Two K^+-selective conductances in single proximal tubule cells isolated from frog kidney are regulated by ATP

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1. The whole-cell and single channel patch clamp techniques were used to identify K^+-selective conductances in single proximal tubule cells isolated from frog kidney and to examine their ATP sensitivity. Whole-cell currents were inhibited by the K^+ channel inhibitors Ba^{2+} and quinidine in a dose-dependent manner. Addition of Ba^{2+} alone, quinidine alone, or both inhibitors together revealed two separate conductances, one of which was blocked by both Ba^{2+} and quinidine (G_{Ba}), the other being sensitive to quinidine alone (G_{quin}).

2. With Na^+-containing Ringer solution in the bath and K^+-containing Ringer solution in the pipette, both currents were selective for K^+ over Na^+. The $K^+ : Na^+$ selectivity ratio of G_{Ba} was around 50 : 1, while that of G_{quin} was 4 : 1. In symmetrical KCl solutions G_{Ba} showed inward rectification, while G_{quin} demonstrated outward rectification.

3. In the absence of pipette ATP, both G_{Ba} and G_{quin} ran down over 10 min. However, when 2 mM ATP was included in the pipette G_{Ba} increased, while G_{quin} remained unchanged.

4. Single channel studies demonstrated that a basolateral K^+ channel shared several of the characteristics of G_{Ba}. It was inhibited by both Ba^{2+} and quinidine, underwent run-down in excised patches in the absence of ATP, and was activated by ATP.

5. We conclude that cells of the frog proximal tubule contain at least two distinct K^+-selective conductances, both of which are regulated by ATP, and which may be involved in pump–leak coupling.

Transepithelial Na^+ movement is described by the Koefoed-Johnson and Ussing double membrane model (Schultz & Hudson, 1986). Na^+ which enters the cell across the apical membrane exits via a basolateral Na^+,K^+-ATPase (Na^+ pump). The obligatory coupling between Na^+ and K^+ movement through the pump results in cellular K^+ accumulation. This K^+ is then lost through a basolateral leak pathway (K^+ conductance). The net result is reabsorption of Na^+, with K^+ merely recycling across the basolateral membrane.

To minimize alterations in both intracellular ionic content, and therefore cell volume, any changes in apical Na^+–substrate cotransport must be matched by parallel changes in the basolateral pump and leak rates; this is the so-called pump–leak mechanism (Schultz & Hudson, 1986). In the renal proximal tubule, one of the means of Na^+ entry across the apical membrane is by Na^+ cotransport, in which the entry of Na^+ is coupled to the uptake of amino acids and sugars. Addition of amino acids to the luminal fluid bathing frog and rabbit proximal tubules causes an initial depolarization of the basolateral membrane potential followed by repolarization (Lapointe, Garneau, Bell & Cardinal, 1990). The depolarization is a consequence of the movement of Na^+ and amino acids into the cell via the electrogenic cotransporters. The subsequent repolarization is accompanied by an increase in the K^+ conductance of the basolateral membrane (Lapointe *et al.* 1990), which helps to rid the cells of K^+ and restores the electrical driving force promoting Na^+ entry.

The intracellular mediator which couples the activity of the basolateral K^+ conductance to changes in apical Na^+ cotransport is unknown. However, several factors have been suggested, e.g. intracellular ATP concentration, pH, Ca^{2+} and cell volume (Messner, Koller & Lang, 1985*a*; Messner, Wang, Paulmichl, Oberleithner & Lang, 1985*b*; Hudson & Schultz, 1988). All of these have been shown to regulate epithelial K^+ channels (Lau, Hudson & Schultz, 1984; Christensen & Zeuthen, 1987; Hurst, Beck, Laprade & Lapoint, 1993).

In a previous study, we measured the activity of single K^+ channels in the frog proximal tubule, and noted that their activity was independent of the presence of substrates, i.e. transport (Robson & Hunter, 1992). This was not in accord with macroscopic studies, in which there was a clear

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Table 1. Composition of extracellular solutions (mM)

Solute	Isolation	Ringer	100 KCl	20 KCl
NaCl	101	97	—	—
KCl	3	3	100	20
$CaCl_2$	—	2	2	2
$MgCl_2$	—	1	1	1
Hepes	10*	10*	10†	10†
Mannitol	—	10	10	150

* Titrated to pH 7·4 with NaOH; † titrated to pH 7·4 with KOH.

upregulation of the basolateral K^+ conductance following an increase in transport rate (see above). The possibility thus remained that there is a K^+ conductance which we did not detect at the single channel level. In this paper we describe the results of whole-cell experiments in which we establish the presence of at least two K^+ conductances in cells of the frog proximal tubule, both of which may be involved in pump–leak coupling.

METHODS

Cell isolation

Single proximal tubule cells were isolated by enzyme digestion from kidneys of *Rana temporaria* as described previously (Hunter, 1989). Frogs were killed by decapitation and the brain and spinal cord destroyed. Kidneys were removed and, to promote dissociation of the tight junctions, perfused with a divalent cation-free Ringer isolation solution (Table 1). The kidneys were then subjected to partial digestion with collagenase and pronase, which resulted in the generation of a mixed population of single cells derived from the whole kidney. Proximal cells were identified from their 'snowman'-like appearance (Hunter, 1990).

Patch experiments

A suspension of single cells was placed in a Perspex bath on the stage of an inverted microscope and standard patch clamp techniques were used to investigate whole-cell and single channel currents (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Voltage protocols were driven from an IBM-compatible computer equipped with a Labmaster TL-1 interface, using the pCLAMP software Clampex (Axon Instruments). Recordings were made using a List EPC-7 amplifier. To reduce stray capacitance and associated noise, patch pipettes were coated with Sylgard (Dow Corning).

Whole-cell experiments

Whole-cell patches were obtained via the basolateral aspect of the cells and currents saved directly onto the hard disk of the computer following low-pass filtering at 1·5 kHz. Average steady-state currents at each potential were derived using IV, an 'in-house' computer program written in Quick Basic 4. Reversal potentials (V_{rev}) of ohmic and rectifying currents were calculated by linear or polynomial regression, respectively. Outward (G_{out}) and inward (G_{in}) chord conductances were derived from outward- and inward-directed currents, respectively. 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 cm^{-2}.

