An ATP-Regulated and pH-Sensitive Inwardly Rectifying K⁺ Channel in Cultured Human Proximal Tubule Cells

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Abstract: Although renal K⁺ channels along the nephron have been explored in various animal species, little is known about the K⁺ channels in human proximal tubule cells. Using the patch-clamp technique, we investigated the properties of an inwardly rectifying K⁺ channel present in the surface membrane of cultured human proximal tubule cells of normal kidney origin. This channel was the most frequently observed K⁺ channel in cell-attached patches, and cytoplasmic ATP was required to maintain channel activity in inside-out patches. Its single channel conductance was about 42 pS for inward currents and 7 pS for outward currents under the symmetrical K⁺ condition. The ATP effect on channel activity was dose-dependently stimulatory within a range of 0.1 to 10 mM, and a nonhydrolyzable ATP analog, AMP-PNP (3 mM), had no effect on channel activity in either the presence or absence of ATP (1 mM). The channel ac-

tivity observed in cell-attached patches was reduced to 30 to 50% of controls by a membrane-permeable nonspecific protein kinase inhibitor, K252a (1 μ M), or a potent protein kinase A inhibitor, KT5720 (500 nM). In contrast, a membrane-permeable cAMP analog, 8Br-cAMP (100 μ M), induced a twofold increase in channel activity. The addition of a catalytic subunit of protein kinase A (PKA-CS, 100 U/ml) to the bath in inside-out patches stimulated channel activity in the presence of 1 mM ATP. Furthermore, the channel activity maintained with 1 mM ATP in inside-out patches was suppressed by internal acidification and enhanced by alkalization. These results suggest that the activity of the inwardly rectifying K⁺ channel in cultured human proximal tubule cells was ATP-dependent and regulated at least in part by cAMP/PKA-mediated phosphorylation processes and intracellular pH. [Japanese Journal of Physiology, 51, 523–530, 2001]

Key words: potassium channel, human kidney, ATP, protein kinase A, intracellular pH.

Until now, many types of K⁺ channels have been identified and characterized electrophysiologically in renal tubules of many animal species. These K⁺ channels contribute a great deal to physiological functions of tubular epithelial cells [1–3]. Furthermore, the molecular cloning of various K⁺ channels has enabled analyses of their structure–function relationships and tissue distribution/localization. Some of these cloned K⁺ channels were reported to be expressed at the mRNA level in kidneys, including the human one [4–8]. However, little information is available regarding the native K⁺ channels in human kidney, though a cGMP-regulated K⁺ channel has recently been identified in immortalized human kidney epithelial cells [9].

With respect to the regulation of renal K⁺ channels, a variety of factors have been shown to be involved. It has been revealed that intracellular ATP is one of the most important factors that couple the K⁺ channel activity to changes in cell metabolism [3, 10]. In the proximal tubule, the ATP-regulated K⁺ channels were detected mainly in the basolateral membranes of several animal species [11–15]. It is generally thought that these K⁺ channels are related with the activity of basolateral Na⁺/K⁺-ATPase and play an important role in the formation of membrane potential in this nephron segment [14–16]. To complicate matters, however, previous reports on the effects of ATP on channel activity varied in individual animals. In rabbit

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[14, 15] and salamander [11] proximal tubules, basolateral ATP-regulated K^+ channels were inhibited by ATP in a dose-dependent manner. In contrast, the effects of ATP were not inhibitory but simply stimulatory on ATP-regulated K^+ channels in the basolateral membrane of frog proximal tubule [13] and the surface membrane of opossum proximal tubule cells [12]. This diversity prompted us to explore how the K^+ channel(s) in human proximal tubule cells would be regulated by intracellular ATP.

In this study, we applied the patch-clamp technique to the cultured human proximal tubule cells of normal kidney origin and identified an inwardly rectifying K^+ channel, which is regulated by ATP, in the surface membrane of these tubule cells. The regulation of this channel, as well as its basic properties, were investigated with special interest in the effects of ATP, protein phosphorylation, and pH.

