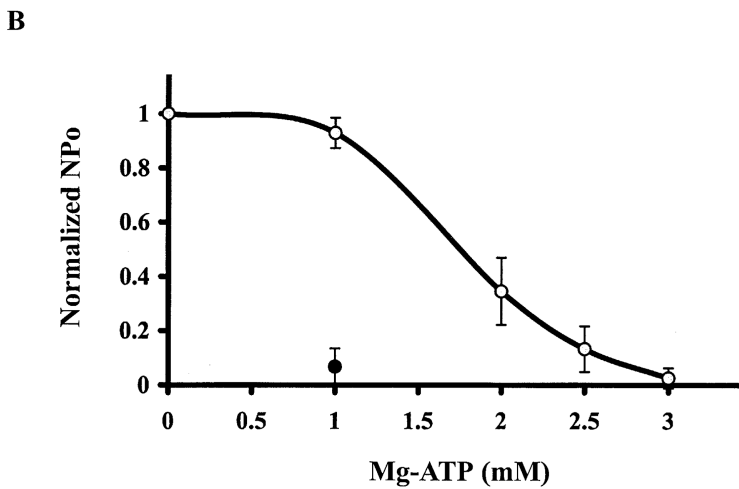
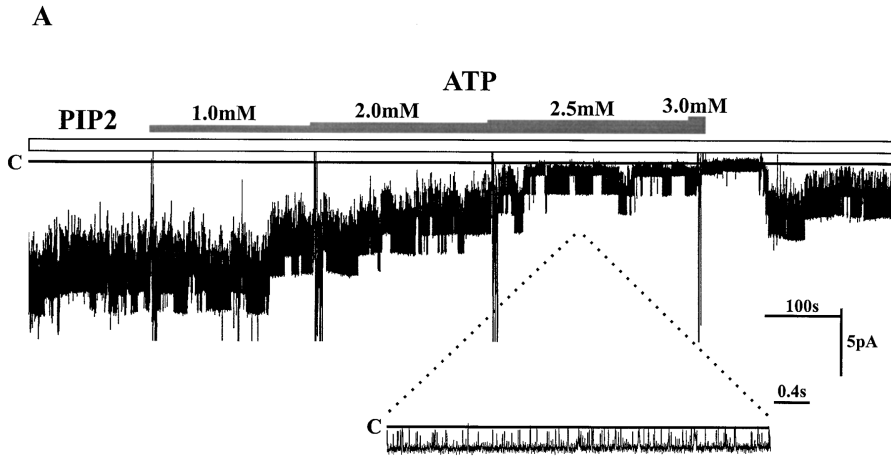


FIGURE 10. PIP<sub>2</sub> reduces Mg-ATP sensitivity of SK channels. (A) Representative SK channel activity recording of the concentration dependence of Mg-ATP inhibition in the presence of 50 μM PIP<sub>2</sub> in the bath solution. 1 mM Mg-ATP had little effect on channel activity in the presence of PIP<sub>2</sub> (B, open circle), whereas this Mg-ATP concentration abolished channel activity in the absence of PIP<sub>2</sub> (B, solid circle). C = channel-closed state.  $-V_{\text{pipette}} = -40$  mV. (B) Mg-ATP concentration-dependence of SK channel activity in the presence of 50 μM PIP<sub>2</sub> (open circles).  $EC_{50} = 1.8$  mM; Curve was fitted to the equation:  $Y = 1/[1 + 10^{(logEC_{50} - X) \cdot Hill}]$ . Hill =  $1.2 \pm 0.1$ .

et al., 1998; Hilgemann et al., 2001), including ROMK. The generation of PIP<sub>2</sub> by ATP-dependent PI kinases has been shown to activate ROMK1, and this effect is enhanced by PKA phosphorylation of the channel protein (Liou et al., 1999). Our observations in principal cells support this view by demonstrating that PIP<sub>2</sub> activates the SK channel and that PKA phosphorylation processes are required to give full SK channel activation by PIPs (Figs. 7–9).