Single channel experiments

Cell-attached and excised inside-out patches were obtained from the basolateral membrane to examine the blocker and ATP sensitivity of the basolateral K^+ channel identified previously in these cells (Hunter, 1991). In all experiments clamp potential was held at 0 mV, currents being driven by an imposed K^+ concentration gradient. Single channel currents were digitized (Sony PCM – modified by the Department of Physiology workshop at Leeds University to extend frequency range to DC) prior to storage on video tape (Panasonic, Model NV-F65HQ). For data analysis, sections of the recordings were sampled into the computer at 500 Hz following low-pass filtering at 300 Hz, using the pCLAMP software Fetchex (Axon Instruments). Channel activity is expressed as NP_o, where N is the number of channels in the patch and P_o is the open probability of channel, and was calculated using SCAP, a single channel analysis program written 'in-house' in Quick Basic 4.

Experimental protocol and solutions

The osmolality of all solutions was measured (Roebling osmometer) and adjusted to within 1 mosmol (kg H_2O)$^{-1}$ of 215 mosmol (kg H_2O)$^{-1}$ with water or mannitol as appropriate. All chemicals were obtained from Sigma, and were of analytical grade.

Whole-cell experiments

Effect of ATP. The bath contained amphibian Ringer solution (Table 1) and the pipette potassium gluconate solution (Table 2). Experiments were performed in the absence of ATP, or with 5 μM or 2 mM Na_2ATP added to the pipette solution. Osmolarity of the 2 mM ATP solution was maintained by omitting 5 mM mannitol. Clamp potential was stepped, at 60 s intervals, from a holding potential of −40 mV to between +10 and −100 mV in 10 mV steps. The ionic selectivity of whole-cell currents was examined initially on achieving the whole-cell configuration, and again after 10 min, by measuring the change in V_{rev} on substitution of bath Na^+ by K^+. To measure the Ba^{2+}-sensitive conductance of cells, 5 mM Ba^{2+} was added to the bath (substitution of mannitol) at time 0 and after 10 min.

Currents with symmetrical K^+ solutions. To examine whether the whole-cell K^+ currents showed rectification, clamp potential was stepped between +40 and −80 mV in 10 mV steps from a holding potential of −40 mV with symmetrical K^+ solutions, 100 KCl (Table 1) and 100 KCl–low Ca^{2+} (Table 2).

Dose response to Ba^{2+} and quinidine. The bath contained Ringer solution and the pipette 100 KCl–low Ca^{2+} solution (Tables 1 and 2, respectively). Whole-cell potential was clamped between +10 and −100 mV in −10 mV steps. Ba^{2+}, ranging from 0·5 μM to 5 mM, or quinidine, ranging from 0·5 μM to 1 mM, were added to the bath (substitution of mannitol). Experiments were performed in the

Table 2. Composition of intracellular solutions (mM)

Solute	100 KCl–low Ca^{2+}	Potassium gluconate	100 NaCl
NaCl	—	—	100
KCl	100	10	—
Potassium gluconate	—	90	—
$MgCl_2$	2	2	2
Hepes	10†	10†	10*
Mannitol	20	20	—
EGTA	0·5	0·5	0·5

* Titrated to pH 7·4 with NaOH; † titrated to pH 7·4 with KOH.

absence of pipette ATP. Although under this circumstance current run-down occurs, only those cells whose currents returned to at least 75% of the initial control level after the dose–response experiment was completed were used. ATP was not used in the pipette as it activates a Cl^- conductance under these conditions (Robson & Hunter, 1994*a*). In some cells, both inhibitors were applied simultaneously to examine the current–voltage relationship of the Ba^{2+}- and quinidine-sensitive currents.

In a second series of experiments the K^+ : Na^+ selectivity ratio of the quinidine-sensitive currents was examined using ionic substitution. The pipette and bath solutions were as described in the previous paragraph. This high-Na^+ bath solution additionally contained 5 mM Ba^{2+} and 100 μM gadolinium (to inhibit non-selective conductances; Robson & Hunter, 1994*b*). The current sensitive to 1 mM quinidine was measured under two circumstances, initially with high Na^+ and then high K^+ (where all the Na^+ was replaced with K^+). The current sensitive to quinidine showed outward rectification (see Results, Dose response to Ba^{2+} and quinidine). This could be a consequence of voltage dependence of single channel P_o. Therefore ionic selectivity was examined using a tail current protocol which allows the measurement of currents over a range of potentials at constant P_o. Clamp potential was stepped initially to +40 mV for 500 ms. This was followed by a second step which was varied between +40 and −100 mV in 20 mV steps. Instantaneous whole-cell current was measured for the second potential step and V_{rev} calculated.

Effect of Ba^{2+} and quinidine together. The inter-relationship between the inhibition observed with Ba^{2+} and quinidine was examined further under symmetrical KCl conditions, 100 KCl solution (Table 1) and 100 KCl–low Ca^{2+} solution (Table 2). Experiments were carried out in the absence of pipette ATP. Cell potential was stepped between +40 and −80 mV in −10 mV increments. Cells were exposed to maximal inhibitory concentrations of Ba^{2+} and quinidine; either 1 mM quinidine, 5 mM Ba^{2+}, or both of the inhibitors were added to the bath, and G_{out} and G_{in} measured.

Isolation of K^+ currents. In a previous study we have shown that ATP activates a Cl^- conductance in these cells (Robson & Hunter, 1994*a*) which could potentially contaminate the K^+ currents. K^+ and Cl^- currents were therefore separated using ionic gradients such that the V_{rev} for each ion species was different. The bath solution was 20 KCl (Table 1) whereas the control pipette solution was 100 KCl–low Ca^{2+} (Table 2). ATP (2 mM) or 5′-adenyl-imidodiphosphate (AMP-PNP; 2 mM; a non-hydrolysable analogue of ATP) was added to the pipette solution as indicated (substitution of mannitol). Under these conditions, clamping to the experimentally derived V_{rev} for Cl^-, +33 mV, yielded K^+ currents, while clamping to that for K^+, −33 mV, gave Cl^- currents. In this way both Cl^- and K^+ currents could be monitored and recorded in the same cell, although this study will describe only changes in the K^+-selective currents. Ba^{2+} (5 mM) and quinidine (1 mM) were used as indices of K^+ conductance and were added to the bath initially on achieving the whole-cell configuration and again after 10 min. The conductance sensitive to Ba^{2+} was designated G_{Ba}. Quinidine was added to the bath in the continued presence of Ba^{2+}. The conductance sensitive to quinidine was designated G_{quin}.

