Dual Effect of Adenosine Triphosphate on the Apical Small Conductance K^+ Channel of the Rat Cortical Collecting Duct

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ABSTRACT We used the patch-clamp technique to study the effects of ATP on the small-conductance potassium channel in the apical membrane of rat cortical collecting duct (CCD). This channel has a high open probability (0.96) in the cell-attached mode but activity frequently disappeared progressively within 1–10 min after channel excision (channel “run-down”). Two effects of ATP were observed. Using inside-out patches, low concentrations of ATP (0.05–0.1 mM) restored channel activity in the presence of cAMP-dependent protein kinase A (PKA). In contrast, high concentrations (1 mM) of adenosine triphosphate (ATP) reduced the open probability (P_o) of the channel in inside-out patches from 0.96 to 0. 1.2 mM adenosine diphosphate (ADP) also blocked channel activity completely, but 2 mM adenosine 5'-[β,γ -imido]triphosphate (AMP-PNP), a nonhydrolyzable ATP analogue, reduced P_o only from 0.96 to 0.87. The half-maximal inhibition (K_i) of ATP and ADP was 0.5 and 0.6 mM, respectively, and the Hill coefficient of both ATP and ADP was close to 3. Addition of 0.2 or 0.4 mM ADP shifted the K_i of ATP to 1.0 and 2.0 mM, respectively. ADP did not alter the Hill coefficient. Reduction of the bath pH from 7.4 to 7.2 reduced the K_i of ATP to 0.3 mM. In contrast, a decrease of the free Mg^{2+} concentration from 1.6 mM to 20 μ M increased the K_i of ATP to 1.6 mM without changing the Hill coefficient; ADP was still able to relieve the ATP-induced inhibition of channel activity over this low range of free Mg^{2+} concentrations. The blocking effect of ATP on channel activity in inside-out patches could be attenuated by adding exogenous PKA catalytic subunit to the bath. The dual effects of ATP on the potassium channel can be explained by assuming that (a) ATP is a substrate for PKA that phosphorylates the potassium channel to maintain normal function. (b) High concentrations of ATP inhibit the channel activity; we propose that the ATP-induced blockade results from inhibition of PKA-induced channel phosphorylation.

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INTRODUCTION

The renal CCD is one of the main sites for K^+ secretion (Giebisch, 1987; Field and Giebisch, 1990). K^+ secretion is accomplished by two separate transport steps (Koeppen et al., 1983). First, K^+ is pumped actively into cells through the Na^+-K^+ -ATPase. In a subsequent step, K^+ diffuses from cell into lumen through K^+ channels along a favorable electrochemical gradient.

Two types of K^+ channels have been found in the apical membrane of mammalian renal CCDs: the "Maxi" K^+ and the small-conductance K^+ channel (Hunter et al., 1984; Frindt and Palmer, 1987, 1989; Geibel et al., 1990; Wang et al., 1990a). The "Maxi" K^+ channel has a low open probability under physiological conditions, and the incidence of the channel (channel density) is very infrequent (Frindt and Palmer, 1989; Wang et al., 1990a).

In contrast, the small-conductance K^+ channel displays a high open probability and a high incidence (32% in control animals) that is significantly increased (64%) in animals on a high potassium diet (Wang et al., 1990a). The conductance of the small K^+ channel is inward-rectifying with an inward slope conductance of 35 pS and outward slope conductance of 15 pS. The channel sustains Rb^+ currents (Frindt and Palmer, 1989), consistent with results of microperfusion studies in which Rb^+ secretion has been demonstrated in the CCDs (Warden et al., 1989). Furthermore, the insensitivity of channel activity to 5 mM tetraethyl ammonium (TEA) is in accord with the lack of effects of similar concentrations of TEA on transepithelial potential of isolated rabbit CCDs (Frindt and Palmer, 1987). These channel properties permit the conclusion that the small-conductance K^+ channel is responsible for the apical K^+ conductance of principal cells and for K^+ secretion into the mammalian renal CCDs. The channel is blocked by Ba^{2+} and is pH sensitive. A previous study showed that the open probability of the small-conductance K^+ channel was sensitive to ATP and to the ATP/ADP ratio (Wang et al., 1990a).

ATP-sensitive potassium channels have been found in various mammalian tissues, including cardiac and skeletal muscle (Noma, 1983; Spruce et al., 1985; Findlay, 1987a; Weik and Neumcke, 1989; Woll et al., 1989; Nichols and Lederer, 1990), hypothalamic neurons (Ashford et al., 1990), pancreatic β -cells (Cook and Hales, 1984; Findlay et al., 1985; Mislér et al., 1986; Ohno-Shosaku et al., 1987; Ribalet and Ciani, 1987), smooth muscle (Standen et al., 1989), and renal tubule cells (Bleich et al., 1990; Wang et al., 1990b). A intensive review of the ATP-sensitive channel was recently published (Ashcroft and Ashcroft, 1990). Studies of cell types other than renal tubule cells show that the blocking effect of ATP does not result from the molecule entering the channel pore (Sturgess et al., 1986), since channel inhibition is not voltage dependent. In cells of the early distal tubule of the *Amphiuma* kidney, ATP alters the calcium sensitivity of a large-conductance K^+ channel (Hunter and Giebisch, 1988). However, the channel described in the present study is not sensitive to Ca^{2+} , and the inhibition by ATP must thus be mediated by a different mechanism. Recently, it was suggested that high concentrations of ATP reduce channel activity by facilitating the binding of a protein kinase inhibitor to cyclic AMP (cAMP)-dependent PKA, thus blocking the activity of PKA. These events could reduce the PKA-induced phosphorylation of the potassium channel that is thought to be essential for maintaining channel opening (Ribalet et al., 1989).

In the present study we investigated the mechanism by which ATP regulates the small-conductance K⁺ channel and found that ATP has a dual effect on the channel activity. The results suggest that (a) ATP in low concentrations is a substrate for PKA that phosphorylates the channel protein and (b) ATP at high concentrations inhibits PKA-induced phosphorylation of the channel.

Parts of this work have been presented in abstract form to the 74th Annual Meeting (1990) of the Federation of American Societies for Experimental Biology, Washington, DC.

