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Inhibition of phosphatidylinositol 3-kinase stimulates activity of the small-conductance K channel in the CCD

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Abstract

We used Western blotting to examine the expression of phosphatidylinositol 3-kinase (PI3K) in the renal cortex and outer medulla and employed the patch-clamp technique to study the effect of PI3K on the ROMK-like small-conductance K (SK) channels in the cortical collecting duct (CCD). Low K intake increased the expression of the 110-kDa α -subunit (p110 α) of PI3K compared with rats on a normal-K diet. Because low K intake increases superoxide levels (2), the possibility that increases in superoxide anions may be responsible for the effect of low K intake on the expression of PI3K is supported by finding that addition of H₂O₂ stimulates the expression of p110 α in M1 cells. Inhibition of PI3K with either wortmannin or LY-294002 significantly increased channel activity in the CCD from rats on a K-deficient (KD) diet or on a normal-K diet. The stimulatory effect of wortmannin on ROMK channel activity cannot be mimicked by inhibition of phospholipase C with U-73122. This suggests that the effect of inhibiting PI3K was not the result of increasing the phosphatidylinositol 4,5-bisphosphate level. Moreover, application of the exogenous phosphatidylinositol 3,4,5-trisphosphate analog had no effect on channel activity in excised patches. Because low K intake has been shown to increase the activity of protein tyrosine kinase (PTK), we explored the role of the interaction between PTK and PI3K in the regulation of the SK channel activity. Inhibition of PTK increased SK channel activity in the CCD from rats on a KD diet. However, addition of wortmannin did not further increase ROMK channel activity. Also, the effect of wortmannin was abolished by treatment of CCD with phalloidin. We conclude that PI3K is involved in mediating the effect of low K intake on ROMK channel activity in the CCD and that the effect of PI3K on SK channels requires the involvement of PTK and the cytoskeleton.

Keywords

ROMK; protein tyrosine kinase; phosphatidylinositol-3,4,5 trisphosphate; renal K secretion; hypokalemia; cortical collecting duct

The cortical collecting duct (CCD) and connecting tubule are responsible for K secretion and aldosterone-sensitive Na absorption (10,25). K secretion requires Na-K-ATPase, which takes K across the basolateral membrane into the cell, and apical K channels, which move K across the luminal membrane along the electrochemical gradient. Molecular biology and patch-clamp experiments have identified several types of K channels in the apical membrane such as the Ca²⁺-dependent maxi-K channel (9), voltage-gated K channel (38), and ROMK (Kir1.1) (12). Although they all could be involved in K secretion (19,37,38), it is generally accepted that ROMK is mainly responsible for K secretion under normal physiological

conditions (11). Furthermore, studies performed in ROMK-null mice have firmly established that ROMK channels are the key component of the SK channels in the native CCD because no SK channel activity was observed in the CCD from ROMK-null mice (20,21).

The regulation of ROMK channels has been extensively investigated. Factors such as dietary K intake play a key role in regulating channel activity in the CCD (11,32): high K intake increases, whereas low K intake decreases, SK channel activity in the CCD. We have previously demonstrated that low K intake stimulates the expression of Src family protein tyrosine kinase (PTK) and that inhibition of PTK increases SK channel activity (33,34). However, detailed mechanisms by which PTK-dependent signal transduction pathways regulate ROMK channel activity are still not completely understood. PTK has been shown to regulate PI3K activity by tyrosine phosphorylation (3,4,15). Moreover, PI3K or PI3K-dependent lipid products such as phosphatidylinositol phosphates are involved in remodeling of the cytoskeleton (6), which has been shown to regulate ROMK channel activity (35). Therefore, we tested the possibility that PI3K may be a part of the signaling molecule interacting with PTK to regulate ROMK channels.