Single channel currents

Sensitivity to Ba^{2+} and quinidine. The sensitivity of the basolateral K^+ channel to 5 mM Ba^{2+} and 1 mM quinidine was examined in cell-attached patches. The bath contained Ringer solution and the pipette 100 KCl solution (Table 1). On obtaining a cell-attached patch either 5 mM Ba^{2+} or 1 mM quinidine was added to the bath solution (with reduction of mannitol to maintain osmolality).

Effect of ATP. The effect of ATP on the basolateral K^+ channel was examined in excised inside-out patches. The bath contained 100 NaCl solution (Table 2) and the pipette 100 KCl solution (Table 1). Under these conditions the driving force for K^+ movement was out of the pipette. Cell-attached patches were obtained in the absence or presence of 2 mM Na_2ATP and excised inside-out patches obtained by moving the pipette away from the cell. To aid the formation of excised patches the base of the chamber was pre-treated with polylysine (1 mg ml^{-1}) so that the cells adhered firmly to the chamber floor. Channel activity was then recorded for 10 min following patch excision.

Statistics

All values are given as means ± S.E.M. Significance was determined using Student's *t* test or ANOVA, as appropriate, and significance assumed at the 5% level.

RESULTS

Whole-cell currents

Effect of ATP

Overall results of the effect of ATP on whole-cell currents are shown in Table 3. With no ATP in the pipette the initial V_{rev} was −50·3 ± 3·8 mV ($n = 14$). This depolarized over a 10 min period to −8·5 ± 3·1 mV and was associated with a significant decrease in whole-cell conductance, as shown in Fig. 1*A* and Table 3. In the presence of 2 mM ATP there was

Table 3. Effect of ATP on whole-cell currents

Time (min)	V_{rev} (mV)	G_{out} (μS cm^{-2})	G_{in} (μS cm^{-2})	ΔV_{rev} (mV)	$Na^+ : K^+$	Ba^{2+} G_{out} (μS cm^{-2})	Ba^{2+} G_{in} (μS cm^{-2})
0 ATP							
0	−50·3 ± 3·8 (14)	44·1 ± 9·6 (14)	32·8 ± 7·3 (14)	+61·7 ± 6·3 (6)	0·07 ± 0·02 (6)	21·7 ± 5·3 (12)	18·5 ± 3·8 (12)
10	−8·5 ± 3·1 (14)*	20·1 ± 6·4 (14)*	18·1 ± 4·9 (14)*	+9·9 ± 4·6 (6)*	0·71 ± 0·11 (6)*	2·9 ± 0·9 (12)*	3·4 ± 0·9 (12)*
2 mM ATP							
0	−46·8 ± 3·3 (8)	53·4 ± 13·0 (8)	37·4 ± 7·8 (8)	+58·6 ± 4·4 (6)	0·07 ± 0·01 (6)	57·8 ± 9·3 (11)	34·0 ± 5·0 (11)
10	−43·9 ± 3·0 (8)	69·7 ± 12·7 (8)*	52·0 ± 7·4 (8)*	+39·0 ± 3·2 (6)*†	0·19 ± 0·02 (6)*†	36·2 ± 6·6 (11)*†	27·4 ± 2·3 (11)†
5 μM ATP							
0	−55·8 ± 5·5 (4)	37·2 ± 17·4 (4)	21·1 ± 6·2 (4)	+63·0 ± 4·9 (4)	0·06 ± 0·02 (4)	36·5 ± 15·9 (4)	19·1 ± 5·2 (4)
10	−20·2 ± 6·8 (4)*	14·2 ± 3·6 (4)	16·4 ± 3·5 (4)	+14·9 ± 3·2 (4)*	0·55 ± 0·07 (4)*	2·8 ± 1·3 (4)*	5·7 ± 2·7 (4)*

ΔV_{rev}, change in V_{rev} on replacement of bath Na^+ with K^+. * Significant difference from time zero; † significant difference from 0 ATP at 10 min. n is given in parentheses.

no significant change in V_{rev}: −46·8 ± 3·3 *vs.* −43·9 ± 3·0 mV ($n = 8$, shown in Fig. 1*B* and Table 3). However, there was a significant increase in whole-cell conductance (Table 3).

In the absence of ATP, cells were initially K^+ selective. The substitution of bath Na^+ by K^+ produced a positive shift in V_{rev} (Table 3). However by 10 min, cells were significantly less K^+ selective (Table 3). In conjunction with this loss of K^+ selectivity the Ba^{2+}-sensitive conductance of the cells also decreased over time. When 2 mM ATP was included in the pipette the loss of K^+ selectivity was significantly less than that in the absence of ATP ($F_{3,20} = 30·3$). The Ba^{2+}-sensitive conductance of these cells at 10 min was significantly greater than in the ATP-free condition.

In contrast to the findings with 2 mM ATP, when 5 μM ATP was included in the pipette whole-cell K^+ currents decayed as in the ATP-free condition (Table 3).

Currents with symmetrical K^+ solutions

Whole-cell currents showed inward rectification in both the absence and presence of pipette ATP. In the absence of ATP, G_{out} was significantly smaller than G_{in}: 52·3 ± 14·5 *vs.* 172·1 ± 33·9 μS cm^{-2} ($n = 8$). This was also the case with currents recorded in the presence of ATP: 97·3 ± 36·2 *vs.* 219·2 ± 43·4 μS cm^{-2} ($n = 6$). There was no significant difference between the conductances recorded in the absence or presence of ATP.

Dose response to Ba^{2+} and quinidine

Whole-cell currents were blocked by Ba^{2+} in a dose-dependent manner (Fig. 2*A*). The maximal Ba^{2+}-sensitive G_{out} was not significantly different from G_{in}: 21·7 ± 5·3 *vs.* 18·5 ± 3·8 μS cm^{-2} ($n = 12$). The V_{rev} of this conductance was −70·0 ± 3·8 mV ($n = 12$; Fig. 4*A*), which corresponds to a $K^+ : Na^+$ selectivity ratio of 51·5 ± 16. A Hill plot