METHODS

Preparation of CCD Tubules

Sprague-Dawley rats of either sex (80–120 g), raised free of viral infections (Taconic Inc., Germantown, NY), were maintained on a high-potassium (10%) diet (Taklad Research Diet, Madison, WI) for 10–14 d before use to increase the density of the small-conductance K⁺ channels. A previous study showed that the characteristics of the small-conductance K⁺ channel of the rat on a high-potassium diet were not different from those obtained in tubules harvested from animals on a normal diet (Wang et al., 1990a). Methods of tubule preparation were similar to those previously described (Wang et al., 1990a). Cortical collecting tubules were dissected at 22°C in HEPES-buffered NaCl Ringer solution, which contained (in millimolar) NaCl 135, KCl 5, MgCl₂ 1.8, CaCl₂ 1.8, glucose 5, and HEPES 10 (pH 7.4 with NaOH), and transferred onto a 5 × 5-mm coverglass coated with CELL-TAK (Biopolymers, Inc., Farmington, CT) to immobilize the tubules. The coverglass was placed in a chamber mounted on an inverting microscope (model IM35, Carl Zeiss, Inc., Thornwood, NY), and the tubules were superfused with HEPES-buffered NaCl Ringer solution. The tubules were cut open with a sharpened micropipette to expose the apical membrane. Two types of cells can be identified in CCDs (O'Neil and Hayhurst, 1985): i.e., principal and intercalated cells. By means of Hoffman modulation optics (Modulation Optics Inc., Greenvale, NY), intercalated cells of rat CCD can be seen to appear irregular and protrude into the lumen. In contrast, principal cells display a regular hexagonal shape and are flat. In this study only the principal cells were patched.

Experimental Media

During the experiments the tubules were superfused with HEPES-buffered Ca²⁺-free Ringer containing (in millimolar) NaCl 135, KCl 5, MgCl₂ 1.8, EGTA 1, glucose 5, and HEPES 10 (pH 7.4 or 7.2). In some experiments MgCl₂ was removed or reduced to 50 μM (20 μM free Mg²⁺) as indicated in the text. The pipette solution contained 140 mM KCl, 1.8 MgCl₂, 1 mM EGTA, and 10 mM HEPES (pH 7.4). Stock solutions of 10 mM ATP, ADP (Sigma Chemical Co., St. Louis, MO), and AMP-PNP (Boehringer Mannheim Diagnostics, Inc., Houston, TX) were made with the corresponding bath solutions and kept at 4°C. The cAMP-dependent PKA, PKA catalytic subunits, and the PKA inhibitor (Sigma Chemical Co., St. Louis, MO) were dissolved in 200 μl of the bath solution before the experiments and kept at 4°C. The concentrated stock solution was diluted by adding appropriate aliquots directly to the bath chamber. Experiments were performed at 37°C.

Patch-Clamp Technique

Patch-clamp experiments were performed according to the methods of Hamill et al. (1981). Patch-clamp electrodes were pulled from glass capillaries (Drummond Scientific Co., Broomall, PA) and had resistances of 4–6 MΩ when filled with 140 mM KCl. Recordings were made using

a patch-clamp amplifier (L/M-EPC7, List Medical, Darmstadt, FRG), and single-channel currents were low-pass filtered at 1 kHz using an eight-pole Bessel filter (902LPF, Frequency Devices, Inc., Haverhill, MA). The recordings were digitized at a sampling rate of 44 kHz using a modified Sony PCM-501ES pulse-code modulator and stored on videotape (Sony SL-2700). For analysis, the data were transferred to an IBM-compatible AT hard disk at a rate of 5 kHz and analyzed using the pClamp software system (Axon Instruments, Inc., Burlingame, CA). Since more than three levels of channel opening were obtained in some of patches, manual analysis was carried out for these multiple level channels to assess channel open probabilities.

Single-channel slope conductance was calculated by recording single-channel currents at a number of different holding potentials (from -30 to 30 mV). Open probability (P_o) of individual K^+ channels was determined from data samples of 1–2-min duration as follows:

$$P_o = (1/N) \cdot \Sigma(t_1 + t_2 + \cdots t_n) \quad (1)$$

where N was the number of channels, i.e., the maximum number of superpositions of current level seen in the patch, and t the fractional open time spent at each of the observed current levels.

Statistics

Data are presented as mean $\pm$ SD. Where appropriate, Student's t tests for paired and unpaired data were used to assess significance of difference.

RESULTS

Effect of Low Concentrations of ATP on Channel Activity

We confirmed in the present experiments that the open probability of the small-conductance K^+ channel in cell-attached patches was close to 1 and was voltage independent (Frindt and Palmer, 1989; Wang et al., 1990a). Frequently, channel activity decreased progressively over a 10-min period after excision. In some instances channel activity disappeared immediately. Occasionally (in 11% of patches), application of a small amount of ATP (0.05–0.1 mM) to the bath maintained channel activity at levels in the cell-attached configuration.

Fig. 1 shows the result from an experiment in which the bath solution was changed from an ATP-containing solution to an ATP-free solution. It is apparent that channel "run-down" took place and that addition of 0.1 mM ATP reversed the decline of channel activity. Similar phenomena were reported in excised patches from pancreatic β cells (Findlay and Dunne, 1986; Misler et al., 1986; Ohno-Shosaku et al., 1987; Ribalet et al., 1989). These observations suggested that phosphorylation of the K^+ channel or of a closely related protein was essential for maintaining normal channel function (Mislser et al., 1986; Ohno-Shosaku et al., 1987; Ribalet and Ciani, 1987). Kozlowski and Ashford (1990) have reported that removal of bath Mg^{2+} prevented the ATP-sensitive K^+ channel of inside-out patches from running down in CRI-G1 insulin-secreting cells. However, the K^+ channel run-down in the present study was Mg^{2+} independent, since total removal ($n = 5$) or reduction of the bath Mg^{2+} ($20 \mu M$) failed to prevent the K^+ channel run-down. Application of a low concentration of ATP was essential to maintain channel activity also in the $20 \mu M$ Mg^{2+} bath (4 of 32 patches, data not shown).

FIGURE 1. Effect of 0.1 mM ATP on channel activity. The channel recording was obtained in an inside-out patch with 140 mM KCl, 1.8 mM MgCl₂, 1 mM EGTA, and 10 HEPES (pH 7.4) in the pipette and 5 mM KCl, 135 mM NaCl, 1.8 mM MgCl₂,

1 mM EGTA, and 10 HEPES (pH 7.4) in the bath. The holding potential was 10 mV and experiments were performed at 37°C. The downward deflection indicates an inward K⁺ currents and the closed state is indicated by C. The ATP concentration in the bath is indicated on the top of the tracing.

