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# Mechanisms Underlying Regulation of a Barium -sensitive $K^+$ Conductance by ATP in Single Proximal Tubule Cells Isolated from Frog Kidney

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**Abstract.** $K^+$ channels play an important role in pump-leak coupling and volume regulation in the renal proximal tubule. Previous experiments have identified a barium-sensitive $K^+$ conductance ($G_{Ba}$) in proximal tubule cells isolated from frog kidneys. In this paper we examine the regulation of $G_{Ba}$ by ATP. $G_{Ba}$ was measured in single cells isolated from frog kidney using the whole-cell patch-clamp technique. $G_{Ba}$ was activated by 2 mM intracellular ATP. This activation was enhanced by inhibition of protein kinase C and attenuated by inhibition of protein kinase A, indicating reciprocal regulation by these kinases. Activation by ATP was reduced in the presence of a hypertonic bath solution, suggesting that cell swelling was required. However, after activation to steady-state, $G_{Ba}$ was not sensitive to cell-volume changes. Hypotonic shock-induced volume regulation was inhibited by barium and quinidine, inhibitors of $G_{Ba}$. The effect of maximal inhibitory concentrations of barium and quinidine on volume regulation was similar and addition of both blockers together did not augment the inhibitory response. $G_{Ba}$ was also activated by ADP, via a mechanism dependent on the presence of $Mg^{2+}$. However, the responses to ADP and ATP were not additive, suggesting that these nucleotides may share a common mechanism of activation. The regulation of $G_{Ba}$ by ATP was biphasic, with a half-maximal activating concentration of 0.89 mM and a half maximal inhibitory concentration of 6.71 mM. The sensitivity to nucleotides suggests that $G_{Ba}$ may be regulated by the metabolic state of the cell. Furthermore, the sensitivity to solution osmolality, coupled with the blocker profile of inhibition of volume regulation, suggests that $G_{Ba}$ could play a role in volume regulation.

**Key words:** ATP-sensitive $K^+$ channel — Proximal tubule — PKA — PKC

## Introduction

The mechanism underlying the reabsorption of $Na^+$ by epithelial cells was first proposed by Koefoed-Johnson and Ussing in the 1960s. In this model, maintenance of a low intracellular $Na^+$ concentration by the basolaterally located $Na^+/K^+$ pump provides the driving force for the uptake of $Na^+$ across the apical membrane of the cell via a number of cell-specific transporters, e.g., $Na^+$ channels, $Na^+$ co-transporters and antiporters. Turnover of the pump subsequently removes $Na^+$ from the cell in exchange for $K^+$, which must then recycle across the basolateral membrane via $K^+$ channels. In addition, there is now evidence that apical $K^+$ channels also play an important role in this model [37]. At steady state the combined activity of these transport mechanisms maintains the intracellular composition and cell volume. However, the rate of transport of $Na^+$ across the apical membrane is dynamic. To prevent changes in cell composition and volume, therefore, the activity of these transport proteins must be precisely matched to the apical transport of $Na^+$. This matching of transport rates at the apical and basolateral membranes is known as pump-leak coupling and results in homocellular regulation [33]. In the renal proximal tubule, stimulation of $Na^+$-coupled transport at the apical membrane is associated with the activation of both basolateral and apical $K^+$ channels [3,24,37]. Acti-

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**Table 1.** Composition of experimental solutions

| | Extracellular | | | Intracellular |
|---|---|---|---|---|
| Concentration (mM) | Isolation | 20KCl | 50 NaCl | 100 KCl-low $Ca^{2+}$ |
| NaCl | 101 | | 50 | |
| KCl | 3 | 20 | 3 | 100 |
| $MgCl_2$ | | 1 | 1 | 2 |
| $CaCl_2$ | | 2 | 2 | |
| HEPES | 10* | 10** | 10* | 10** |
| Mannitol | | 150 | 84 | 15 |
| EGTA | | | | 0.5 |
| $Na_2ATP$ | | | | 2 |

*Titrated to pH 7.4 with NaOH
**Titrated to pH 7.4 with KOH

vation of the basolateral $K^+$ channel is dependent on changes in intracellular ATP, with stimulation of apical $Na^+$-coupled transport decreasing ATP and leading to channel activation [36]. The mechanism regulating apical $K^+$ channel activity is unknown. However, activation of both of these $K^+$ conductances forms part of the pump-leak coupling mechanism.

In addition to pump-leak coupling, many epithelial cells also demonstrate volume regulation. Such volume regulation falls into two categories; regulatory volume increase (RVI) is seen in response to cell shrinkage, while regulatory volume decrease (RVD) follows cell swelling. Both these regulatory mechanisms rely on the fact that the movement of osmotically active solute is accompanied by an obligatory flux of water. Thus, RVI is a consequence of the accumulation of $Na^+$ and $Cl^-$ by the cell, while RVD involves the activation of solute efflux pathways for $K^+$, $Cl^-$ and $HCO^-_3$ [20]. The loss of $K^+$ from the cell typically occurs as a consequence of the activation of $K^+$ channels [1, 4, 25]. A previous study has demonstrated that single proximal tubule cells isolated from *Rana temporaria* are also capable of RVD in response to cell swelling initiated by hypotonic shock [28]. The RVD response of cells was not homogeneous, as some cells showed RVD in HEPES-buffered solutions, while other cells had an absolute requirement for $HCO^-_3$. Thus it appears that within the proximal tubule there are two cell populations as defined by the anion dependence of RVD, $HCO^-_3$-independent cells and $HCO^-_3$-dependent cells. In the $HCO^-_3$-independent cells, RVD was inhibited by the $K^+$-channel blocker $Ba^{2+}$, suggesting that $K^+$ channels may play an important role in RVD in these cells.