MATERIALS AND METHODS

Cell culture. Renal proximal tubule epithelial cells (RPTECs) of normal human kidney origin were purchased from Clonetics (Walkersville, MD, USA). It is guaranteed that more than 90% of the cells are positive for gamma-glutamyl transferase, a marker protein specific to the proximal tubule [17]. These cells were provided as cryopreserved secondary culture and used at passages 3–6 in this study. The cells were plated on 25 cm² flasks at a density of 6.25×10^4 cells/flask and cultured for 4 to 7 d in a humidified atmosphere of 5% CO₂/95% air at 37°C. The culture medium used was renal epithelial cell growth medium (Clonetics), which was changed every other day. On the day of the patch-clamp experiment, the cultured cells at 70 to 80% confluence were dispersed with trypsin/EDTA. The cells were then resuspended in the culture medium and seeded on collagen-coated coverslips (Iwaki, Tokyo, Japan) in $\phi 35$ mm culture dishes at a density of 1×10^5 cells/dish. After 3- to 7-h incubation, the coverslips were transferred to an open bath heating chamber (Warner, Hamden, CT, USA).

Solutions. The control bath solution contained (in mM) 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 5 glucose, and 10 HEPES. Patch pipettes were filled with the KCl solution, which contained (in mM) 145 KCl, 1 MgCl₂, 1 EGTA, and 1 HEPES. The same KCl solution was used as the bath solution for inside-out patches. For the experiment determining the K^+ selectivity, 115 mM NaCl was substituted for 115 mM KCl in the bath solution. All these solutions were titrated to pH 7.3 by using 5 N NaOH or KOH. KCl solutions of pH 6.8 and 7.8 were also employed to examine the

pH effect on channel activity.

Test substances. MgATP, AMP-PNP, and 8Br-cAMP were purchased from Sigma (St. Louis, MO, USA). K252a and KT5720 were from Calbiochem (La Jolla, CA, USA). The catalytic subunit of protein kinase A (PKA-CS) was from Promega (Madison, WI, USA). K252a and KT5720 were dissolved in dimethyl sulfoxide (DMSO) as stock solutions, whereas others were dissolved in water. These stock solutions were diluted with appropriate bath solutions and added to the bath. The final concentration of DMSO in the bath ranged from 0.047 to 0.054%, which had no apparent effect on the channel activity.

Patch-clamp technique. The cell-attached and inside-out configurations of the patch-clamp technique were employed to determine the activity of the K^+ channel in RPTECs. All patch experiments were performed at 33°C. This setting of temperature was defined most appropriate for the patch-clamp experiment applying to RPTECs, since higher temperatures impaired the cell viability and lower ones caused the giga-seal formation to be difficult. Patch pipettes were fabricated from glass capillaries (Warner, Hamden, CT, USA), with the resistance ranging from 3 to 5 M Ω when filled with the KCl solution. The pipette-holding potential (V_p) was set at 0 mV for cell-attached patches and +50 mV for inside-out patches, unless otherwise stated. Current signals were recorded with a patch-clamp amplifier (Axopatch 200B; Axon, Foster City, CA, USA) and stored on a DAT recorder (RD-120TE; TEAC, Tokyo, Japan). The recorded signals were then low-pass filtered (3611 Multifunction Filter; NF Electronic Instruments, Tokyo, Japan) at 500 Hz and digitized at a rate of 10 kHz through an interface (Digidata 1200A; Axon). The acquired data were analyzed with an acquisition/analysis software (pCLAMP6; Axon) on an IBM-compatible personal computer. Current traces of downward deflections represented inward currents. Single channel conductance was determined by linearly fitting the current-voltage curve between two appropriate potentials. To examine the gating kinetics, dwell-time histograms were fitted with the Levenberg-Marquardt least square fitting algorithm. The best fitting was validated by using a fit-comparison option of the pCLAMP6 software. Channel activity was determined by NP_O , which was calculated from amplitude histograms as

$$NP_O = \sum_{n=1}^N n \cdot t_n$$

where N is the maximum number of channels observed in the patch, P_O is the open probability, n is the

number of channels observed at the same time, and t_n is the fractional time spent at each current level. For convenience, normalized channel activity was calculated by dividing $NP_{O_{exp}}$ with $NP_{O_{cont}}$ to compare the channel activity in experimental conditions with controls. $NP_{O_{cont}}$ and $NP_{O_{exp}}$ are the channel activities under control and experimental conditions, respectively. $NP_{O_{cont}}$ was determined from a 20 s sampling period just before the test substances were added. $NP_{O_{exp}}$ was determined from a 10 s sampling period 15 to 90 s after adding substances.