Effect of PKA on Channel Activity

Fig. 2 summarizes results that demonstrate the effect of ATP and PKA on K⁺ channel activity. We observed that a low concentration of ATP (0.05–0.1 mM) alone frequently failed to restore channel activity in inside-out patches (Fig. 2, trace 2), suggesting that in the majority of inside-out patches a component that induces phosphorylation of the channel using ATP as substrate is absent. Inspection of Fig. 2 demonstrates that application of 0.1 mM cAMP to the bath partially restored channel activity (trace 3). This indicates that a cAMP-dependent protein kinase could be involved in restoring channel activity. This hypothesis was tested directly by exploring the effect of exogenous PKA. Fig. 2 illustrates that in the presence of both 0.1

FIGURE 2. Effect of PKA on channel activity. The channel recording was made in an inside-out patch with the same pipette and bath solutions as described in Fig. 1. The holding potential was 10 mV and experiments were performed at 37°C. Trace 1: ATP-free bath. Trace 2: 30 s after application of 0.1 mM ATP in the bath. Trace 3: 20 s after adding 0.1 mM cAMP. Trace 4: immediate response to 1 μ M cAMP-dependent PKA. Trace 5: 10 min after using PKA. Traces 6 and 7: 5 and 60 s, respectively, after adding 10 μ M PKA inhibitor to the bath. Trace 8: 30 s after changing the bath solution to the ATP and enzyme-free Ringer solution. The channel closed state is indicated by C.

mM ATP and 0.1 mM cAMP adding 1 μ M cAMP-dependent PKA to the bath dramatically increased the channel's open probability (traces 4 and 5). Thus, when 1 μ M PKA (or 20 U/ml of PKA catalytic subunit) was added within 60 s after channel run-down, restoration of channel activity was seen in 25 of 27 membrane patches. However, when the enzyme was added after 60 s, only 4 of 22 membrane patches with run-down channels responded to the PKA or PKA catalytic subunit. Traces 6 and 7 of Fig. 2 demonstrate that the effect of exogenous PKA on channel activity was partly antagonized by 20 μ M PKA inhibitor, and that removal of the exogenous PKA and ATP led to channel run-down trace (8). PKA was also able to restore channel activity of two run-down channels in the presence of 0.1 mM ATP and 20 μ M free Mg^{2+} (data not shown).

Although the exogenous PKA-induced phosphorylation modulated the activity of the small-conductance K^+ channels, the effect of a native enzyme that similarly could affect the channel remained to be demonstrated. Fig. 3 shows a representative experiment in which 20 μ M PKA inhibitor was added to the bath in an inside-out patch in the presence of 0.1 mM ATP. In four experiments 20 μ M PKA inhibitor reduced the channel open probability from 0.96 to 0.63 (Table I). This result suggests strongly that endogenous PKA plays an important role in the modulation of channel activity. Ribalet et al. (1989) reported that the blocking effect of the PKA inhibitor on ATP-sensitive channel was ATP concentration dependent, and that the effect of the inhibitor increased with ATP concentration. Accordingly, we investigated the effects of the PKA inhibitor on channel activity in the presence of 0.1, 0.4, or 0.6 mM ATP (Table I). As expected, application of 20 μ M PKA inhibitor in inside-out patches to the bath reduced the channel open probability by 32–34% of the control value, but increasing the concentration of ATP did not enhance the inhibiting effect. The exogenous PKA inhibitor induced blockade of the small-conductance K^+ channel is not ATP concentration dependent at ATP concentrations above 0.1 mM.

Blocking Effect of ATP and ADP on Channel Activity

Whereas ATP at low concentrations serves as a substrate for cAMP-dependent PKA that phosphorylates the channel to maintain channel activity, high concentrations of ATP (>0.1 mM) inhibit the small-conductance K^+ channel in excised patches from the apical membrane of rat CCD. Fig. 4 shows a representative tracing from four experiments using inside-out patches in which the effects of progressively increasing ATP concentrations from 0.1 to 0.2, 0.3, 0.6, 0.8, and 1 mM were tested. Half-maximal inhibition of channel activity was observed at 0.5 mM ATP and complete inhibition was accomplished by 1 mM ATP (lower right part of Fig. 4). The half-maximal dose of channel inhibition in the present study was higher than that reported in some nonrenal cells (Ohno-Shosaku et al., 1987; Ribalet and Ciani, 1987), but is well within the physiological range of renal tubular ATP concentration (Uchida and Endou, 1988; Torikai, 1989). The ATP-induced inhibition was full reversible, since changing the bath to 0.1 mM ATP solution restored channel activity.

The main characteristic of the channel kinetics during ATP inhibition was the appearance of long periods of closures between the "normal" opening and closing events (Fig. 5). Inspection of the data in Fig. 5 shows that in control conditions the channels of inside-out patches displayed one open state with a mean lifetime of 25 ms

FIGURE 3. Effect of PKI on channel activity. A channel recording was made in inside-out patch with the same pipette solution as in Fig. 1 and the same bath solution as in Fig. 1, except for the presence of 0.1 mM ATP in the bath. The upper part of the figure shows the slow time course of the recordings and the four parts indicated by a short bar were displayed in fast time resolution (lower part of the figure). 20 μ M PKA inhibitor was added to the bath as indicated in the figure and the holding potential of the patch was 15 mV. The closed state of the channel is indicated by C.

and one closed state with a mean lifetime of 0.9 ms. Addition of 0.4 mM ATP led to inhibition of channel activity but the channel still displayed one open state with a mean lifetime of 25 ms. However, after ATP addition the channel showed two closed states: i.e., the short closure with the same lifetime of 0.9 ms as in control condition

TABLE I
Effect of the Exogenous PKA Inhibitor on the Channel Open Probability (P_o) at Low or High Concentrations of ATP

	Concentration	
	(0.05–0.1 mM ATP)	(0.4–0.6 mM ATP)
Decrease of the P_o (%)	34 ± 2 ($n = 4$)	32 ± 2 ($n = 3$)

and, in addition, a new long-lasting state of closure in the range between 40 and 2,700 mS. Since the events of the long closure were too few to make an exponential curve fitting, we could only calculate the arithmetic mean closed time of 600 mS for this case.