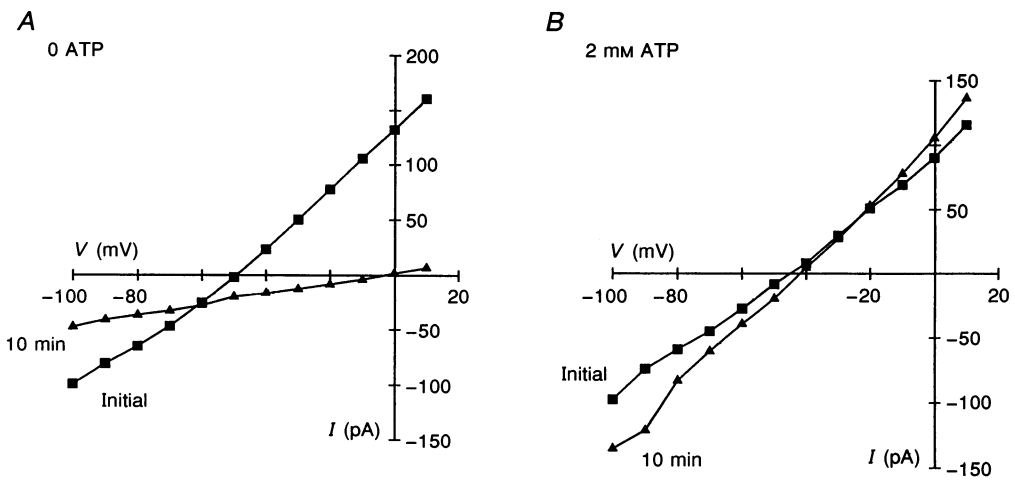

Figure 1. Whole-cell *I–V* curves in the presence and absence of ATP

Typical *I–V* curves recorded initially on achieving the whole-cell configuration (■) and again after 10 min (▲) in the absence (*A*) and presence (*B*) of ATP.

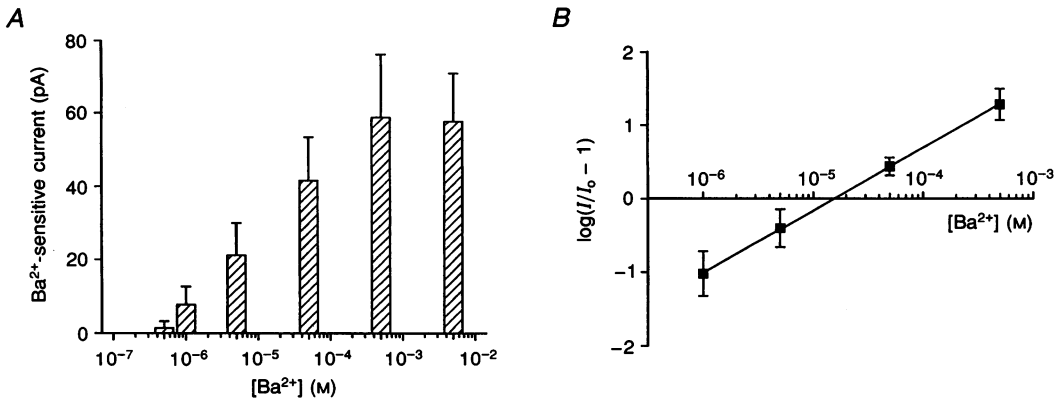

Figure 2. Dose response to Ba^{2+}

A, Ba^{2+}-sensitive current recorded at 0 mV with varying concentrations of Ba^{2+}. *B*, Hill plot of the data shown in *A*. *I* is the current sensitive to a particular concentration of Ba^{2+}; I_o is the maximal Ba^{2+}-sensitive current. The line through the data is the best fit linear regression; $r^2 = 0{\cdot}99$.

(Fig. 2*B*) of the currents at 0 mV gave a mean Hill coefficient of $1{\cdot}0 \pm 0{\cdot}2$ ($n = 8$). The block was voltage dependent. The half-maximal blocking concentration (K_d) for Ba^{2+} at 0 mV ($21{\cdot}8 \pm 5{\cdot}6$ μM) was significantly greater than that at −60 mV ($8{\cdot}3 \pm 1{\cdot}9$ μM, $n = 8$). The electrical distance (δ) sensed by the blocking Ba^{2+} ions was estimated to be 0·2 from the Woodhull equation (Woodhull, 1973):

$$K_{d(V)} = K_{d(0)}\exp(z\delta FV/RT)$$

where $K_{d(V)}$ is the K_d measured at a particular potential, *V*, of −60 mV, $K_{d(0)}$ is the K_d measured at 0 mV, $z = 2$ (the valency of Ba^{2+}), and *R*, *T* and *F* have their usual values.

Whole-cell currents were also blocked by quinidine in a dose-dependent manner (Fig. 3). A Hill plot of the currents at 0 mV (Fig. 3*B*), gave a mean Hill coefficient of $0{\cdot}87 \pm 0{\cdot}12$ ($n = 16$). Block was not voltage dependent. The K_d for inhibition at 0 mV, $15{\cdot}9 \pm 2{\cdot}6$ μM ($n = 16$), was not significantly different from that at −60 mV, $19{\cdot}6 \pm 4{\cdot}5$ μM ($n = 16$).

The quinidine-sensitive G_{out} (recorded in the presence of 5 mM Ba^{2+}) was significantly larger than G_{in}: $8{\cdot}1 \pm 2{\cdot}4$ *vs.* $1{\cdot}2 \pm 0{\cdot}4$ μS cm^{-2} ($n = 6$). The V_{rev} of this conductance was $-30{\cdot}4 \pm 2{\cdot}7$ mV ($n = 6$; Fig. 4*B*), which corresponds to a $K^+ : Na^+$ selectivity ratio of $3{\cdot}7 \pm 0{\cdot}4$ ($n = 6$). From the tail current protocol, substitution of bath Na^+ with K^+ gave a change in the V_{rev} of quinidine sensitive currents of $+28{\cdot}9 \pm 3{\cdot}2$ mV ($n = 5$), giving a $K^+ : Na^+$ selectivity ratio of $3{\cdot}5 \pm 0{\cdot}5$.