Statistics. The data are expressed as mean $\pm$ SE from 6 to 16 different patches. A statistical comparison was made with Student's *t*-test or ANOVA in conjunction with a Bonferroni *t*-test. A *p* value less than 0.05 was considered to be significant.

RESULTS

Basic properties of the K⁺ channel in human proximal tubule cells

In cell-attached patches under control conditions, one type of K⁺ channel was predominantly observed. Its typical single channel recording is shown in Fig. 1A. The mean P_O of this K⁺ channel was 0.84 ± 0.03 ($n=22$). Analyses of dwell-time histograms revealed that the gating kinetics of this K⁺ channel was characterized by one closed and two open time constants (Fig. 1B). The open state with a slow time constant ($\tau_{o2}=46.7$ ms, 88%) predominated over that with a fast one ($\tau_{o1}=1.5$ ms, 12%).

The current-voltage (*I-V*) relationship was examined in inside-out patches, where 1 mM ATP was present in the bath (see below), under the symmetrical ionic condition. As shown in Fig. 2, this K⁺ channel

exhibited an inward rectification. It is evident that the amplitude of inward currents is greater than that of outward currents (Fig. 2A). Although large outward spikes were voltage-dependently observed in almost all patches examined, their origins were not resolved. Data were pooled from experiments similar to that of Fig. 2A, and currents were plotted as a function of $-V_p$ to yield an *I-V* curve (Fig. 2B). The inward slope conductance measured from -90 to -30 mV of $-V_p$ was 42.4 ± 2.8 pS ($n=8$), and the outward slope conductance from $+15$ to $+60$ mV was 7.1 ± 1.4 pS ($n=8$). An asymmetrical K⁺ condition was also employed to estimate the K⁺ selectivity of this channel. Reducing the internal K⁺ concentration to 30 mM, from 145 mM, caused a shift of the *I-V* curve, resetting the reversal potential at $+31$ mV (Fig. 2B). Based on this observation, we calculated that the K⁺-to-Na⁺ permeability ratio (P_K/P_{Na}) was 8.1, using the Goldman-Hodgkin-Katz voltage equation.

Effect of internal ATP on channel activity

As shown in Fig. 3A, channel activity rapidly declined ("channel rundown") following excision of the patch membrane into the inside-out configuration. When ATP was added to the bath immediately after rundown, channel activity could be restored and was further enhanced by increasing ATP. The amplitude of a single channel current was unaffected with graded doses of ATP. The dose-response relationship of ATP is summarized in Fig. 3B. ATP stimulated channel activity in a dose-dependent manner, with a maximum plateau observed at 3 to 10 mM. No inhibitory effect was observed within the dose range we employed.

To examine whether the ATP-dependent channel activation involved ATP-hydrolysis, we tested the ef-

Fig. 1. Basic electrical behavior of the inwardly rectifying K⁺ channel in RPTECs. **A:** A typical current trace recorded from a cell-attached patch under the basal condition. The V_p was held at 0 mV. The dotted line indicates the closed channel level. The nick on the left corresponds to the open channel level. **B:** Analyses of gating kinetics. Dwell time histograms were obtained from the current recording shown in A, with the time intervals being logarithmically binned [35]. Histograms were fitted as described in MATERIALS AND METHODS to give one closed time constant of $\tau_c=2.6$ ms and two open time constants of $\tau_{o1}=1.5$ ms and $\tau_{o2}=46.7$ ms.

Fig. 2. Current-voltage relationships of the K^+ channel in inside-out patches.