A previous study has identified two $K^+$ conductances found in all proximal tubule cells isolated from *Rana temporaria*, $G_{Quin}$ and $G_{Ba}$ [31]. Both conductances are activated by ATP. However, whereas $G_{Quin}$ is inhibited by quinidine and is an outward rectifier, $G_{Ba}$ is sensitive to both quinidine and $Ba^{2+}$ and shows inward rectification. The activity of $G_{Ba}$ is maintained in whole-cell patches in the presence of 2 mM ATP, via a hydrolysis-independent mechanism. By contrast, activation of $G_{Ba}$ above the initial level achieved initially in the whole-cell configuration is dependent on ATP hydrolysis. Given the role of ATP in pump-leak coupling, the following study examined further the mechanisms by which ATP regulates $G_{Ba}$. In addition, the volume sensitivity of $G_{Ba}$ was also examined.

## Materials and Methods

### Cell Isolation

Single proximal tubule cells were isolated by enzyme digestion of whole kidneys taken from *Rana temporaria* as described previously [13]. Briefly, frogs were killed by stunning, and the brain and spinal cord destroyed prior to removal of the kidneys in accordance with UK legislation. Kidneys were superfused with isolation solution (Table 1) and then injected with collagenase and pronase. The kidneys were minced and incubated in enzyme solution for 10 min at room temperature. After this time, tissue fragments were triturated and enzyme digestion halted by a series of centrifugation and resuspension steps. Cells were resuspended in ice-cold isolation solution. At the end of the last centrifugation step cells were resuspended in the appropriate control solution, either 20 KCl or 50 NaCl (Table 1) and stored under ice. Proximal tubule cells were identified by their 'snowman' appearance.

### Cell Length Experiments

Cell length was measured with a photodiode array-based system as described previously [28]. Cells were initially superfused with 50 NaCl (Table 1) and hypotonic shock imposed by removal of 40 mM mannitol. This was repeated in the presence of 5 mM $Ba^{2+}$ (substitution of mannitol), 1 mM quinidine (substitution of mannitol) [31] or 5 mM $Ba^{2+}$ together with 1 mM quinidine.

### Patch Experiments

Single cells were placed in a Perspex bath on the stage of an inverted microscope (Nikon Diaphot or Olympus 1X70) and standard patch-clamp techniques employed to investigate whole-cell currents [12]. Voltage protocols were driven from an IBM-compatible computer equipped with either a Labmaster TL–1 or Digidata interface (Axon Instruments, Foster City, CA) using the pClamp software, Clampex (Axon Instruments). Recordings were made using a List EPC-7 amplifier. To reduce stray capacitance, patch pipettes were coated with Sylgard (Dow Corning Corp., USA).

Whole-cell clamp was obtained via the basolateral aspect of the cells and currents were saved directly onto the hard disk of the computer following low-pass filtering at 5 kHz. Average steady-state currents at each potential were derived using Excel 2000. Cell area was calculated from the capacity transients seen in response to a 20 mV potential step, with membrane capacitance assumed to be 1 μF per $cm^2$. The mean cell capacitance was 48.4 ± 1.64 pF

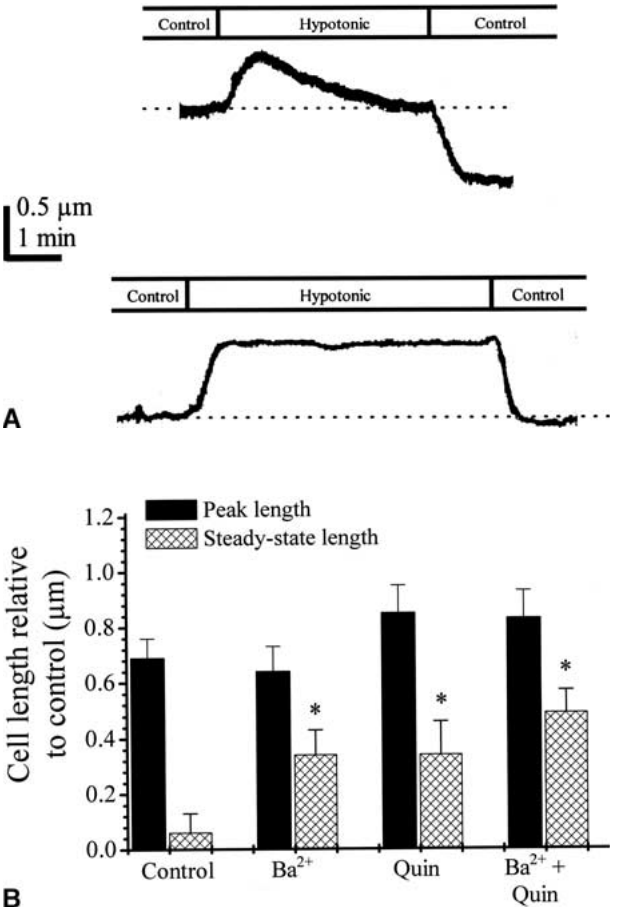


**Fig. 1.** Effect of hypotonic shock on proximal cell length in the absence and presence of different $K^+$ channel inhibitors. (*A*) Typical traces obtained from a regulating (*upper*) and non-regulating (*lower*) cell, (*B*) Peak length and steady-state lengths expressed relative to control, with control length given as zero. The * indicates a significant difference from the control circumstance.

($n$ = 100), while the mean series resistance was 0.12 ± 0.01 μS ($n$ = 100).