Effect of Ba^{2+} and quinidine together

The outward currents were inhibited by both Ba^{2+} and quinidine. The Ba^{2+}-sensitive G_{out} was $18{\cdot}4 \pm 5{\cdot}6$ μS cm^{-2} ($n = 17$). This was significantly smaller than the G_{out} inhibited by quinidine, $52{\cdot}8 \pm 11{\cdot}7$ μS cm^{-2}. Addition of Ba^{2+} and quinidine together gave no further inhibition: Ba^{2+}- plus quinidine-sensitive G_{out} was $47{\cdot}6 \pm 11{\cdot}5$ μS cm^{-2} ($n = 17$; $F_{2,48} = 3{\cdot}7$; Fig. 5*A*). Doubling the Ba^{2+} concentration to 10 mM failed to elicit any further inhibition

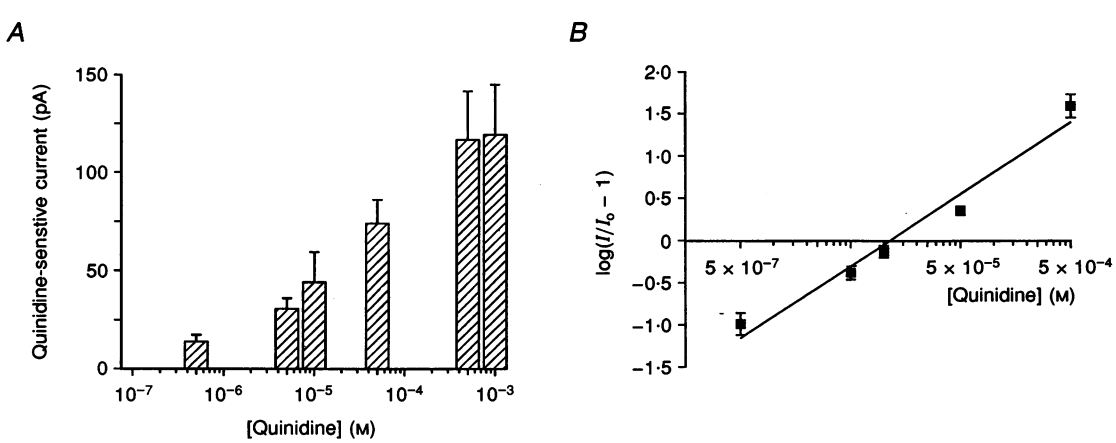

Figure 3. Dose response to quinidine

A, quinidine-sensitive current recorded at 0 mV with varying concentrations of quinidine. *B*, Hill plot of the data shown in *A*. *I* is the current sensitive to a particular concentration of quinidine; I_o is the maximal quinidine-sensitive current. The line through the data is the best fit linear regression; $r^2 = 0{\cdot}97$.

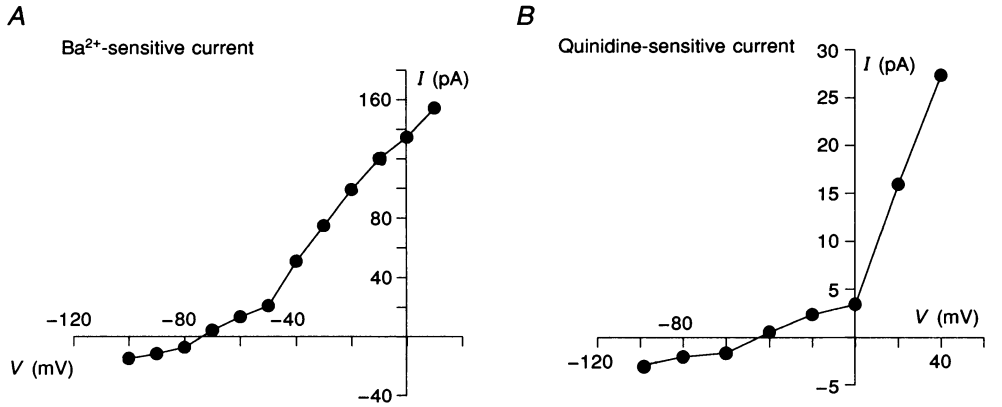

Figure 4. Blocker-sensitive currents

The whole-cell current sensitive to 5 mM Ba^{2+} (*A*) or 1 mM quinidine (*B*, measured in the presence of Ba^{2+}). The bath contained 97 mM NaCl (Table 1) and the pipette 100 KCl (Table 2). The theoretical equilibrium potential for K^+ was −88 mV. Each figure is taken from a different cell. The lines through the data points have no theoretical significance.

of the outward currents at +40 mV: 154·7 ± 29·9 *vs.* 153·8 ± 30·9 pA ($n = 10$). We may have predicted this result, since we calculated that at +40 mV around 99% of the channels should be blocked by 5 mM Ba^{2+} (using the $K_{d(0)}$ value of 21·8 μM; Woodhull, 1973). In contrast, Ba^{2+} alone inhibited most of the inward current. The reduction of G_{in} by Ba^{2+} was not significantly different from that seen when both quinidine and Ba^{2+} were added together: 148·2 ± 19·3 *vs.* 151·4 ± 19·8 μS cm^{-2} ($n = 17$). Addition of quinidine on top of Ba^{2+} gave no further reduction in inward current (Fig. 5*A*). Addition of quinidine alone had very little effect on inward current flow through G_{Ba} due to relief of block at negative potentials. The initial G_{in} on stepping clamp potential was significantly smaller than that recorded 500 ms after stepping the clamp potential: 171·7 ± 29·4 *vs.* 198·0 ± 9·5 μS cm^{-2} ($n = 16$). Therefore, quinidine inhibits mainly outward currents, whereas Ba^{2+} inhibits mainly the inward currents.

If we assume that there are two conductances (see Discussion), one of which is sensitive to Ba^{2+} and quinidine, the other sensitive to quinidine alone, we can plot the corresponding *I–V* curves as shown in Fig. 5*B*. The Ba^{2+}-sensitive conductance showed inward rectification in symmetrical solutions. G_{out} was significantly smaller than G_{in}: 18·4 ± 5·6 *vs.* 148·2 ± 19·3 μS cm^{-2} ($n = 17$). In contrast, the quinidine-sensitive conductance (measured by addition of quinidine in the presence of Ba^{2+}) showed outward rectification. G_{out} was significantly larger than G_{in}: 29·4 ± 6·7 *vs.* 4·2 ± 1·1 μS cm^{-2} ($n = 17$).