METHODS

Preparation of CCDs

Pathogen-free Sprague-Dawley rats of both sexes (50–60 g) were used in the experiments and purchased from Taconic Farms (Germantown, NY). After 1-wk recovery from travel stress, the rats were placed on either normal rat chow (1.1% wt/wt) or a K-deficient diet (KD) for 6–7 days. Rats were killed by cervical dislocation, and their kidneys were removed immediately. Several thin slices (<1 mm) of the kidney were cut with a razor blade and placed on ice-cold Ringer solution until dissection. The dissection was carried out at room temperature, and two watchmaker forceps were used to isolate the single CCD. The animal use protocol has been submitted and approved by New York Medical College animal committee. To immobilize the tubules, they were placed onto a 5 × 5-mm cover glass coated with polylysine (Sigma, St. Louis, MO). The cover glass was transferred to a chamber (1,000 µl) mounted on an inverted Nikon microscope. The tubules were superfused with HEPES-buffered NaCl solution, and the temperature of the chamber was maintained at 37 ± 1°C by circulating warm water around it. To gain access to the apical membrane, a sharp pipette was used to cut the tubules. Only principal cells were used for the study.

Patch-clamp technique

The K current was recorded with an Axon 200A patch-clamp amplifier and was low-pass filtered at 1 kHz using an eight-pole Bessel filter (902LPF, Frequency Devices, Haverhill, MA). Data were digitized by Axon interface (Digidata 1200) and stored on the hard drive of an IBM-compatible Pentium computer (Gateway 2000). We used the pClamp software system 6.04 (Axon Instruments, Burlingame, CA) to analyze the data. Channel activity was defined as NP_o , which was calculated from data samples of 60-s duration in the steady state as follows

$$NP_o = \sum (t_1 + t_2 + \dots + t_i) \quad (1)$$

where t_i is the fractional open time spent at each of the observed current levels. The patch pipettes were drawn with a Narishige PP-81 puller and were polished with a homemade fire polisher before the patch-clamp experiments.

Preparation of M1 cells

M1 cells, a mouse CCD line, were purchased from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% FBS. Before H₂O₂ treatment, the cells were cultured in medium containing 1% FBS for 16 h, followed by incubation for an additional 30 min in a solution containing (in mM) 22 HEPES (pH 7.4), 124 NaCl, 5 KCl, 1 MgCl₂, 1.5 CaCl₂, 0.16 HPO₄, 0.4 H₂PO₄, 5 NaHCO₃, and 5.6 mM H₂O₂ (100 μ M final concentration) was added directly to the cells in HEPES buffer for 120 min, followed by the incubation in the control medium for an additional 2 h. The viability of M1 cells treated with H₂O₂ as determined by the trypan blue dye exclusion method was ~90% of the corresponding control cells. After treatment with H₂O₂, the cells were washed twice with ice-cold phosphate-buffered saline and incubated for 30 min in RIPA lysis buffer.

Western blotting

The tissue-dissected renal cortex and outer medulla or M1 cell culture were homogenized. Proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline, rinsed, and washed with 0.05% Tween 20-Tris-buffered saline buffer. We used ECL (Amersham Pharmacia Biotech) to detect the protein bands, and the intensity of the corresponding band was determined with Alpha DigiDoc 1000 (Alpha Innotech, San Leandro, CA). The antibody of the 110-kDa subunit of PI3K was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Changes in PI3K expression were normalized by comparison to β -actin expression.

Experimental solution and statistics

The pipette solution contained (in mM) 140 KCl, 1.8 MgCl₂, and 10 HEPES (pH = 7.4). The bath solution for cell-attached patches was composed of (in mM) 140 NaCl, 5 KCl, 1.8 CaCl₂, 1.8 MgCl₂, and 10 HEPES (pH = 7.4). For inside-out patches, we added 50–100 μ M ATP to prevent channel run-down. D-myo-phosphatidylinositol 3,4,5-trisphosphate (D-myo-PIP₃) was purchased from Echelon Bioscience (Salt Lake City, UT), whereas LY-294002, wortmannin, herbimycin A, and U-73122 were obtained from Biomol (Plymouth Meeting, PA). Phalloidin was obtained from Sigma and dissolved in methanol. Data are shown as means $\pm$ SE, and a paired Student's *t*-test was used to calculate the significance between the control and experimental groups. Statistical significance was taken as $P < 0.05$.