In addition to ATP, ADP also inhibited the small-conductance K^+ channel. Fig. 6 illustrates a representative recording obtained from an inside-out patch in which an increase of the ADP concentration from 0 to 0.2, 0.6, 0.8, and 1.2 mM in the bath

FIGURE 4. The inhibitory effect of ATP on channel activity. A channel recording was registered at 10-mV holding potential in an inside-out patch with the same bath and pipette solution as in Fig. 1. The dose-response curve for ATP is in the lower right part of the figure. The closed state of the channel is indicated by C.

progressively reduced the open probability of the channel. From four such experiments the concentration of 50% inhibition of the channel was 0.6 mM ADP; 1–1.2 mM ADP totally blocked channel activity. Like the ATP effect, the ADP-induced inhibition of channel activity was full reversible (Fig. 6).

Since 0.05 mM ATP was added to the bath to prevent the channel from running down during the study of the blocking effect of ADP, it was difficult to analyze the kinetics of ADP inhibition without the interference of ATP. To avoid this complica-

tion, the effect of 0.05 mM ATP on the channel activity was ignored when kinetic information of ADP inhibition was analyzed.

To compare the inhibition kinetics of ATP and ADP we used the Lineweaver-Burk equation:

$$1/v = \{K_i/V_{i(\max)}\} \cdot \{1/[S]\} + \{1/V_{i(\max)}\} \quad (2)$$

where $V_{i(\max)}$ is 100% inhibition of the channel activity, S is concentration of ATP, and v is the percentage of channel inhibition calculated by the equation:

$$1 - (P/P_o), \quad (3)$$

FIGURE 5. Histograms of open- and closed-duration of the K⁺ channel in an inside-out patch in the 50 μM ATP bath (the upper panels) and in the 0.4 mM ATP-containing bath (the lower panels). Please note the histogram of long closed duration which is absent in the control condition.

where P is the open probability of the channel in the presence of inhibitor (ATP or ADP) and P_o is the control value. The K_i (the concentration needed for 50% inhibition of the channel activity) of ATP and ADP was almost identical (Fig. 7 A). The Hill plot showed that the Hill number for both ATP and ADP was 3 (Fig. 7 B). These experiments imply that the binding sites for ATP and ADP are the same, and that both ATP and ADP have the same affinity for these binding sites. Although this binding site did not discriminate between ATP and ADP, addition of as much as 2 mM of the ATP analogue AMP-PNP to the bath reduced the channel open probability only by some 10% (from 0.96 to 0.87) in three inside-out patches (data not shown).

FIGURE 6. The inhibitory effect of ADP on channel activity. A channel recording was registered at 10-mV holding potential in an inside-out patch with the same bath and pipette solution as in Fig. 1, except for the presence of 50 μ M ATP in the bath. The dose-response curve for ADP is in the lower part of the figure. The closed state of the channel is indicated by C.

Effect of the ATP/ADP Ratio on Channel Activity

In common with other ATP-sensitive channels (Dunne and Petersen, 1986; Kakei et al., 1986; Misler et al., 1986), we observed that the ATP-induced inhibition can be modulated by ADP. Although ADP inhibits the channel activity of inside-out patches with almost the same K_i as ATP, the effects of ATP and ADP are not additive. In the previous study we have shown that 1 mM ATP-induced inhibition of channel activity was almost fully relieved by acute application 0.5 mM ADP to the bath of an inside-out patch (Wang et al., 1990a). Fig. 8 shows the effect of ATP on the

FIGURE 7. (A) Lineweaver-Burk plot of ATP and ADP. The plot was made by using reciprocal values of ATP (○) or ADP (△) concentration ($1/S$) vs. the reciprocal of the percentage of the channel inhibition ($1/v$) that was calculated by using the equation: $1 - P/P_0$, where P_0 is the channel open probabilities in the control condition (50–100 μ M ATP in the bath) and P is the value in the presence of ATP or ADP, respectively. The slope of the plot represents the value of dose for half-maximal inhibition (K_i). (B) Double logarithmic Hill plot for ATP (○) and ADP (△). The meaning of P and P_0 is the same as in A.

small-conductance K^+ channel in the presence of 0.2 mM ADP in the bath. 1 mM ATP, a concentration that in the absence of ADP fully blocks channel activity, reduced the open probability of the excised apical K^+ channel only by 50% of the control value in the presence of ADP. Table II summarizes the results of experiments in which the inhibitory action of ATP was evaluated at different ADP concentrations. In the presence of 0.2 mM ADP, 1.8–2.2 mM ATP was needed for full inhibition of channel activity, whereas in the presence of 0.4 mM ADP 2.0 mM ATP in the bath was needed to reduce the channel open probability to 50% of the control value. This

FIGURE 8. The effect of ATP on channel activity in the presence of 0.2 ADP. A channel recording was registered at 10-mV holding potential in an inside-out patch with the same bath and pipette solutions as in Fig. 1. The dose response curve for ATP at fixed 0.2 mM ADP is in the lower part of the figure. The closed state of the channel is indicated by C.

compares with an ATP concentration of 0.5 mM in the absence of ADP to achieve half-maximal inhibition.

A Lineweaver-Burk plot showed that, in the presence of 0.2 mM or 0.4 mM ADP, the K_i for ATP inhibition was significantly increased (Fig. 9A). In contrast to the change of K_i , a Hill plot of these data suggested that the binding sites for ATP were not altered in the presence of ADP, since the Hill coefficient for ATP was still 3 (Fig. 9B). To test whether ATP and ADP were two different, exclusive inhibitors for the channel, we used Dixon plots of $1/v$ (v is the percentage of the control value of P_o) versus the concentration of ATP at two fixed concentrations of ADP (Segel, 1976). Fig. 9C demonstrates that the curves shift in a parallel fashion at fixed ADP concentrations of 0.2 and 0.4 mM. This indicates that ATP and ADP cannot combine simultaneously with the target protein.