A: Representative current traces recorded from the same inside-out patch under the symmetrical K^+ condition in which both bath and pipette contained 145 mM K^+ . The bath solution contained 1 mM ATP to maintain channel activity. The V_p was varied as depicted on the panel. Closed and open channel levels are indicated by dotted lines and nicks, respectively. The large outward spikes observed at $-V_p = +30$ and $+45$ mV seemed to originate in another channel. **B:** Current-voltage curves were obtained from 8 inside-out patches under the symmetrical K^+ condition (closed circles) and 6 inside-out patches under the asymmetrical condition, where bath K^+ was reduced to 30 mM by substituting Na^+ (open circles).

Fig. 3. Dose-dependent stimulation of channel activity by internal ATP.

A: Changes in the channel activity in response to graded doses of internal ATP. Current traces were recorded from the same inside-out patch under the symmetrical ionic condition with the V_p held at $+50$ mV. In this patch, K^+ current disappeared 42 s after patch excision, and 0.1 mM ATP was added to the bath 10 s after the channel rundown. A stepwise addition of ATP up to 10 mM was done every 30 to 40 s. Closed and open channel levels are indicated by dotted lines and nicks, respectively. **B:** The dose-response curve of internal ATP was plotted by using data from 9 inside-out patches.

fect of a nonhydrolyzable ATP analog, AMP-PNP, on channel activity in inside-out patches. In the absence of ATP, channel activity was declined as mentioned above, and the addition of AMP-PNP (1 to 3 mM) failed to restore the channel activity (data not shown). Furthermore, we explored the effect of AMP-PNP in the presence of 1 mM ATP. As shown in Fig. 4, channel activity maintained with 1 mM ATP in an inside-out patch was not affected by AMP-PNP (3 mM).

Regulation by cAMP/PKA-mediated processes

Since it was revealed that the nonhydrolyzable ATP analog had no effect on channel activity in these experiments, we speculated that the ATP action might be induced by phosphorylation processes of the channel

and/or its related proteins. To test this hypothesis, a series of experiments were performed. First the effects of protein-kinase inhibitors, which could permeate through the membrane, were examined in cell-attached patches. The results are shown in Fig. 5. A nonspecific protein kinase inhibitor, K252a (1 μ M), caused a marked reduction of channel activity in a reversible manner (Fig. 5A). The addition of a PKA-specific inhibitor, KT5720 (500 nM), similarly suppressed the activity (Fig. 5B). The suppressive effects of these inhibitors are summarized in Fig. 5C, which shows that K252a of 1 μ M and KT5720 of 500 nM caused 73 and 56% reductions of channel activity, respectively. No significant difference was noted between the suppressive effects of K252a and KT5720.

We next examined the effect of cAMP in cell-

Fig. 4. Effect of a nonhydrolyzable ATP analog, AMP-PNP, on the channel activity maintained with ATP. The current traces shown were recorded from the same inside-out patch under the symmetrical ionic condition with the V_p held at +50 mV. The top trace represents current recording at the initial control period, during which 1 mM ATP alone was present in the bath. The lower three traces represent current recordings at the indicated periods after 3 mM AMP-PNP was added to the bath in the presence of 1 mM ATP. Closed and open channel levels are indicated by dotted lines and nicks, respectively. Similar results were obtained from 8 additional inside-out patches.

attached patches, using a membrane-permeable analog, 8Br-cAMP. As shown in Fig. 6A, 100 μ M 8Br-cAMP reversibly stimulated the channel. The channel activity in response to 8Br-cAMP was about twofold higher than the control activity (Fig. 6C). Finally, the direct effect of PKA was explored in inside-out patches (Fig. 6, B and D). When 100 U/ml PKA-CS was added to the bath in the presence of a submaximal dose of ATP (1 mM), the channel activity exhibited a fourfold increase on average. The stimulatory effect of PKA-CS was also reversible.