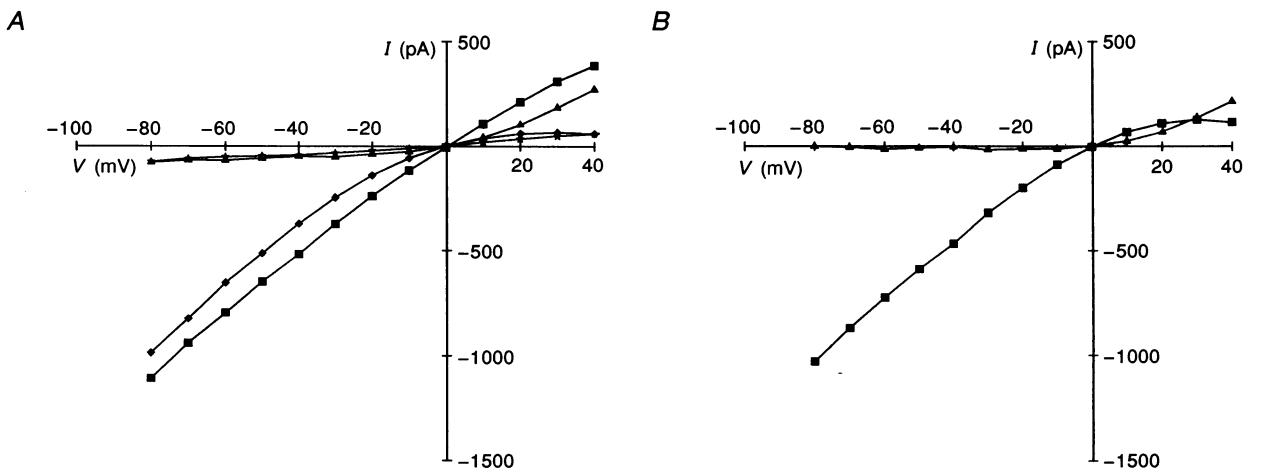

Figure 5. Blocker-sensitive currents in symmetrical KCl solutions

Typical *I–V* curves from the same cell. *A*, currents recorded either under the control circumstance (■), Ba^{2+} alone (▲), quinidine alone (◆) or both inhibitors (★). *B*, Ba^{2+}-sensitive current (■) and quinidine-sensitive current (▲, measured in the presence of Ba^{2+}) from the cell shown in *A*.

Isolation of K^+ currents

G_{Ba}. In the absence of pipette ATP the initial G_{Ba}, measured at +33 mV, was 29·0 ± 7·1 μS cm^{-2} ($n = 12$). After 10 min this had fallen to 12·3 ± 3·9 μS cm^{-2} (Fig. 6). With 2 mM ATP the initial G_{Ba} increased significantly over 10 min from 55·1 ± 8·6 to 85·1 ± 9·1 μS cm^{-2} ($n = 25$). In contrast, when 2 mM AMP-PNP was included in the pipette solution there was no significant change in G_{Ba} over 10 min: 37·4 ± 6·7 *vs.* 35·4 ± 5·3 μS cm^{-2} ($n = 18$).

G_{quin}. In the absence of ATP the initial G_{quin} was 43·2 ± 11·7 μS cm^{-2} ($n = 7$). By 10 min it had fallen to 7·3 ± 2·5 μS cm^{-2} (Fig. 6). When 2 mM ATP was present there was no significant change in G_{quin} over 10 min: 54·7 ± 14·1 *vs.* 57·0 ± 14·7 μS cm^{-2} ($n = 20$). When 2 mM AMP-PNP was included in the pipette solution G_{quin} decayed as in the ATP-free condition: 40·2 ± 10·4 *vs.* 5·9 ± 1·9 μS cm^{-2} ($n = 8$).

For outward currents recorded at +33 mV, G_{Ba} was significantly larger than G_{quin}: 67·2 ± 20·2 *vs.* 43·2 ± 11·7 μS cm^{-2}, respectively ($n = 10$).

Single channel currents

The basolateral K^+ channel was inhibited by both Ba^{2+} and quinidine (Fig. 7). Addition of these inhibitors to the bath reduced both single channel open probability and single channel current (Fig. 8). Channel activity was sensitive to ATP. In the absence of ATP, 10 min after excising the inside-out patch there was no detectable channel activity: $NP_o = 0{\cdot}00 \pm 0{\cdot}00$ ($n = 7$; Fig. 9). However, with 2 mM ATP in the bath channel activity was increased significantly to 0·140 ± 0·009 ($n = 7$; Fig. 9).

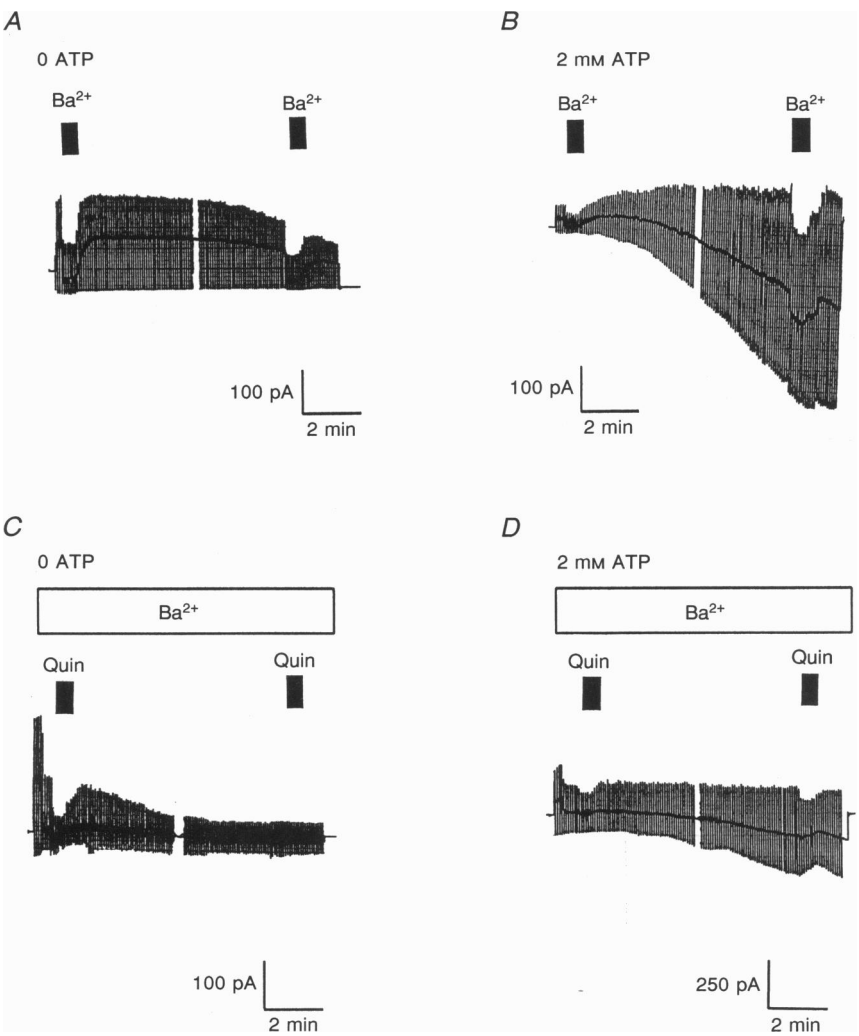

Figure 6. Changes in G_{Ba} (upper) and G_{quin} (lower) with time

Typical current traces recorded with either 0 ATP in the pipette (*A* and *C*) or 2 mM ATP (*B* and *D*). Black boxes indicate when either 5 mM Ba^{2+} or 1 mM quinidine were added transiently to the bath. Note that 5 mM Ba^{2+} was present continuously in *C* and *D*.