Role of Mg²⁺ in the ATP Effect on Channel Activity

Removal of Mg²⁺ in pancreatic β cells produces a striking reduction in the ability of total ATP concentration to inhibit channel activity (Dunne et al., 1987; Ashcroft and

TABLE II
Dose of ATP or ADP Concentration (K_i) for 50% Decrease of the Channel Open Probability in Comparison to Control Values

	K_i (pH 7.4)	K_i (pH 7.2)
ATP	0.5 ± 0.04 mM (4)	0.3 ± 0.02 mM (4)*
ATP (20 μ M Mg ²⁺)	1.6 ± 0.1 mM (3)*	—
ADP	0.6 ± 0.04 mM (4)	—
ATP/0.2 mM ADP	1.0 ± 0.06 mM (4)*	—
ATP/0.4 mM ADP	2.0 ± 0.10 mM (3)*	—

Free Mg²⁺ concentration is 1.6 mM in all experiments; the exception is indicated in the table. Values are mean $\pm$ SD; the number of observations is indicated in the parenthesis. * $P < 0.001$.

Kakei, 1989). We have tested the effect of reducing the free Mg²⁺ concentration from 1.6 mM to 20 μ M in the apical K⁺ channel of rat CCD on ATP-induced inhibition. Fig. 10 summarizes three such experiments. It is apparent that, in the bath containing low free Mg²⁺, the K_i for ATP was significantly increased to values close to 1.6 mM (Table II), and that 3–3.5 mM ATP was needed to block the channel fully. However, the Hill coefficient was not altered in the low concentration of free Mg²⁺ (Fig. 10, A and B). This is similar to the results in rat ventricular myocytes in which ATP·Mg is a more effective channel inhibitor than ATP⁴⁻ and ATPH³⁻ (Findlay, 1988a, b). We have also studied the modulatory effect of ADP on the ATP-induced channel inhibition in low concentrations of Mg²⁺. Fig. 11 displays a representative recording of three experiments. At a free Mg²⁺ concentration of 20 μ M, 1 mM ADP almost fully relieved the inhibition of channel activity by 3 mM ATP.

pH Modulation of Channel Activity by ATP

We had demonstrated in a previous study that the small-conductance apical K⁺ channel was exquisitely pH sensitive and that a decrease of bath pH from 7.4 to 6.9

FIGURE 9. (A) Lineweaver-Burk plot of ATP with 0, 0.2, or 0.4 ADP. The plot was made by using the reciprocal of ATP concentration ($1/S$) at different fixed ADP concentrations (○, ADP-free; ●, 0.2 mM ADP; and △, 0.4 mM ADP) vs. the reciprocal of the percent of the channel inhibition ($1/v$) that was calculated by using the equation: $1 - P/P_0$, the meanings of P_0 and P are the same as in Fig. 7 A. (B) Double logarithmic Hill plot for ATP at different fixed ADP concentrations. The meanings of the symbols, P , and P_0 are the same as in Fig. 7 A. (C) Dixon plot using ATP concentration at two fixed ADP concentration (0.2 mM ADP, ●; and 0.4 mM ADP, △) vs. the reciprocal of the ratio of P/P_0 (v).

in inside-out patches led to almost complete cessation of channel activity (Wang et al., 1990a). Since the channel was also ATP-sensitive, we tested in the present study whether the inhibitory effect of protons and ATP was synergetic. Fig. 12 shows a representative recording of four such experiments. Channel activity of an inside-out patch at bath pH 7.2 was decreased in comparison with that at bath pH 7.4. Increasing the bath ATP concentrations from 0.05 to 0.2, 0.4, and 0.6 mM in the acid solution led to a steeper decline of the relation between open probability of the K⁺ channel and [ATP] than that observed at pH 7.4 in the bath solution. Application of

FIGURE 10. (A) Lineweaver-Burk plot of ATP at 20 μ M free Mg^{2+} bath (Δ) and the 1.6 mM free Mg^{2+} bath ($\circ$). The plot was made by using the reciprocal of ATP concentration (1/S) vs. the reciprocal of the percent of the channel inhibition (1/v) that was calculated by using the equation: $1 - P/P_0$. The meanings of P and P_0 are the same as in Fig. 7 A. (B) Double logarithmic Hill plot for ATP at 20 μ M free Mg^{2+} bath (Δ) and containing 1.6 mM free Mg^{2+} bath ($\circ$).

0.6–0.7 mM ATP resulted in full inhibition of channel activity at pH 7.2, compared with a concentration of 1 mM ATP which was necessary at pH 7.4 to block the channel fully. Proton modulation of the response of ATP-sensitive channel to ATP was also reported in heart muscle (Lederer and Nichols, 1989) and in skeletal muscle (Davies, 1990).

Inspection of the Lineweaver-Burk and Hill plots, illustrated in Fig. 13, A and B, indicates that, in a pH 7.2 bath, the K_i for ATP inhibition is reduced (Table II)

without a change in Hill's coefficient. This indicates that the affinity of ATP to the binding sites is increased at low pH without changing the number of binding sites.

Investigation of the Mechanism of ATP Inhibition

Inspection of Fig. 3 suggested that the PKA inhibitor-induced blockade is characterized by an additional longtime closure between "normal" channel activity. This behavior is very similar to the kinetics of ATP-blocked channels. A possible explanation of the ATP-induced inhibition of the channel is that ATP inhibits the PKA-

FIGURE 11. Effect of ADP on ATP-induced channel inhibition. A channel recording was made in inside-out patch with 5 mM KCl, 135 mM NaCl, 50 μ M MgCl_2 (20 μ M free Mg^{2+}), 1 EGTA, and 10 HEPES (pH 7.4) in the bath. The upper part of the figure shows the slow time course of the recordings and the three parts indicated by a short bar were displayed in fast time resolution (lower part of the figure). The concentration of ATP and ADP in the bath is indicated in the top of the figure and the holding potential of the patch was 40 mV. The closed state of the channel is indicated by C.

induced phosphorylation. Inside-out patches were used to test this hypothesis, and Fig. 14 A is a typical recording out of five such experiments. It is apparent that four channels were active in control conditions. An increase of bath ATP concentration to 1.1 mM totally inhibited channel activity. However, the ATP-induced blockade of the channel could be partly overcome by addition of 20 U/ml of the exogenous PKA catalytic subunit. After wash-out the channel activity returned to the control level. A greater increase of bath ATP to 2.1 mM blocked the channel again. However, in the presence of 2.1 mM ATP, addition of the same amount of the PKA catalytic subunit failed to restore the channel activity. This indicates that high concentrations of ATP not only inhibited the endogenous but also exogenous PKA-induced phosphorylation

FIGURE 12. The inhibitory effect of ATP on channel activity at pH 7.2. A channel recording was registered at 10-mV holding potential in an inside-out patch with the same bath and pipette solution as in Fig. 1. The bath pH is indicated in the figure. The concentration response curve for ATP at pH 7.2 is in the lower part of the figure. The closed state of the channel is indicated by C.

of the channel. The ATP inhibition was fully reversible, since channel activity was restored when the bath was changed to control solution.