RESULTS

We first examined the effect of low K intake on the expression of the p110 α subunit of PI3K, a catalytic subunit of the kinase. Figure 1A is a Western blot showing that low K intake significantly increases the expression of the p110 α subunit of PI3K by $220 \pm 20\%$ ($n = 4$). We previously demonstrated that low K intake increased the production of superoxide anions and the related species, which stimulated c-Src expression (2). Thus we examined whether increases in superoxide anions and the related products were also responsible for stimulating the expression of PI3K. We incubated M1 cells with 100 μ M H₂O₂ for 2 h followed by the culture in the control medium for an additional 2 h. Figure 1B is a typical Western blot showing that the addition of H₂O₂ increased the expression of the p110 α subunit ($95 \pm 9\%$, $n = 3$) in M1 cells. This suggests that increases in superoxide anions are at least partially responsible for stimulation of PI3K expression induced by low K intake.

After observing that low K intake stimulates the expression of p110 α , we examined the effect of LY-294002 (10 μ M), an inhibitor of PI3K (31), on ROMK-like SK channel activity. Figure 2 is a typical recording showing the effect of LY-294002 on SK channels in

the CCD from rats on a KD diet. As reported previously, SK channel activity was low in the CCD from rats on a KD diet. Inhibition of PI3K increased SK channel activity, defined as NP_o , from 0 to 3.5 in this particular patch. To determine whether the effect of LY-294002 was the result of inhibition of PI3K, we used wortmannin, another specific inhibitor of PI3K (1). Figure 3 is a typical recording demonstrating that application of wortmannin (100 nM) increased the activity of SK channels in the CCD from rats on a KD diet. Under control conditions, no channel activity was observed in this patch; inhibition of PI3K opened two SK channels in the patch. Because the open probability of the SK channel is close to 1, an increase in SK channel activity must result from opening the previously silent K channels or the insertion of new K channels. Also, we observed the positive response (increases in channel number) to wortmannin and LY-294002 in 7 of a total of 14 patches in the CCD from rats on a KD diet.

Figure 4 is a bar graph summarizing results from 14 patches regarding the effect of PI3K inhibitors on SK channel activity. Under control conditions, the mean NP_o was 0.47 ± 0.04 in the CCD from rats on a KD diet, and inhibition of PI3K with wortmannin (100 nM) increased channel activity by 120% and significantly raised NP_o to 1.1 ± 0.12 ($P < 0.01$). To determine whether the effect of wortmannin on SK channels was affected by K intake, we examined the effect of PI3K inhibitors on SK channel activity in the CCD from rats on a control K diet (1.1%). Data summarized from Fig. 4 show that inhibition of PI3K modestly increased channel activity by 40% from 1.38 ± 0.2 to 1.92 ± 0.2 ($P < 0.05$) ($n = 13$). In addition, the positive response to wortmannin was observed in 5 of a total of 13 patches.

Stimulation of PI3K was expected to decrease PIP_2 concentrations or increase PIP_3 levels. Because PIP_2 has been shown to activate ROMK channels (13,18), it is possible that inhibition of PI3K increases the availability of PIP_2 , which activates channel activity. To test this possibility, we tested the effect of U-73122, an inhibitor of phospholipase C. If the effect of wortmannin on SK channel activity was the result of increasing PIP_2 levels, U-73122 should be able to mimic the effect of wortmannin because inhibition of phospholipase C was also expected to increase PIP_2 levels. Thus we examined the effect of U-73122 on the SK channel activity in the CCD from rats on a KD diet and found that application of U-73122 (1 μ M) has no effect on channel activity ($n = 8$) (data not shown).

To test whether PIP_3 could directly inhibit SK channels, we examined the effect of exogenous PIP_3 on SK channel activity in inside-out patches to determine whether PIP_3 could inhibit SK channel activity in the CCD. Figure 5 is a typical channel recording demonstrating that addition of the PIP_3 analog $D-myo-PIP_3$ (1 μ M) had no effect on channel activity ($n = 3$).