Whole-cell patches were obtained with 20 mM KCl in the bath (Table 1) and 100 KCl-low $Ca^{2+}$ (Table 1) in the pipette. Whole-cell potential was held at 0 mV and then transiently stepped to +33 mV followed by a second step to −33 mV for 200 ms duration. This protocol was repeated at 5–second intervals over a 10–minute recording period. Currents at −33 mV were $Cl^-$ selective, while currents at +33 mV were $K^+$- selective. Data on the $Cl^-$ -selective current have been published previously [29,30]. The $K^+$-selective current comprises two different conductances, one that is blocked by $Ba^{2+}$ and one that is not. Therefore, $I_{Ba}$ was defined as the current sensitive to 5 mM $Ba^{2+}$(a maximal inhibitory concentration) [31]. Changes in $I_{Ba}$ were taken as the difference between the $Ba^{2+}$-sensitive current measured initially on achieving the whole-cell configuration and that recorded after ten minutes. $I_{Ba}$ was observed in all cells tested.

To investigate the mechanisms underlying the ATP-dependent activation of $I_{Ba}$, 2 mM ATP was included in the pipette solution (100 KCl low $Ca^{2+}$, Table 1). A number of activators and inhibitors of protein kinases, protein phosphatases and G-proteins were tested in the presence of 2 mM ATP. To activate protein kinase C (PKC), cells were pre-incubated with 100 mM phorbol-12–myristate-13–acetate (PMA) for 10 min prior to whole-cell clamp, and PMA was included in the bath and pipette solutions. To inhibit PKC, whole-cell recordings were obtained with 10 μM PKC-ps in the pipette. To activate protein kinase A (PKA), intact cells were exposed to 100 μM dibutyryl cAMP (dbcAMP) and 100 μM 3–isobutyl-1–methylxanthine (IBMX) for five minutes and then whole-cell clamp was obtained. During whole-cell recordings, dbcAMP and IBMX were present in both the pipette and bath solutions. To inhibit PKA, whole-cell patches were obtained with 1 μM protein kinase inhibitor (PKI) in the pipette. Protein phosphatase activity was examined by inclusion of either 25 units/ml of exogenous alkaline phosphatase or 10 mM $F^-$ (a non-specific phosphatase inhibitor) in the pipette. G protein activity was altered by inclusion of either 10 μM GTPγS or 1 mM $F^-$ (for activation) in the pipette.

The activation of $I_{Ba}$ in response to 2 mM pipette ATP was also examined in the presence of a hypertonic bath Ringer's (addition of 40 mM mannitol). In addition, in a separate series of experiments, $I_{Ba}$ was allowed to activate to steady state and then the magnitude of $I_{Ba}$ was determined with hypertonic (addition of 40 mM mannitol), control or hypotonic (removal of 40 mM mannitol) bath Ringer's.

### Chemicals and Solutions

The osmolality of all solutions was measured (Roebling osmometer) and adjusted to within 1 mosmol per kg water of 215 mosmol per kg water with water or mannitol as appropriate. Chemicals were obtained from Sigma, and were of analytical grade. Alkaline phosphatase and protein kinase C ps were obtained from Calbiochem, UK.

### Statistics

Results are given as means ± 1 SEM, with the number of experiments in parentheses ($n$). Significance was tested using ANOVAs or Fisher's exact probability test as appropriate, and significance assumed at the 5% level. Statistical analysis of fractional data was carried out after these data had been normalized by taking logarithms.

## Results

### Cell-length Experiments

As observed in a previous study, two different responses to hypotonic shock were observed (Fig. 1*A*). In 54 % of cells tested (27 from 50), cell length increased to a peak, followed by recovery back towards the control level. In the remaining cells (23 from 50) cell length increased to a peak that was maintained until the cells were placed back in control Ringer's. The initial length of the regulating cells was 21.6 ± 0.29 μm ($n$ = 27). On exposure to a hypotonic shock, length increased by 0.69 ± 0.07 μm to a peak, followed by a subsequent decrease of 0.62 ± 0.1 μm. After regulation, cell length was not significantly different from the initial level. In the regulating cells, placing cells back in control Ringer's post RVD gave a further decrease in length (Fig. 1*A*), consistent with the loss of solute from the cell during RVD. Exposure to a hypotonic shock in the presence