If a small dose of PKA failed to restore the channel activity owing to the high concentration ATP the inhibited the PKA-induced phosphorylation of the channel, it would be expected that increasing the enzyme concentration could overcome the ATP inhibition. Fig. 14 *B* summarizes the results from three such experiments in which augmenting the dose of PKA catalytic subunit in the bath at a high concentration of ATP of 2.1 mM progressively raised the open probability of the K^+ channels in

FIGURE 13. (A) Lineweaver-Burk plot of ATP at bath pH 7.2 (Δ) and 7.4 ($\circ$). The plot was made by using reciprocal of ATP concentration ($1/S$) vs. the reciprocal of the percent of the channel inhibition ($1/v$) that was calculated by using the equation: $1 - P/P_o$. The meanings of P and P_o are the same as in Fig. 7 A. (B) Double logarithmic Hill plot for ATP at different bath pH (7.4, $\circ$; and 7.2, Δ).

inside-out patches. This suggests that ATP is a competitive inhibitor of the PKA-induced phosphorylation of the channel.

DISCUSSION

ATP and Channel Phosphorylation

ATP-sensitive K^+ channels were first found in ventricular cells of the heart (Noma, 1983), then in skeletal muscle cells (Spruce et al., 1985), pancreatic β cells (Cook and

Hales, 1984; Findlay et al., 1985; Kakei et al., 1986; Misler et al., 1986; Ribalet and Ciani, 1987), amphibian renal tubule cells (Hunter and Giebisch, 1988), arterial smooth muscle (Standen et al., 1989), and recently in mammalian renal tubule cells (Bleich et al., 1990; Wang et al., 1990b). Similar to ATP-sensitive K⁺ channels, in heart muscle and pancreatic β -cell (Findlay and Dunne, 1986; Findlay, 1987a; Ohno-Shosaku et al., 1987; Kozlowski and Ashford, 1990), channel activity of inside-out patches in the present study decreases very rapidly (run-down). Kozlowski and Ashford (1990) have recently observed in CRI-G1 insulin-secreting cells that

FIGURE 14. (A) Effect of PKA on ATP-induced channel inhibition. Channel recording was made in an inside-out patch at 10-mV holding potential with the same pipette solution and the same bath composition as described in Fig. 1. ATP concentration of the bath is indicated on the top of the figure and the closed state is indicated by C. 20 U/ml of PKA catalytic subunit was added as indicated by the arrow. (B) The dose-response curve for PKA catalytic subunit in the presence of 2.1 mM ATP in the bath. Channel open probability of three inside-out patches is plotted vs. the concentration of PKA catalytic subunit (logarithmic scale).

channel run-down can be prevented by perfusing patch membranes with Mg²⁺-free bath solutions. However, run-down of this small-conductance K⁺ channel of rat CCD principal cells is not abolished in Mg²⁺-free bath.

We show in this study that low concentrations of ATP in the bath (<0.1 mM) are essential for maintaining channel activity of inside-out patches. ATP also maintains ATP-sensitive K⁺ channel activity in the presence of Mg²⁺ in an insulin secreting cell line (Findlay and Dunne, 1986; Ribalet et al., 1989), mouse pancreatic β cells (Ohno-Shosaku et al., 1987), and heart muscle cells (Findlay, 1987a). It was

suggested that phosphorylation is involved in the refreshment of channel activity by ATP (Ashcroft and Ashcroft, 1990), since Mg^{2+} is required and neither nonhydrolyzable ATP analogue AMP-PNP nor ADP can substitute for ATP (Misler et al., 1986; Findlay, 1987a; Ohno-Shosaku et al., 1987; Ribalet et al., 1989). Our results of the present study support this view. First, addition of a small amount of ATP (0.05–0.1 mM) to the bath sustained channel activity in 11% of the inside-out patches for >10 min. Secondly, application of exogenous PKA further increased the restoration of channel activity of a “run-down” patch. The time-dependent PKA effect could result from the irreversible conformation change of the channel protein after longtime closure. Similar phenomena have been observed in another ATP-sensitive K^+ channel (Ribalet et al., 1989). Thirdly, the key role of PKA in maintaining the function of the small-conductance K^+ channel was further established by the studies in which use of the PKA inhibitor not only blocked the exogenous PKA-induced effect on the channel activities, but also inhibited the endogenous enzyme activities; the channel open probability was significantly reduced after adding PKA inhibitor to the bath in inside-out patches in which the channel openings were sustained without the exogenous PKA.

In addition to the apical K^+ channel of rat CCD, the PKA-induced phosphorylation modulates the activity of K^+ channels in insulin secreting cells (Dunne, 1989; Ribalet et al., 1989), cardiac ventricular myocytes (Walsh and Kass, 1988), the cells of giant axons of squid (Augustine and Bezanilla, 1990) and neuron cells in *Aplysia* (Scholz and Byrne, 1988).

The PKA-induced modulation of the small-conductance K^+ channel could play an important role in the regulation of K^+ secretion of the rat collecting duct. Schafer and Troutman (1986) have demonstrated that addition of arginine vasopressin (ADH) to the bath solution increased Rb^+ flux from the bath to lumen, an indication that K^+ secretion had been stimulated in the perfused rat CCD tubule. The conductive nature of this ADH-induced K^+ flux was established by the barium sensitivity. In addition, Gapstur et al. (1988) observed in rat collecting tubules that ADH enhanced cAMP binding to regulatory subunits of the cAMP-dependent PKA. These observations are consistent with our results: the ADH-induced rise of cAMP production could stimulate the activity of the cAMP-dependent PKA which in turn would accelerate phosphorylation of the small-conductance K^+ channel in apical membranes of collecting duct cells. An increase of channel activity, or “switching on” of silent channels, could result in the observed augmentation of net K^+ secretion.