After confirming that PIP_3 had no inhibitory effect on SK channel activity, we explored the possible interaction between PTK and PI3K by examining whether the effects of wortmannin and herbimycin A were additive. The CCDs were first incubated with wortmannin-containing solution for 20–30 min. In the continuous presence of wortmannin, 1 μ M herbimycin A was applied to the patch. Figure 6 is a typical recording showing that inhibition of PTK increases SK channel activity. Data summarized in Fig. 7 show that in the presence of wortmannin, inhibition of PTK increased NP_o from 1.1 ± 0.12 to 2.1 ± 0.2 ($n = 16$). In contrast, blocking PTK not only increased channel activity from 0.48 ± 0.04 to 2.19 ± 0.2 ($n = 15$) but also completely abolished the stimulatory effect of inhibiting PI3K, because the effect of wortmannin on SK channels is absent in the presence of herbimycin A (Fig. 7). This suggests that PI3K-induced inhibition of SK channels requires activated PTK and that PTK can regulate SK channels through a PI3K-independent signaling pathway.

Because PI3K or PI3K-dependent lipid products have been shown to regulate cellular function such as remodeling the cytoskeleton and vesicle trafficking (6), we examined the role of the cytoskeleton in mediating the effect of inhibiting PI3K. We have previously shown that phalloidin, an agent that stabilizes F-actin, abolished the stimulatory effect of inhibiting PTK on SK channels (35). Figure 8 summarizes data from 10 experiments, in which the effect of wortmannin on SK channels was examined in the CCD treated with 10 μ M phalloidin. It is apparent that wortmannin failed to increase channel activity in the presence of phalloidin. Thus this suggests that the effect of PI3K on SK channels requires the involvement of the cytoskeleton.

DISCUSSION

In the present study, we have demonstrated that expression of the P110 α subunit of PI3K is upregulated by low K intake and that inhibition of PI3K increases SK channel activity in the CCD. Although we did not measure the activity of PI3K in the present study, we speculate that low K intake should also increase the activity of PI3K as a consequence of enhanced expression of the kinase, because inhibition of PI3K had a larger effect on SK channels in rats on a KD diet than that in those on a control K diet. This suggests that PI3K is active and involved in the regulation of SK channels under physiological conditions. We used wortmannin and LY-294002 to demonstrate the role of PI3K in the regulation of SK channels in the CCD. Although wortmannin is generally considered to be a specific inhibitor of PI3K, it has also been shown to block the activation of mitogen-activated protein kinases (MAPK) by insulin (36). However, the observation that the effect of wortmannin on SK channels can be mimicked by LY-294002, another PI3K inhibitor which has a different structure from wortmannin, strongly suggests that the stimulatory effect of wortmannin is the result of inhibiting PI3K.

Three classes of PI3K are identified based on their lipid substrate preference. However, only class I PI3K is able to phosphorylate PI (4,5) phosphate to PI (3,4,5)-phosphate (29). There are four members of class I PI3K in mammalian cells: α -, β -, γ -, and δ -isoforms. However, PI3K α - and β -isoforms are more ubiquitously expressed in mammalian cells than δ - and γ -isoforms, which are mainly expressed in the hemopoietic cells (29,30). We demonstrated that low K intake increases the expression of the p110 α -subunit. Because p110 α is a catalytic subunit, increased expression of the P110 α -subunit suggests that K depletion may increase PI3K activity.

The mechanism by which low K intake stimulates the expression of PI3K is not completely understood. The observation that the addition of H₂O₂ increased the expression of the P110 α -subunit suggests that increases in superoxide anions and related species are partially responsible for the stimulation of PI3K expression induced by low K intake. Also, we previously demonstrated that low K intake increases superoxide anion levels, which stimulate the expression of Src family PTK (2). Thus this suggests that increases in superoxide anion levels induced by low K intake are responsible for stimulation of expression of PI3K and PTK. Relevant to our observation is the report that H₂O₂ stimulates PI3K activity in A6 cells (23). Also, it has been shown that most of the growth factors can activate PI3K (6). In this regard, low K intake has been reported to increase levels of growth factors such as IGF (8). It is possible that increases in superoxide anion and growth factors during low K intake may be responsible for stimulating the expression of PI3K.