DISCUSSION

The sensitivities of the currents to Ba^{2+} and quinidine, and the differential effects of ATP and AMP-PNP on these currents indicate that single proximal tubule cells of the frog contain at least two separate K^+-selective conductances. Multiple K^+ channel types, with differing blocker sensitivities, have been reported in a number of cells, e.g. turtle colon and sheep parotid secretory cells (Germann, Lowy, Ernst & Dawson, 1986; Ishikawa & Cook, 1993). The blocker sensitivity of the frog proximal tubule reported here, where one conductance was inhibited by Ba^{2+} and quinidine, and the second by quinidine alone, is in direct contrast to turtle colon, in which two types of channel were inhibited by Ba^{2+}, only one being inhibited by quinidine (Germann *et al.* 1986).

Several studies have demonstrated the existence of more than one K^+ channel in the renal proximal tubule. However, the blocker sensitivities of these channels is not well documented. In *Necturus* proximal tubule two stretch-activated K^+ channels have been found on the basolateral membrane and a Ca^{2+}-activated K^+ channel on the apical membrane (Filipovic & Sackin, 1991; Filipovic & Sackin, 1992). In rabbit proximal tubule two K^+ channel species have been observed on the apical membrane and one type on the basolateral membrane (Merot, Bidet, Le Maout, Tauc & Poujeol, 1989; Beck, Hurst, Lapoint & Laprade, 1993). Our earlier single channel study in frog isolated proximal cells revealed only one type of K^+ channel, situated on the basolateral membrane (Hunter, 1991). Obviously, other K^+ channels could be present which either exist on the apical

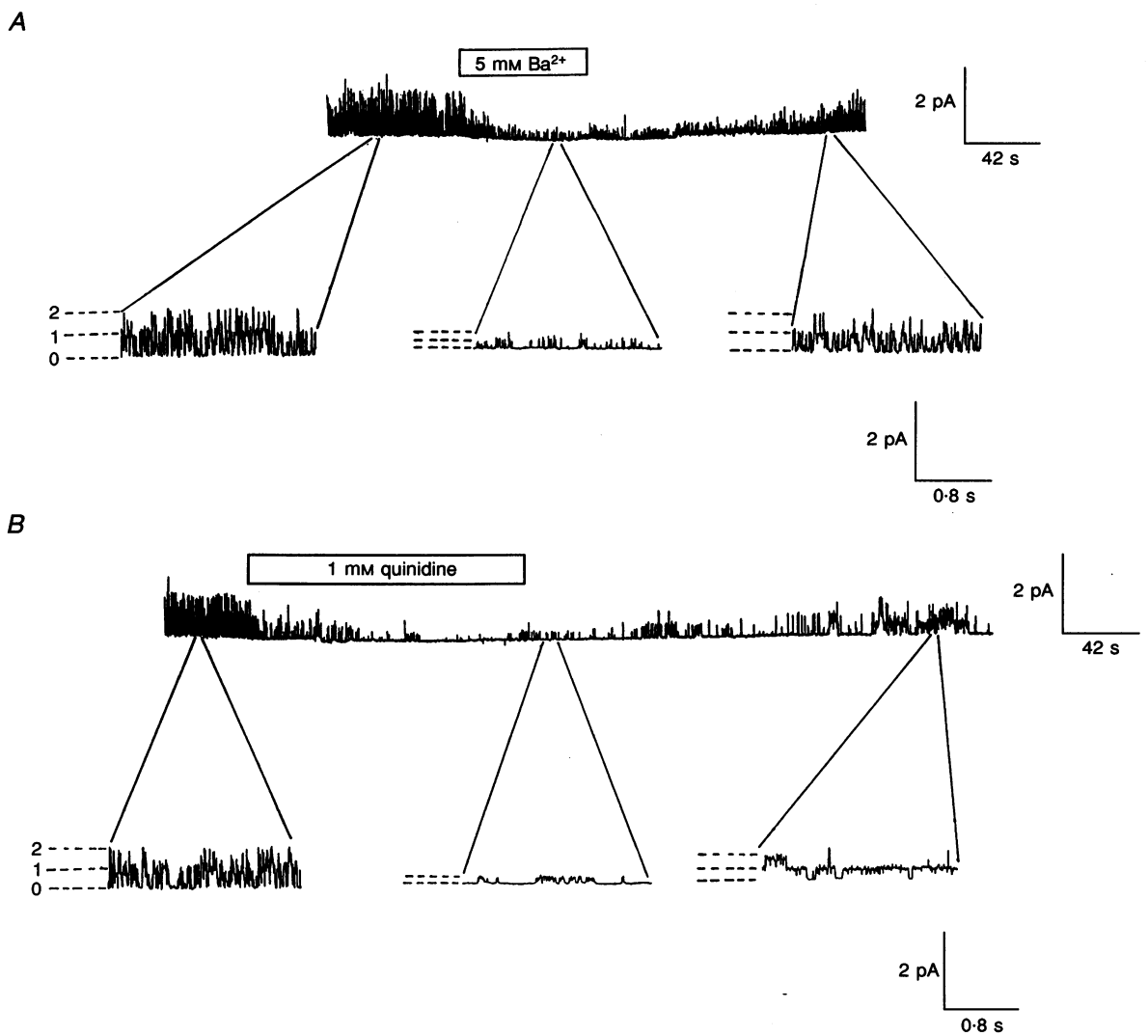

Figure 7. Typical single channel records taken from a cell-attached patch of the basolateral membrane showing the effect of adding Ba^{2+} (*A*) and quinidine (*B*) to the bath

The open boxes indicate when the blocker was present in the bathing solution. The continuous lines point to sections of the recording which are shown on an expanded time scale. The dotted lines represent discrete channel openings: 0 represents the closed channel level, 1 represents 1 channel open, etc. Both *A* and *B* were taken from the same patch recording.