Sensitivity to internal pH

The effects of varying internal pH on channel activity were examined in inside-out patches (Fig. 7). All the bath solutions contained 1 mM ATP. Channel activity observed in the control KCl solution of pH 7.3 was completely suppressed when the bath was replaced with an acid solution of pH 6.8. In contrast, raising the internal pH to 7.8, from 7.3, caused activation of this channel. Both the inhibitory and stimulatory effects of internal pH were reversible. Changes in internal pH induced no appreciable alteration in the

Fig. 5. Channel inhibition by membrane-permeable inhibitors of protein kinases. Representative current traces showing channel inhibitions by 1 μ M K252a, a nonspecific protein kinase inhibitor (A) and 500 nM KT5720, a specific inhibitor of PKA (B). Current recordings were performed in cell-attached patches at $V_p=0$ mV, and each chemical was added to the bath. Dotted lines indicate the closed channel level. Nicks on the left denote open channel levels. C: Summary of the effects of protein kinase inhibitors. Data were obtained from 8 cell-attached patches for K252a and 12 cell-attached patches for KT5720. ** Significant difference at $p<0.01$ compared with respective initial control levels.

current amplitude (Fig. 7A). The summarized data of pH effects on channel activity are shown in Fig. 7B and clearly demonstrate that changes in internal pH significantly affected channel activity.

DISCUSSION

Our study revealed that an inwardly rectifying K⁺ channel with an inward conductance of 42 pS was present in cultured human proximal tubule cells. In cell-attached patches under the control condition, it was the most frequently observed K⁺ channel and was usually highly active, with the mean P_o being 0.84. Therefore it is likely that this K⁺ channel would be a major contributor to the formation of the membrane potential in these cells.

Fig. 6. Channel activation by 8Br-cAMP and PKA-CS.

A: Changes in the channel activity in response to a membrane permeable cAMP analog, 8Br-cAMP, added to the bath at 100 μ M. Currents were recorded from a cell-attached patch at $V_p=0$ mV. The dotted line indicates the closed channel level. Nicks on the left denote open-channel levels. **B:** Direct stimulatory effect of PKA-CS added at 100 U/ml to the bath in which 1 mM ATP was present. Currents were recorded from an inside-out patch under the symmetrical ionic condition with the V_p held at +50 mV. **C:** Summary of the effects of 8Br-cAMP on channel activity in cell-attached patches ($n=16$). **D:** Summary of the effects of PKA-CS on channel activity maintained with 1 mM ATP in inside-out patches ($n=10$). * Significant difference at $p<0.05$ compared with respective initial control levels. The control for PKA-CS denotes the channel activity in the presence of 1 mM ATP alone.

At present, little is known about the native K^+ channels in human kidney tubules, except one intriguing report by Hirsch *et al.* [9]. They identified and characterized a novel nonrectifying K^+ channel of 65 pS in immortalized human proximal tubule cells, which was regulated by cGMP. Again, they reported that this K^+ channel was the most prominent one in these cells. We could not detect this 65 pS K^+ channel in RPTECs, however, at least in control cell-attached patches. This discrepancy is probably due to differences in the culture systems employed. The immortalized human proximal tubule cells were established by continuously exposing human kidney cortex explants

to $NiSO_4$ [18]. This procedure brought about chromosomal abnormalities in these cells, though they still retained histochemical and functional characteristics of proximal tubule cells. Thus it is possible that K^+ channels observed in the immortalized cells are not expressed in normal cells.

The activity of the inwardly rectifying K^+ channel identified in this study was affected by internal ATP concentrations (Fig. 3) and pH values (Fig. 7). The susceptibility to ATP and pH is a common feature of K^+ channels in the basolateral membrane of proximal tubule cells [1]. Thus it seems very likely that the inwardly rectifying K^+ channel in the surface membrane of RPTECs would correspond to the K^+ channel of basolateral origin, though we did not perform precise morphological analyses.