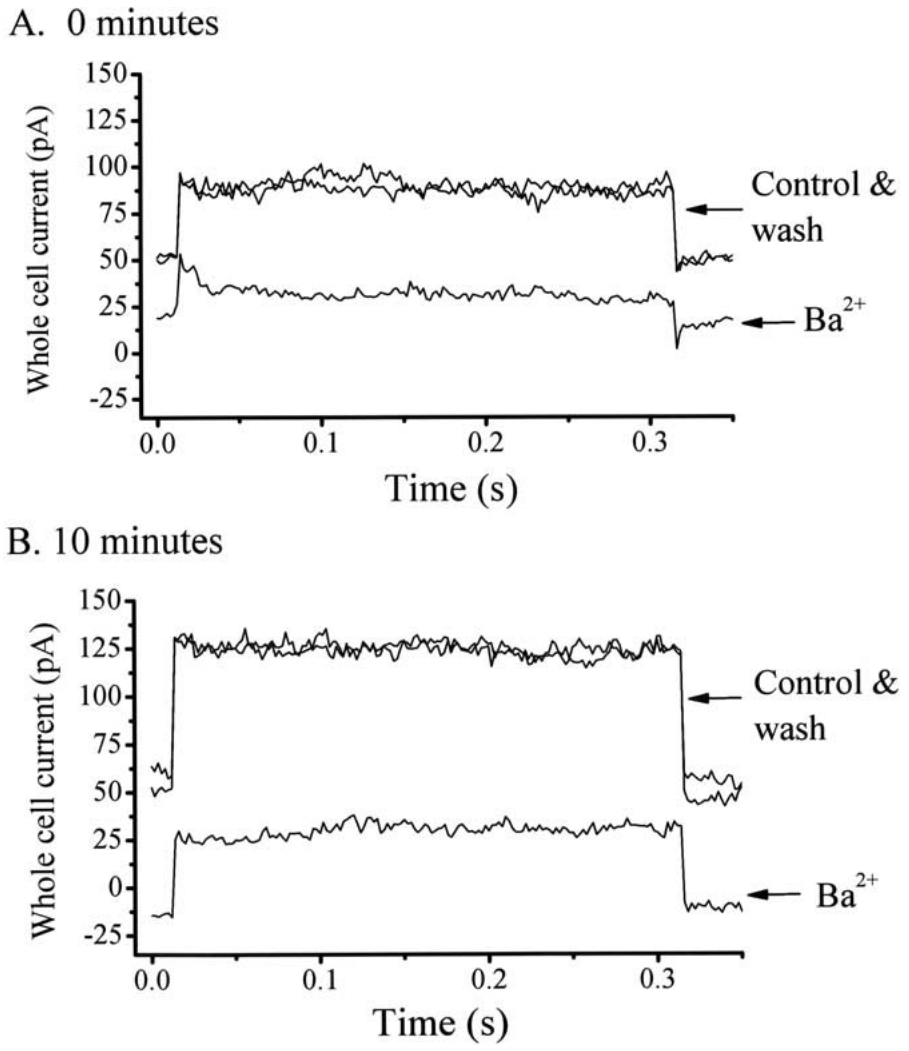


**Fig. 2.** Typical whole-cell traces obtained from the same cell initially on achieving the whole-cell configuration (*A*) and after 10 min (*B*) with 2 mM ATP in the pipette. Clamp potential was stepped from 0 to +33 mV and then back to 0 mV. Each figure shows the total current recorded under the control circumstance, in the presence of 5 mM $Ba^{2+}$ and on washout of $Ba^{2+}$ from the bath.

of $Ba^{2+}$, quinidine or $Ba^{2+}$ plus quinidine produced the same pattern of regulators versus non-regulators and there was no significant difference between the numbers of cells showing RVD between the groups. The number of cells demonstrating volume regulation were: 63 % (12 from 21 cells), 68 % (11 from 21 cells) and 53% (10 from 19 cells), respectively. There was also no significant difference between the initial cell lengths of all groups, 21.6 ± 0.29 μm ($n$ = 27), 20.6 ± 0.62 μm ($n$ = 12), 22.1 ± 0.78 μm ($n$ = 11), and 22.4 ± 0.93 μm ($n$ = 10) under the control circumstance and in the presence of $Ba^{2+}$, quinidine or $Ba^{2+}$ plus quinidine, respectively. In the presence of the $K^+$-channel blockers, hypotonic shock also increased cell length to a peak followed by regulation (Fig. 1*B*). The increase to peak between the groups was not significantly different. However, the degree of regulation was inhibited under all conditions compared to control, $F_{3,55}$ = 5.47; there was no significant difference in effectiveness of the different $K^+$-channel blockers.

## Regulation of $G_{Ba}$ by ATP

The initial $I_{Ba}$ with 2 mM ATP in the pipette was 1.72 ± 0.25 pA/pF ($n$ = 35). This was not significantly different from any of the other experimental conditions, $F_{15,166}$ = 1.65.

As demonstrated in a previous study, with 2 mM ATP in the pipette, the $Ba^{2+}$-sensitive current increased over 10 minutes, Figs. 2 to 4. The initial $I_{Ba}$ was 1.72 ± 0.25 pA/pF ($n$ = 35), whereas after ten minutes, $I_{Ba}$ had risen to 2.86 ± 0.35 pA/pF, a mean increase of 1.15 ± 0.19 pA/pF.

The increase in $I_{Ba}$ over 10 minutes in response to ATP was significantly different between the experimental conditions, $F_{15,166}$ = 10.52.

### *Role of Phosphorylation*

Activation of $I_{Ba}$ was enhanced when PKC-ps was included in the pipette solution ($n$ = 9). Conversely,