Inhibitory Effect of ATP and ATP/ADP Ratio

Inhibitory effects of ATP on K^+ channels were reported in cardiac muscle cells (Noma, 1983; Findlay, 1987a; Lederer and Nichols, 1989; Nichols and Lederer, 1990), pancreatic β cells (Cook and Hales, 1984; Findlay et al., 1985; Kakei et al., 1986; Misler et al., 1986; Ohno-Shosaku et al., 1987; Ribalet et al., 1989) and skeletal muscle cells (Spruce et al., 1985; Woll et al., 1989; Davies, 1990). The ATP concentration for 50% inhibition (K_i) of the channel activity (0.5 mM) in the present study is higher than that of the ATP-sensitive channels observed in pancreatic β cells, cardiac muscle, and skeletal muscle, i.e., type I ATP-sensitive K^+ channel (Ashcroft and Ashcroft, 1990). However, the K_i of the present investigation is well below that of

the other four types of ATP-sensitive K⁺ channel (Ashcroft and Ashcroft, 1990). In addition to ATP, ADP also blocks the small-conductance K⁺ channel and the K_i for ADP (0.6 mM) and the Hill coefficient of 3 are almost identical to the values for ATP. This suggests that ATP and ADP occupy the same binding sites with similar affinity. On the other hand, the ATP analogue (AMP-PNP) is much less potent (10% of ATP effect) in depressing the channel. In other cells it was reported that the ATP-sensitive K⁺ channels were also inhibited by other adenine nucleotides such as ADP, AMP and ATP analogues (Noma, 1983; Ribalet and Ciani, 1987; Spruce et al., 1987). However, ADP is less effective than ATP in cardiac muscle cells and the pancreatic β cells (Noma, 1983; Ribalet and Ciani, 1987), and AMP-PNP is equipotent in inhibiting K⁺ channels of skeletal muscle cells (Spruce et al., 1987). These differences between renal K⁺ channels and those in other tissues could result from a different allosteric structure of the binding protein of the different tissues.

Although both ATP or ADP alone inhibit the channel with similar affinity, the efficiency of ATP to block the channel is significantly reduced in the presence of ADP. A similar phenomena was observed in insulin-secreting β cells (Dunne and Petersen, 1986; Kakei et al., 1986; Misler et al., 1986; Ribalet and Ciani, 1987), in rat ventricular myocytes (Findlay, 1988) and in renal cells of the rabbit thick ascending limb of Henle's loop (Bleich et al., 1990; Wang et al., 1990b). The effect of ADP on ATP-induced inhibition of channel activity is Mg²⁺ dependent in rat ventricular cells and pancreatic β cells, since ADP fails to relieve ATP-induced inhibition of channel activity in inside-out patches in the absence of Mg²⁺ in the bath (Findlay, 1987b, 1988a; Lederer and Nichols, 1989). We were unable to study the effect of ADP on ATP-induced inhibition of channel activity in the complete absence of Mg²⁺, because of frequent channel run-down. However, at Mg²⁺ free concentration as low as 20 μ M, 1 mM ADP could still fully relieve channel inhibition induced by 3 mM ATP. This suggests that the modulatory effect of ADP on ATP-induced inhibition is not dependent on free Mg²⁺ concentration in the range between 20 μ M and 1.6 mM.

The observation that ADP only reduced the K_i value for ATP without affecting $V_{i(max)}$ indicates that ADP is a competitive inhibitor of ATP. Three results suggest that the binding sites of the target protein could be occupied by either three molecules of ATP or three molecules of ADP, but not by mixture of one ATP and two ADP or two ATP and one ADP molecules, respectively, i.e., that these agents imply mutual exclusive inhibition on the binding sites of the K⁺ channel. First, half-maximal inhibition of the channel activity could be induced by combinations of either 1 mM ATP/0.2 mM ADP (1:0.2) or 2 mM ATP/0.4 mM ADP (2:0.4). Secondly, using Dixon plots of $1/v$ versus the concentration of ATP, the curves obtained at two fixed concentrations of ADP are parallel (Segal, 1976). Thirdly, the Hill coefficient for ATP is not changed at different concentrations of ADP. This hypothesis can well explain that in the presence of ADP, the sensitivity of the channel to ATP is reduced, since the binding of three ATP molecules can be achieved only by increasing ATP concentration in the presence of ADP.

Since the intracellular ATP concentration of rat CCDs in physiological conditions is in the range of 2–3 mM (Uchida and Endou, 1988; Torikai, 1989), one would expect that the small-conductance K⁺ channel remains closed at this relatively high intracellular ATP concentration. However, instead of closure the channel open probability is

close to 1. Two important aspects of channel regulation may account for this apparent paradox. First, it is likely that the ratio of ATP/ADP is a more important regulator of the apical K^+ channel than the absolute ATP concentrations. In a previous study (Wang et al., 1990a) we have shown that 0.5 mM ADP can fully restore channel activity blocked by 1 mM ATP. In the present study inhibition of channel activity by 3 mM ATP was almost totally abolished by 1 mM ADP. It was reported that the ratio of ATP/ADP of cortical renal tubules of rabbit kidney in control condition is in the range between 3.1 and 5.8 (Balaban et al., 1980). If the same ratio obtains in the rat CCD principal cells, channel open probability would be expected between 50% and 90%. Secondly, the sensitivity of the small-conductance K^+ channel of CCD principal cells to ATP is reduced in the presence of cAMP-dependent PKA (see Fig. 14). Thus, continuous cAMP production and stimulation of PKA would be expected to increase the K_i value for ATP. Accordingly, these channel modulators are likely to maintain the high open probability of the apical small-conductance K^+ channels of rat CCD principal cells despite of relatively high intracellular ATP concentrations.

The modulatory effect of the ratio of ATP/ADP on channel activity could also play an important physiological role in K^+ channel regulation of rat CCD principal cells. Homeostasis of intracellular potassium concentrations requires that the K^+ influx through the $Na^+-K^+-ATPase$ must match the conductive efflux across the apical membrane. Intracellular ATP and ADP concentrations and their ratio could play an important role in maintaining the balance between cellular metabolism and K^+ secretion in principal cells of the rat CCD. Thus, the stimulation of $Na^+-K^+-ATPase$ activity in the basolateral membrane could consume more ATP and produce more ADP. The reduction of ATP concentration and decrease of the ATP/ADP ratio would then act to increase the open probability of the small-conductance K^+ channel on the apical membrane, raising potassium secretion into the lumen. Reduced turnover rates of the $Na^+-K^+-ATPase$ would induce the opposite effect. Indeed, Balaban et al. (1980) have observed in rabbit cortical tubule that inhibition of $Na^+-K^+-ATPase$ by 0 K^+ solution increases the ratio of ATP/ADP from 3 to 6, that could lead to a significantly reduction of open probability of the ATP-sensitive K^+ channel. However, direct measurement of transport-related ATP levels and their effects upon K^+ channel activities in rat CCD tubule will be necessary to test this hypothesis.