The observation that inhibition of PTK can completely abolish the effect of wortmannin on SK channels suggests that PI3K-induced inhibition of SK channels depends on the PTK-signal transduction pathway. It has been reported that PI3K activity is regulated by PTK through tyrosine phosphorylation (4,15). The catalytic activity of p110 α is suppressed by

association with an 85-kDa regulatory subunit (p85 α). Because the p85 α regulatory subunit has two SH2 domains, it can interact with tyrosine-phosphorylated proteins (6). It has been demonstrated that the association of tyrosine-phosphorylated substrates with the p85 subunit leads to activation of PI3K (15). In addition, the p85 subunit has been demonstrated to be a substrate of PTK and tyrosine phosphorylation of p85 relieves its inhibitory activity on PI3K (4). Thus it is conceivable that Src family PTK can stimulate PI3K activity by increasing tyrosine phosphorylation of the p85 subunit or by phosphorylating a substrate protein that interacts with p85 subunits and, accordingly, activates PI3K. Thus inhibition of PTK leads to inactivation of PI3K, and it then abolishes the stimulatory effect of wortmannin on SK channels. Also, the observation that the stimulatory effect of inhibiting PTK can still be observed in the presence of the PI3K inhibitor indicates that PTK-induced inhibition of ROMK channels can be induced by an additional PI3K-independent pathway.

There are at least four mechanisms by which PI3K may regulate the activity of ROMK channels: 1) PI3K may regulate ROMK channel activity by phosphorylation of ROMK or related proteins because PI3K is also a serine/threonine kinase (14); 2) PI3K may inhibit ROMK channel activity by decreasing PIP₂ concentrations, which are essential for maintaining ROMK channels in the open state; 3) PI3K or PI3K-dependent lipids may inhibit channel activity by stimulating PKC or recruiting PTK enzymes; and 4) PI3K could regulate cell functions such as vesicle trafficking through the FYVE domain (30) or cytoskeleton remodeling by regulation of small G proteins (6).

Relevant to the first possibility is the observation that PI3K phosphorylated IRS-1, the insulin receptor substrate (16). However, it is generally believed that kinase activity of PI3K serves mainly for autoregulation of PI3K activity, such that phosphorylation of the P85 subunit leads to inhibition of catalytic activity of the P110 subunit (5). However, we could not completely exclude the possibility that PI3K inhibits SK channels through protein phosphorylation.

For the second possibility, stimulation of PI3K should lead to decreasing PIP₂ levels, which have been shown to stimulate ROMK channel activity (18). Although this possibility is not supported by the observation that inhibition of phospholipase C did not increase channel activity, data may not be conclusive because phospholipase C may not be active in our experimental setting. However, it is unlikely that wortmannin-induced stimulation of SK channel activity results from an increase in PIP₂ concentrations because stimulation of PI3K, which phosphorylates PIP₂ to PIP₃, would not alter the total content of PIP₂ and PIP₃. It has been reported that PIP₃ and PIP₂ were equally potent in stimulation of ATP-sensitive K channels (27) and epithelial Na channels (22). Thus even if PIP₂ levels fall, PIP₃ should be able to maintain SK channel activity. Although direct addition of PIP₃ did not increase SK channel activity in the present experiment, this may be due to the fact that channel activity was saturated by PIP₂ produced by ATP in the bath solution. Also, it is unlikely that PIP₃ can inhibit SK channels because direct application of the exogenous PIP₃ analog had no effect on SK channel activity in inside-out patches. Thus the inhibitory effect of PI3K on SK channels is indirect and requires the intracellular component. Relevant to this is the finding that the Src family PTK inhibited ROMK channel activity in the intact cell but had no effect on channel activity in excised patches (33).

PIP₃ has been shown to regulate the activity of several types of PKC, including atypical isoforms of PKC and PKC α (24,26). Moreover, PI3K has been reported to directly associate with PKC- δ and - ϵ (7). In this regard, we have previously shown that low K intake increased PKC- ϵ expression and that inhibition of PKC abolished the internalization of ROMK channels induced by stimulation of PTK (28). Although it is possible that PIP₃ may also stimulate PKC activity, which inhibits SK channel activity in the CCD, it is unlikely that the

stimulatory effect of wortmannin on SK channel activity is the result of blocking PKC activity, because we have previously observed that inhibition of PKC had no significant effect on SK channel activity (28). PI3K-dependent lipid products, including PIP₃, have been shown to play an important role in recruiting Bruton's tyrosine kinase (Btk) to the plasma membrane by facilitating binding of PIP₃ to the pleckstrin-homology domain of Btk (17). Thus it is possible that PI3K-dependent lipid products may also stimulate the recruitment of PTK to the plasma membrane and enhance tyrosine phosphorylation. Although it is not known whether Btk is also expressed in the CCD, we cannot rule out this possibility. Further experiments are needed to examine this possibility.