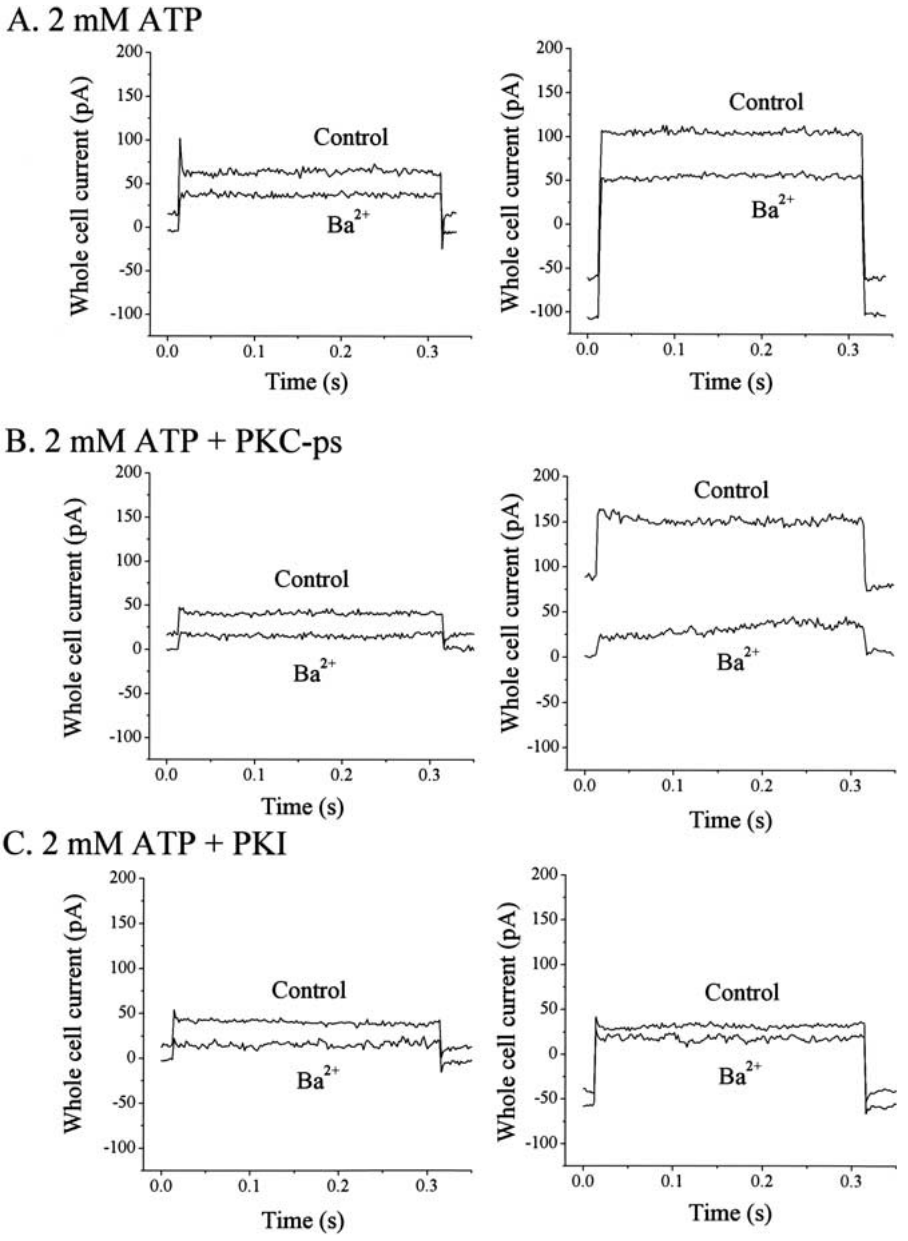


**Fig. 3.** Effect of inhibition of PKA and PKC. Typical traces recorded on stepping from 0 mV to +33 mV in the presence of ATP (*A*), ATP + PKCps (*B*) and ATP + PKI (*C*). The left-hand figures indicate currents recorded at time 0, while the right-hand figures indicate currents recorded from the same cells at 10 min. Note the shift in current at 0 mV with ATP and ATP + PKI, which represents a change in $Cl^-$ conductance.

activation was attenuated when PMA ($n$ = 9), alkaline phosphatase ($n$ = 7) or PKI ($n$ = 15) were included in the pipette solution. dbcAMP plus IBMX ($n$ = 6) and 10 $F^-$ ($n$ = 15) were without effect on activation. These data are shown in Figs. 3 and 4.

*Role of G-proteins*

GTPγs attenuated the activation with ATP. In the presence of ATP plus GTPγs, the initial $I_{Ba}$ was 1.56 ± 0.34 pA/pF ($n$ = 15). However, at 10 min, activation was significantly reduced compared to ATP alone (1.94 ± 0.44 pA/pF). 1 mM $F^-$ was without effect. In the presence of ATP plus 1 mM $F^-$ the initial $I_{Ba}$ was 1.59 ± 0.32 pA/pF ($n$ = 11). At 10 min $I_{Ba}$ was 2.00 ± 0.29 pA/pF.

*Other Nucleotides*

With 2 mM ADP in the pipette, $I_{Ba}$ increased over 10 min, a mean increase of 0.85 ± 0.17 pA/pF ($n$ = 13). This was not significantly different from the response elicited by 2 mM ATP. The activation observed with ADP was abolished when $Mg^{2+}$ was omitted from the pipette solution ($n$ = 5). 5 mM ADP ($n$ = 7) also increased $I_{Ba}$, however, activation was not significantly different from that observed with 2 mM ADP. In contrast, 2 mM AMP ($n$ = 4) was associated with a fall in $I_{Ba}$ over time. Finally, the