Given that ATPγS is a substrate for PKA phosphorylation of ROMK1 channels (Jeong and Nikiforov, 1999; Liou et al., 1999), the inability of ATPγS to maintain channel activity in the absence of Mg-ATP indicates that PKA phosphorylation of SK is insufficient to sustain channel activity in the absence of PIP<sub>2</sub>. In addition, PKI reduced channel activity in the presence of Mg-ATP (Fig. 8 A) where PI kinases would be active and generating PIP<sub>2</sub>. This latter observation is consistent with the modulation of PIP<sub>2</sub> sensitivity of ROMK1 by PKA in oocytes (Liou et al., 1999). Moreover, PIP<sub>2</sub> antibodies abolished channel activity in the presence of a concentration of Mg-ATP sufficient to sustain PKA phosphorylation processes. Furthermore, after dephos-

phorylation by cAP, Mg-ATP did not fully reactivate channel activity in the presence of PKI. Together, our observations on SK channels strongly support the notions that PIP<sub>2</sub> can reactivate channels in the absence of PKA, but the sensitivity of PIP<sub>2</sub>-mediated reactivation is modulated by PKA phosphorylation. Thus, our observations in SK channels are fully consistent with the interactions of PIP<sub>2</sub> and PKA in the regulation of ROMK1 expressed in oocytes. However, we were unable to consistently fully inhibit channel activity by any one of several PI kinase inhibitors. It is possible that the inhibitor concentrations were insufficient to completely abolish PI kinase activity or that insufficient phosphatase or phospholipase activity was present so that a low level of PIPs remained in the membrane even in the presence of the PI kinase inhibitors.

Although the physiological actions of PIPs in the regulation of SK channel activity are incompletely understood, we have shown two circumstances where PIPs can modulate Mg-ATP inhibition. First, extracellular Mg-ATP can inhibit SK channels in intact principal cells by activation of P<sub>2</sub>Y<sub>2</sub> receptors in mouse (Lu et al., 2000) and rat (Fig. 9). This appears to be due to P<sub>2</sub>Y<sub>2</sub>-

mediated activation of phosphatases (Lu et al., 2000). The partial reactivation of the purinergic receptor-inhibited SK channels by bath Mg-ATP in excised patches (Fig. 9 B) is likely due to the action of PI kinase, since a similar degree of reactivation was observed with PIP<sub>2</sub> in the absence of Mg-ATP. These latter observations would be consistent with P<sub>2</sub>Y<sub>2</sub>-activated phosphatases reducing the concentration of PIPs in the plasma membrane. The further reactivation of these channels by addition of PKA suggests that the P<sub>2</sub>Y<sub>2</sub>-activated phosphatases also dephosphorylate ROMK, which is then rephosphorylated by addition of PKA. Furthermore, increase in SK channel activity by PKA is consistent with the necessity of PKA phosphorylation process for full activity of PIP<sub>2</sub>.

Second, while low concentrations of ATP (<1 mM; K<sub>d</sub> ≈ 0.5 mM) are important for channel activation by phosphorylation processes (via PI kinase, PKA, and potentially other kinases), SK channels are inhibited by higher concentrations of ATP (>1 mM; Wang and Giebisch, 1991a). The activity of SK channels in vivo and in intact cells in vitro is clearly maintained under conditions in which the cytosolic ATP is 2–4 mM, a concentration higher than that known to block SK channels in excised membrane patches. The data presented in Fig. 10 demonstrate that PIP<sub>2</sub> shifts the ATP sensitivity in SK channels to the right and thereby help to maintain native channel activity at the relatively high cell ATP concentrations. PIPs also modulate the ATP sensitivity of the pancreatic β-cell K<sub>ATP</sub> channel, Kir6.2 (Shyng and Nichols, 1998; Enkvetchakul et al., 2000; Loussouarn et al., 2001). Recently, we have shown that PIPs compete with ATP for binding to the carboxy terminus of ROMK and Kir6.1 channels (MacGregor et al., 2001) and this could provide a mechanism for PIPs maintaining SK channels in the presence of high cytosolic concentrations of ATP. Finally, given the recent observations by Marx and coworkers (Marx et al., 2002) of a macromolecular signaling complex for the sympathetic regulation of outward K<sup>+</sup> current in heart, it is possible that all of the proteins regulating SK channel activity (PKA, lipid kinases, phosphatases) are similarly compartmentalized into a macromolecular complex.

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