It has been reported that some K^+ channels in various nephron segments require ATP to be active [11–13, 19–21]. Their activities rapidly decline (run down) after excision of the patch membrane into the inside-out configuration in the absence of internal ATP. With regard to the mechanism of rundown, several reports suggested that it would result from dephosphorylation processes induced by membrane-bound phosphatases [22, 23]. These ATP-regulated K^+ channels are divided into two types in terms of the mode of ATP action, i.e., ATP-sensitive (K_{ATP}) and ATP-dependent K^+ channels [24]. The former requires submillimolar concentrations of ATP to maintain their activities, but millimolar ATP causes channel inhibition [11, 19–21]. In contrast, the latter is not inhibited by millimolar ATP [12, 13]. It has been suggested that the channel activation by ATP after rundown would result from rephosphorylation by membrane-bound protein kinases [21, 23, 25], whereas the ATP-induced inhibition of K_{ATP} channels would be caused by the direct binding of ATP to the channel or associated protein without phosphorylation [26, 27].

As described above, the activity of the inwardly rectifying K^+ channel in RPTECs rapidly disappeared upon excision of the patch membrane and was thereafter restored when ATP was added to the bath. ATP stimulated channel activity in a dose-dependent manner, even at the highest dose of 10 mM. These data suggest that the K^+ channel in RPTECs was not an ATP-sensitive K^+ channel, but an ATP-dependent channel, although a possibility that the K^+ channel had direct ATP-binding sites could not be excluded. Moreover, evidence that a nonhydrolyzable ATP analog, AMP-PNP, did not affect channel activity either in the presence or absence of ATP indicates the requirement of ATP-hydrolysis for channel activation. Thus together with reports by other investigators [21,

Fig. 7. Effects of internal pH on channel activity. **A:** Variations in the channel activity in response to different internal pH. Current traces were recorded from the same inside-out patch under the symmetrical ionic condition in the presence of 1 mM ATP. The V_p was held at +50 mV. Closed and open channel levels are indicated by dotted lines and nicks, respectively. **B:** Summary of the effects of internal pH. Data were obtained from 9, 11, and 10 inside-out patches for pH 6.8, 7.3, and 7.8, respectively. *,** Significant differences at $p < 0.05$ (*) and $p < 0.01$ (**) compared with respective initial control levels.

23, 25], it was strongly suggested that the effect of ATP was mainly mediated by phosphorylation processes where ATP served as a phosphoryl group donor. The suppressive effect of a nonspecific protein kinase inhibitor in cell-attached patches also supported the notion that protein kinase-mediated phosphorylation would play an important role in the channel regulation. Furthermore, a PKA-specific inhibitor suppressed the channel activity and 8Br-cAMP stimulated it in cell-attached patches (Figs. 5, 6). Thus it is conceivable that the activity of the K⁺ channel in RPTECs was regulated at least in part by PKA-mediated phosphorylation. This was finally confirmed by the direct channel activation by PKA-CS in inside-out patches in the presence of ATP (Fig. 6). The stimulatory effect of PKA is consistent with the previous reports for K⁺ channels in proximal tubule cells of other species, such as salamander [28] and opossum [25].

Many investigators have reported that protein kinases could modulate activities of renal K⁺ channels [3]. Although the precise molecular mechanisms of phosphorylation processes are not fully understood at present, several new findings have come about. Recently it was reported that interaction between ROMK1 channel and a membrane lipid, phosphatidylinositol 4,5-bisphosphate, would be essential for the channel activation by PKA [29]. Another report showed that A-kinase anchoring protein mediates the effect of PKA on the ROMK channel [30]. The relevance of these findings to the K⁺ channel in RPTECs is subject to further investigation.

Regarding the mechanisms of the pH effect, we could provide no direct evidence in this study. Intracellular protons might directly interact with the channel, since putative proton binding sites were identified

in the ROMK channel protein [31, 32]. Alternatively, pH effects may indirectly be produced via modulation of the activities of protein kinases and phosphatases. Like ATP, intracellular pH is regarded as one of the important mediators that enable functional cross-talk between apical and basolateral membranes of proximal tubule cells [16, 33]. The interrelation between ATP regulation and intracellular pH has also been suggested [34]. Thus pH- and ATP-mediated regulation of the inwardly rectifying K⁺ channel in RPTECs strongly suggests that this channel would be a physiologically important K⁺ channel in human proximal tubule cells.

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