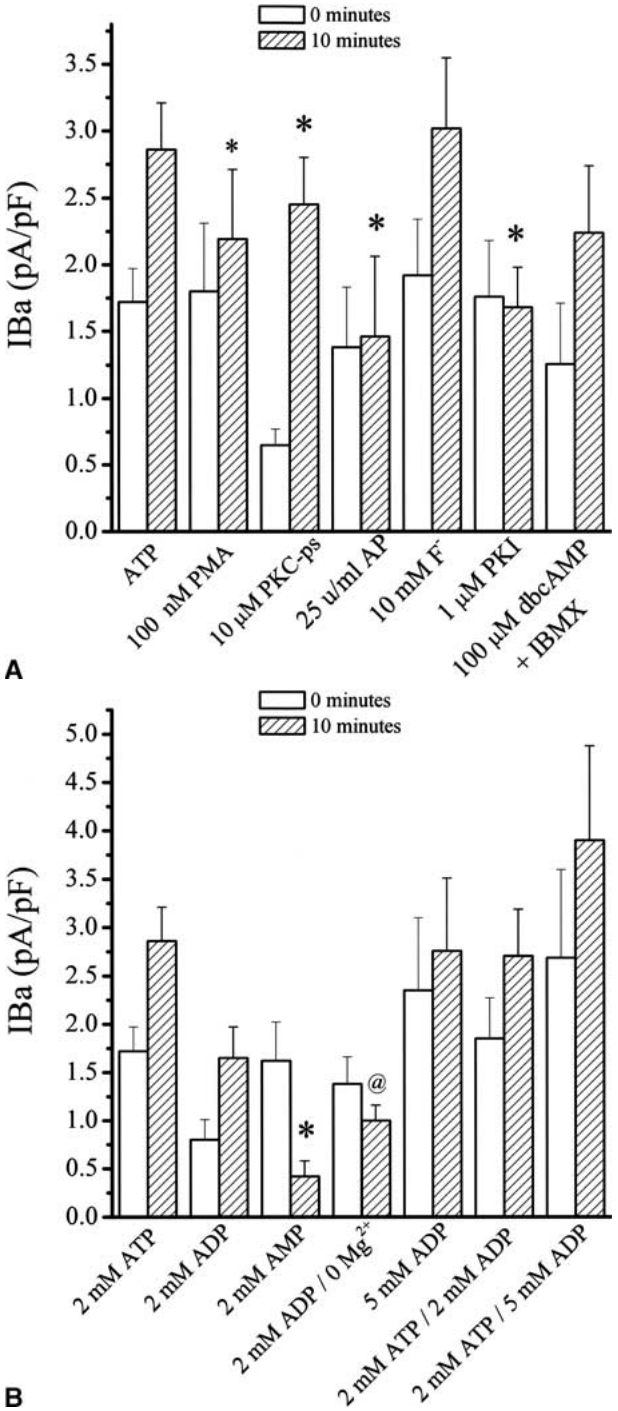


**Fig. 4.** Mechanism of action of ATP on $G_{Ba}$. $I_{Ba}$ recorded at +33 mV at time 0 (*white bars*) and after 10 min (*hatched bars*). 2 mM ATP was present in the pipette for all experimental conditions. * indicates a significant difference from 2 mM ATP, while @ indicates a significant difference from 2 mM ADP.

responses to 2 mM ATP, 2 mM ADP or 5 m ADP were not additive. Activation of $I_{Ba}$ with 2 mM ATP together with 2 mM ADP ($n$ = 15) or 5 mM ADP ($n$ = 6) was not significantly different from ATP alone. These data are shown in Fig. 4.

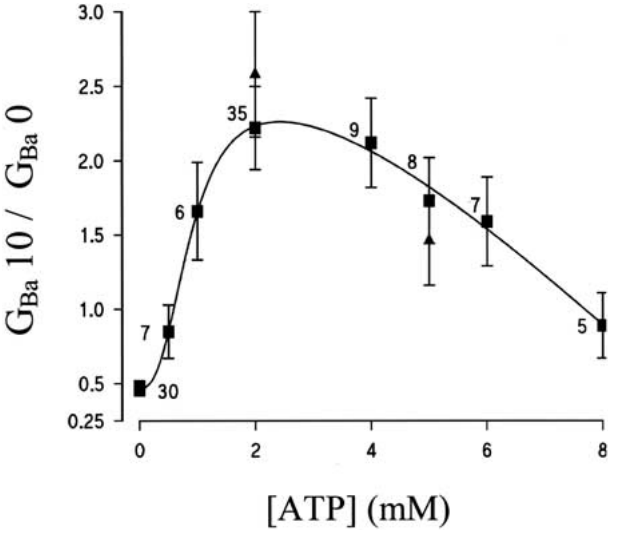


**Fig. 5.** Dependence of $G_{Ba}$ on pipette [ATP]. Changes in $G_{Ba}$ were recorded with 20 mM KCl in the bath and 100 mM KCl-low $Ca^{2+}$ in the pipette. Each point (■) is the mean (± SEM of at least 5 cells (the number of experiments is given next to. each point). The line through the data is the best fit ($r^2$ = 0.995) to a two-binding-site model given in the text (Eq. 1). For comparison, data showing the effect of 2 and 5 mM ADP are also shown (▲).

*Hypertonic Bath Ringer's*

When cells were continuously exposed to a hypertonic bath Ringer's, the initial $I_{Ba}$ was 2.91 ± 0.64 pA/pF ($n$ = 10). This was not significantly different from the control circumstance. However, the activation typically seen with ATP was attenuated, with a mean fall in $I_{Ba}$ over 10 min of 1.47 ± 0.42 pA/pF ($n$ = 10). After activation to steady state, neither hypertonic nor hypotonic shock had any effect on $I_{Ba}$ (2.53 ± 1.02 pA/pF, 2.20 ± 0.71 pA/pF and 2.48 ± 0.91 pA/pF in control, hypertonic and hypotonic solutions, respectively; $n$ = 6).