However, the observation that the effect of wortmannin was absent in the presence of phalloidin suggests that the effect of PI3K is, at least in part, the result of stimulating remodeling of the cytoskeleton. We have previously demonstrated that remodeling of the cytoskeleton is required for the insertion of ROMK channels induced by inhibiting PTK in the CCD (35). Now, we show that the cytoskeleton is also involved in mediating the effect of PI3K on SK channels. This suggests that the effect of inhibiting PI3K may also enhance the insertion of SK channels into the plasma membrane.

The present study indicates that the PI3K plays a role in mediating the effect of low K intake on ROMK channels in the CCD. We hypothesize that low K intake stimulates the expression of PTK and PI3K. Activation of PTK stimulates PI3K, which inhibits SK channels by controlling cytoskeleton remodeling. In addition, PTK can inhibit SK channels by a PI3K-independent pathway and PI3K is partially responsible for mediating the effect of PTK. Besides inhibition of ROMK channels, PI3K has been shown to increase Na transport in A6 cells (23). Thus a PI3K-dependent pathway could serve as a switch molecule that stimulates Na absorption while suppressing K secretion in the CCD. We conclude that low K intake increases the expression of the P110 α -subunit of PI3K and that PI3K is involved in mediating PTK-induced inhibition of apical SK channels in the CCD.

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Fig. 1.

A: Western blot showing expression of the p110 α -subunit of phosphatidylinositol 3-kinase (PI3K) in the renal cortex and outer medulla from rats on a control and K-deficient (KD) diet for 7 days. Arrow indicates the PI3K band. *B:* Western blot demonstrating the effect of H₂O₂ (100 μ M) on the expression of the p110 α -subunit in M1 cells. The same membrane was stripped and exposed to actin antibody. IB, immunoblot.

Fig. 2.

Channel recording showing the effect of Ly-294002 on apical ROMK-like small-conductance K (SK) channels in the cortical collecting duct (CCD) from rats on a KD diet. The *top* trace shows time course of the experiment, and 3 parts of the recording are extended to show the fast time resolution. The channel-closed state is indicated by C or a dotted line, and a bold horizontal bar indicates the channel-open level. The experiment was performed in a cell-attached patch, and the holding potential was 0 mV.

Fig. 3.

Channel recording showing the effect of wortmannin on apical SK channels in the CCD from rats on a KD diet. The top trace demonstrates the time course of the experiment, and 2 parts of the recording are extended to show the fast time resolution. The experiment was performed in a cell-attached patch, and the holding potential was 0 mV.

Fig. 4. Effect of PI3K inhibitors on SK channel activity (NP_o) in the CCD from rats on a KD or a control K diet (1.1%). Significantly different: ** $P < 0.01$ and * $P < 0.05$.

Fig. 5. Effect of *D*-myo-phosphatidylinositol 3,4,5-trisphosphate (PIP₃; 1 μ M) on apical SK channel activity in the CCD. The experiment was carried out in an inside-out patch. Two parts of the trace are extended to show the fast time resolution.

Fig. 6. Channel recording showing the effect of herbimycin A (Herb-A) on apical SK channels in the presence of wortmannin in the CCD from rats on a KD diet. The *top* trace demonstrates the time course of the experiment, and 3 parts of the recording are extended to show the fast time resolution. The experiment was performed in a cell-attached patch, and the holding potential was 0 mV.

Fig. 7. Effect of wortmannin, herbimycin A (HA), and wortmannin+HA on apical SK channels in the CCD from rats on a KD diet. *Significantly different ($P < 0.05$) from the control value (KD). #Significantly different ($P < 0.05$) from wortmannin alone.

Fig. 8.

Effect of wortmannin on SK channel activity in the presence or absence of 10 μ M phalloidin. The experiments were performed in cell-attached patches.