Proton Modulation of ATP-induced Inhibition

The small-conductance K^+ channel in the apical membrane of principal collecting duct cell is a pH-sensitive channel. Decrease of the bath pH from 7.4 to 6.9 in inside-out patches almost completely blocked channel activities (Wang et al., 1990a). We observed in the present study that in the acid pH range the K^+ channel is more sensitive to ATP than in control condition, since the K_i for ATP is significantly reduced from 0.5 to 0.3 mM. Modulation of ATP-sensitive K^+ channels by intracellular protons has been reported in skeletal muscle in which, however, a decrease of intracellular pH markedly reduced the inhibitory effect of ATP on the channels on excised patches (Davies, 1990). The discrepancy between two opposite types of K^+ channel correlates with different functions. A reduced ATP sensitivity of the K^+ channel of skeletal muscles in acid pH could serve to maintain normal excitability of muscle cells during metabolic exhaustion. On the other hand, an increase in the ATP

sensitivity of the apical K⁺ channel of CCD tubule will augment the inhibitory effect of protons in acid pH, thus rendering K⁺ channels more sensitive to changes of the intracellular pH. Modulating the inhibitory effect of ATP by cell pH changes might thus contribute to the regulation of K⁺ secretion in acid-base disturbances.

Mechanism of ATP Inhibition

ATP-induced inhibition is not due to physical obstruction (Sturgess et al., 1986) or channel phosphorylation, since the ATP-induced inhibition is not voltage dependent, and other nucleotides such as ADP can also block channel activity. Dunne et al. (1987) and Ashcroft and Kakei (1989) have proposed that the free ionized forms, ATP⁴⁻ and ATPH³⁻, in pancreatic β -cells are the real active inhibitory species for the ATP-sensitive K⁺ channel. Thus, in the presence of Mg²⁺, low concentrations of ATP are unable to block channel activity, since only few molecules of ATP⁴⁻ and ATPH³⁻ are available. However, an increase of total ATP concentrations results in raising the total concentration of ATP⁴⁻ and ATPH³⁻ and lead to inhibition of channel activity. Consistent with this view is the observation that K_i for ATP is dramatically reduced in the absence of Mg²⁺.

On the other hand, Findlay (1988) has shown that the unionized species of ATP·Mg is a stronger inhibitor of channel activity in heart muscle than ATP⁴⁻ and ATPH³⁻. Similar to the ATP-sensitive K⁺ channel of the ventricular cells, the apical small-conductance K⁺ channel of rat CCD principal cells is also more sensitive to ATP·Mg than ATP⁴⁻ and ATPH³⁻. This view is supported by our demonstration that K_i for ATP increases from 0.5 mM in the presence of 1.6 mM free Mg²⁺ to 1.6 mM in the presence of 20 μ M free Mg²⁺. Therefore, it is unlikely that the ATP·Mg-ATP⁴⁻ mechanism significantly contributes to channel inhibition by high concentrations of ATP in the apical small-conductance K⁺ channel of rat CCD.

With respect to the mechanism of K⁺ channel inhibition by ATP, the following consideration are relevant. Direct binding measurements demonstrate that a peptide (inhibitor protein) is able to bind to the PKA catalytic subunit in the presence of ATP, thus inactivating the enzyme (Whitehouse and Walsh, 1983). Probably the protein inhibitor-cAMP-dependent protein kinase interaction is enhanced significantly, if ATP molecules first bind to the PKA (Whitehouse et al., 1983). Indeed, Ribalet et al. (1989) have recently shown that the efficiency of the inhibitor protein (the same protein as that used by Whitehouse and Walsh) to block ATP-sensitive K⁺ channel of insulin-secreting cells is increased at high ATP concentrations. However, we did not observe that the blocking effect of the inhibitor protein was enhanced in the bath solution containing a high ATP concentration. This discrepancy could result from tissue difference, since the ATP concentration for maintaining the K⁺ channel opening in insulin-secreting cells was ten times lower (5–10 μ M) than the K⁺ channel in the present study (50–100 μ M). The half-maximal concentration needed for ATP to form ATP-PKA, the protein inhibitor, was in the range of 20–60 nM (Whitehouse and Walsh, 1983). Therefore in our experimental conditions even the low ATP concentration (50–100 μ M) may already be at saturating levels to form the ATP-PKA-inhibitor protein. However, it is extremely difficult in the present studies to further reduce bath ATP concentrations to study the synergetic effect of ATP on the

exogenous PKA inhibitor protein, since the channel activities could hardly be sustained at an ATP concentration of less than 50 μ M.

Three observations strongly suggest that the ATP-induced inhibition of K⁺ channel activity resulted from the blockade of PKA-induced phosphorylation of the channel protein: First, ATP-induced channel inhibition was partly relieved by adding exogenous PKA catalytic subunits. Secondly, addition of the same amount of the exogenous PKA catalytic subunits that was able to override 1 mM ATP-induced inhibition failed to overcome 2.1 mM ATP-induced inhibition of the channel. Thirdly, increase of enzyme concentrations progressively restored the channel openings, even in the presence of 2.1 mM ATP. The mechanism by which ATP interferes with the PKA-induced phosphorylation is not clear. One possibility is that PKA activity could be antagonized by some channel-associated internal PKA inhibitor which shows lower affinity to ATP than the peptide used by Whitehouse and Ribalet or alternatively, by a close channel-associated dephosphorylation enzyme, and that this process was accelerated by the presence of ATP. The interfering effect of a high concentration of ATP on the PKA-induced channel phosphorylation also can explain the relieving effect of ADP on the ATP-induced channel inhibition, since both ATP and ADP are mutually exclusive inhibitors and occupation of one of three sites by ADP interferes with the further binding of ATP. Thus, ATP-induced inhibition is relieved.

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