ATP Sensitivity of $G_{Ba}$

$G_{Ba}$ shares many of the properties with the ATP-sensitive $K^+$ channels identified on the basolateral membrane of *Ambystoma* and rabbit proximal tubules. However, whereas these channels are inhibited by millimolar concentrations of ATP, $G_{Ba}$ is activated by 2 mM ATP. To determine whether $G_{Ba}$ was also inhibited by ATP, experiments have investigated the dose dependence of the response of $G_{Ba}$ to ATP. Whole-cell patches were obtained with 20 KCl (Table 1) in the bath and 100 KCl-low $Ca^{2+}$ (Table 1) in the pipette. The concentration of $Na_2ATP$ in the pipette was varied between 0.5, 1, 2, 4, 5, 6 and 8 mM. Pipette solution osmolality and pH were maintained by removal of mannitol and addition of KOH, respectively. $G_{Ba}$ was recorded at

+33 mV, initially on achieving the whole-cell configuration and again after 10 minutes. $G_{Ba}$ was regulated by ATP in a biphasic manner (Fig. 5). The data were described by a two-binding-site model (Eq.1):

$$\frac{G_{Ba}10}{G_{Ba}0} = G_{max} \times \left[\frac{1}{1+\left[\frac{[ATP]}{K_a}\right]^{-P}}\right] \times \left[1-\left[\frac{1}{1+\left[\frac{[ATP]}{K_i}\right]^{-N}}\right]\right] \quad (1)$$

where the $G_{Ba}0$ is $Ba^{2+}$-sensitive conductance initially on achieving the whole-cell configuration, $G_{Ba}10$ is the $Ba^{2+}$-sensitive conductance after 10 minutes, $G_{max}$ is the maximum fraction obtained, [ATP] is the concentration of ATP (mM), $K_a$ is [ATP] for half-maximal activation (mM), $K_i$ is [ATP] for half-maximal inhibition (mM), $P$ is the Hill coefficient for activation and $N$ is the Hill coefficient for inhibition. The conductance initially increased as the ATP concentration was raised from 1 to 4 mM, but decreased thereafter. The following best-fit values were obtained ($r^2 = 0.995$): $K_a = 0.89$ mM, $P = 2.6$; $K_i = 6.71$ mM, $N = 2.1$; the maximum $G_{Ba}$ was 2.6 times the initial $Ba^{2+}$-sensitive conductance.

## Discussion

As described previously, $G_{Ba}$ was activated by inclusion of 2 mM ATP in the pipette [31]. This activation has been shown to be dependent on two separate processes; maintenance of $G_{Ba}$ occurs via an allosteric action of ATP, while activation is dependent on ATP hydrolysis. The current study shows that regulation of $G_{Ba}$ by ATP is biphasic. This is a characteristic property of ATP-sensitive $K^+$ channels ($K_{ATP}$) [14,26,27], which are activated by low ATP concentrations, via a mechanism dependent on ATP hydrolysis, and inhibited by high concentrations of ATP. $K_{ATP}$ channels are composed of two types of subunits, a channel subunit (a member of the inwardly rectifying $K^+$ channel family, typically Kir6.x) together with the sulfonylurea receptor (SUR) [16]. Different SUR subunits have been shown to confer altered ATP sensitivity to $K_{ATP}$ channels. The concentration of ATP producing half-maximal activation of the Kir6.2 and SUR1 complex is only 10 μM [15]. However, coupling Kir6.2 with SUR2 produces $K^+$ channels that are half-maximally activated by 300 μM ATP [23]. $G_{Ba}$ was half-maximally activated by 0.89 mM ATP, which is greater than these previously published values. In addition, the concentration of ATP giving half-maximal inhibition of $G_{Ba}$ also higher [27]. The identity of the renal proximal tubule $K_{ATP}$ channel is not known. One candidate in rabbit proximal tubule is Kir6.1 together with SUR2A and SUR2B [6]. Interestingly, SUR2 subunits confer a reduced sensitivity of both Kir6.2 and Kir6.1 to ATP [23, 39]. Functionally, with respect to $K_{ATP}$, the sensitivity to ATP of $G_{Ba}$ is shifted to the right, with millimolar levels needed for activation. How does this shift in ATP-sensitivity fit with a possible role in ATP-mediated pump-leak coupling? In the rabbit proximal tubule, the ATP concentration varies between 2 to 4 mM in response to $Na^+$ cotransport stimulation or inhibition of the basolateral $Na^+$, $K^+$-ATPase [2] and a fall in ATP over this range is associated with relief of block of $K_{ATP}$ channels [36]. However, over the same concentration range the activity of $G_{Ba}$ is not altered (Fig. 5). If the response of the frog proximal tubule is similar to rabbit, this would suggest that changes in $Na^+$ cotransport and $Na^+/K^+$ ATPase activity would not be expected to regulate $G_{Ba}$, precluding a role for this conductance in ATP-mediated pump-leak coupling. By contrast, $G_{Ba}$ does seem to be sensitive to changes in cell volume. Concentrations of $Ba^{2+}$ and quinidine that maximally inhibit $G_{Ba}$ attenuated hypotonic shock-induced volume regulation by the cells. The degree of inhibition for each individual blocker was similar, and addition of both inhibitors together did not increase the degree of inhibition. This blocker profile is consistent with a role for $G_{Ba}$ in RVD. In support of this, activation of $G_{Ba}$ by ATP in whole-cell patches was diminished in the presence of a hypertonic bath solution. This indicates that activation of $G_{Ba}$ by ATP is dependent on cell swelling. However, imposing changes in cell volume after activation to steady state did not modulate $G_{Ba}$. This suggests that $G_{Ba}$ is not directly sensitive to changes in cell volume and is in contrast to a number of other renal proximal tubule $K^+$ channels [18,32]. A previous study has demonstrated that frog proximal tubule RVD is dependent on the activation of PKC [28]. However, this cannot be the mechanism by which the volume sensitivity of $G_{Ba}$ is mediated, as stimulation of PKC is associated with conductance inhibition. Another requirement for frog proximal tubule RVD is the presence of $Ca^{2+}$ [28]. The direct $Ca^{2+}$ sensitivity of $G_{Ba}$ is not known, although in the current study $Ba^{2+}$-sensitive currents were observed with a low intracellular $Ca^{2+}$ concentration. Other renal inwardly rectifying $K^+$ channels are either inhibited by intracellular $Ca^{2+}$ or insensitive to $Ca^{2+}$ [22, 24]. Given the fact that the activation of $G_{Ba}$ by ATP was dependent on a PKA-mediated phosphorylation event, another possibility is that the volume sensitivity of $G_{Ba}$ is mediated via PKA. However, a previous study demonstrated that stimulation of PKA had no effect on RVD in the frog proximal tubule

[28]. This would suggest that PKA does not play a role in the volume sensitivity of $G_{Ba}$, although the effect of inhibition of PKA on RVD was not examined in the earlier study. Taken together, these data suggest that $G_{Ba}$ plays a role in volume regulation in the frog renal proximal tubule.

Like other $K_{ATP}$ channels, activation of $G_{Ba}$ by ATP is a consequence of a phosphorylation event, as inclusion of alkaline phosphatase in the pipette together with ATP prevented activation. A previous study has shown that ATP can maintain the activity of $G_{Ba}$ in the absence of hydrolysis [31] and therefore in this experiment, the magnitude of $G_{Ba}$ was maintained throughout the experiment even in the absence of phosphorylation. In addition, activation is dependent on PKA-mediated phosphorylation, as an inhibitor of this kinase reduced the activation of $G_{Ba}$. However, stimulating PKA using dbcAMP and IBMX was without effect, suggesting maximal activation of PKA under the experimental conditions. $G_{Ba}$ is inhibited by PKC as PKCps enhanced activation, whilst the PKC inhibitor, PMA, was inhibitory. Therefore, the net activation observed with 2 mM ATP is a consequence of the interplay between PKA-mediated activation and PKC-mediated inhibition. Interestingly, activation of PKC has previously been shown to play an important role in the activation of a volume-sensitive $Cl^-$ conductance in whole-cell patches [29,30]. Activation of the $Cl^-$ conductance in whole-cell patches occurs at the same time as ATP-mediated activation of $G_{Ba}$, confirming the dual regulation of $G_{Ba}$ under the experimental conditions. The duality of $K^+$-channel regulation by these kinases is a feature of many different $K^+$ channels, e.g., apical channels in the distal nephron, basolateral channels in the proximal tubule, $K_{ir}2.1$ and $K^+$ channels found in gallbladder and $T_{84}$ cells [7, 9, 24, 35, 38]. The ATP-dependent activation of $G_{Ba}$ was also sensitive to G-protein activation, as in the presence of GTPγS activation was attenuated. This is similar to regulation of the inwardly rectifying $K^+$ channels in endothelial cells and enterocytes [8, 17]. These properties are consistent with $G_{Ba}$ being a member of the ATP-sensitive $K^+$-channel family. The action of PKC, PKA and G-proteins on $G_{Ba}$ could be important in the regulation of proximal tubule function by hormones. Both dopamine and nitric oxide have been shown to inhibit $Na^+$ transport via a G-protein-coupled and PKC-dependent mechanism [5, 21]. By contrast, in the renal cortex catacholamines have been shown to activate the $Na^+$-$K^+$ ATPase via a PKA-dependent mechanism [10]. Given the role of $K^+$ channels in generation of the membrane potential and the importance of the membrane potential in driving $Na^+$ uptake, any hormone-induced changes in the activity of $G_{Ba}$ would be expected to also have effects on $Na^+$ reabsorption.

The effect of other nucleotides on $G_{Ba}$ also suggests that $G_{Ba}$ belongs to the $K_{ATP}$ channel family. ADP activated $G_{Ba}$ and the degree of activation was not significantly different from that observed with ATP. The effect of ADP was dependent on the presence of $Mg^{2+}$. Activation by ADP is a well-known property of ATP-sensitive $K^+$ channels [11, 19, 34], and is probably mediated by binding of MgADP to a sulfonylurea receptor coupled to the $K^+$ channel protein [11, 23], A previous study has demonstrated that SUR2A and SUR2B are expressed in rabbit proximal tubule [6], although their expression in amphibian renal tissue has not been studied. The effects of ATP and ADP were not additive, and individually they gave similar levels of activation (Fig. 5), suggesting a common mechanism of activation. Given the adenosine nucleotide sensitivity of $G_{Ba}$ and its regulation by PKA and PKC, the possibility exists that it could be regulated by the metabolic status of the cell and may therefore play a role in cellular homeostasis. Depletion of ATP would be expected to lead to inhibition of the $K^+$ conductance and preservation of intracellular $K^+$.

These data demonstrate that the $Ba^{2+}$-sensitive $K^+$ conductance in the renal proximal tubule of the frog is regulated by the nucleotides ATP and ADP. The regulation by ATP shows a characteristic biphasic response, with lower concentrations activating and higher concentrations inhibiting the conductance. However, in comparison to other $K_{ATP}$ channels, $G_{Ba}$ has a lower sensitivity to ATP. Regulation by ATP is dependent on two mechanisms, an allosteric mechanism and a mechanism dependent on ATP hydrolysis and phosphorylation. The regulation by phosphorylation occurs through PKA-mediated activation and PCK-mediated inactivation of the conductance. The net response to changes in ATP thus depends on the overall activity of these two kinases. In addition, activation of $G_{Ba}$ by ATP was dependent on cell swelling. Taken together with the blocker profile of inhibition of RVD, this suggests that $G_{Ba}$ plays a role in volume regulation and is important in cellular homeostasis.

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