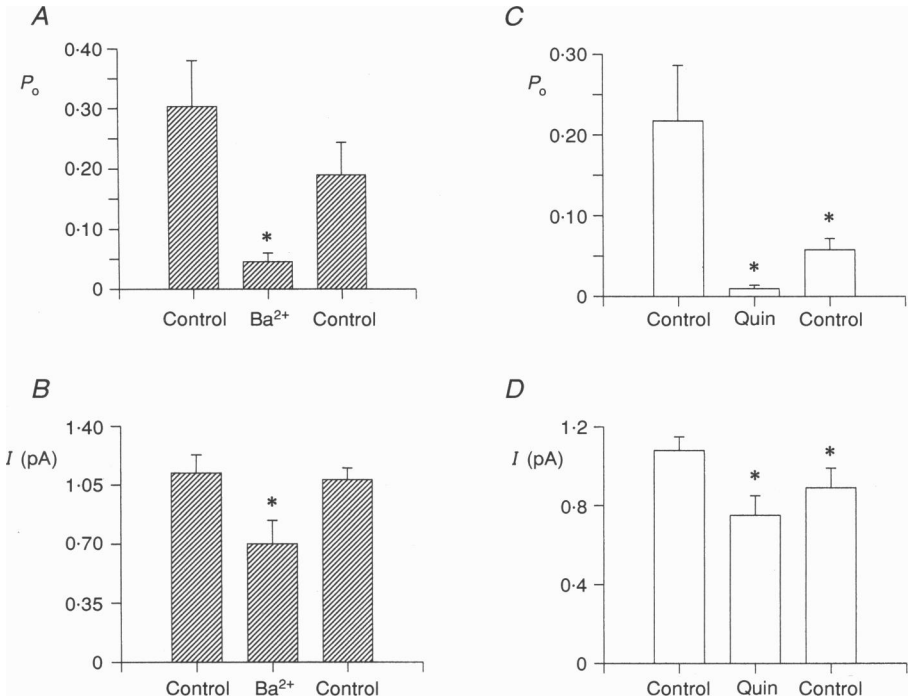

Figure 8. Effect of Ba^{2+} and quinidine on P_o (*A* and *C*) and single channel current (*B* and *D*)
* Significant difference from initial control level.

membrane, or which may also reside in the basolateral membrane, but with such small single channel currents that they are not resolved by the patch clamp technique.

Characteristics of G_{Ba}

The data indicate that G_{Ba} is a K^+ conductance. It was 50 times more selective for K^+ over Na^+. This is within the reported selectivity range for other renal K^+ channels, e.g. 10–70 times selective for K^+ over Na^+ (Kawahara, Hunter & Giebisch, 1987; Gitter, Bayenbach, Christine, Gross, Minuth & Fromter, 1987). The very fact that the conductance was inhibited by Ba^{2+} also suggests that G_{Ba} is a K^+ conductance. Ba^{2+} is a well-known blocker of K^+ channels (Hagiwara, Miyazaki, Moody & Patlak, 1978) and has been shown to inhibit a number of K^+ channels in renal proximal tubule e.g. two channels on the apical membrane of cultured rabbit proximal tubule cells and a channel on the basolateral membrane of *Necturus* proximal tubule (Kawahara *et al.* 1987; Merot *et al.* 1989).

Inhibition by Ba^{2+} was both dose and voltage dependent. The K_d of 21·8 μM at 0 mV was within the range of other epithelial K^+ channels, e.g. 1, 100 and 135 μM (De Wolf & Van Driessche, 1986; Ishikawa & Cook, 1993). Hyperpolar-

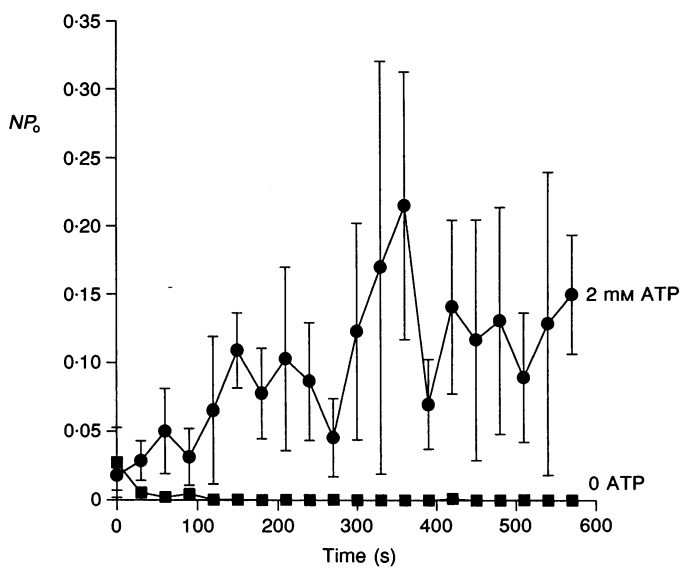

Figure 9. Effect of ATP on NP_o of the basolateral K^+ channel
Mean NP_o is shown against time after excision for patches exposed to either 0 ATP (■) or 2 mM ATP (●).

ization increased the degree of block, presumably by attraction of positively charged Ba^{2+} ions into the channel. Using the Woodhull equation, we calculate that the Ba^{2+} ions sensed approximately 20% of the electrical field across the membrane, i.e. the blocking site was located around 20% of the way from the extracellular face of the channel pore, similar to that in other K^+ channels (De Wolf & Van Driessche, 1986). Similar to a study in guinea-pig enterocytes, the Hill coefficient of 1·0 suggests that only one Ba^{2+} ion is necessary for block (Tatsuta, Ueda, Morishima & Okada, 1994).

Characteristics of G_{quin}

Quinidine has been shown to block K^+ channels in a variety of cell types (Merot *et al.* 1989; Garcia-Diaz, 1991). Once again, the K_d (16 μM) is within the range reported for other K^+ channels, e.g. 41 and 6 μM (Kehl, 1991; Synders, Knoth, Roberds & Tamkun, 1992). However, in contrast to that seen in a number of voltage-gated K^+ channels (Kehl, 1991; Synders *et al.* 1992) inhibition was not voltage dependent. At pH 7·4, quinidine molecules would be expected to carry a positive charge (Kehl, 1991; Synders *et al.* 1992). So, the lack of voltage dependence indicates that quinidine does not sense the potential across the membrane and may inhibit by acting at the extreme outside of the pore or at a site distant from the channel pore. As with Ba^{2+}, the Hill coefficient of 0·87 indicates that only one molecule of quinidine was necessary for blockade (Synders *et al.* 1992; Kehl, 1991). Despite the lack of voltage dependence when cells were superfused with Ringer solution, in symmetrical KCl solutions relief of block was observed with a large inward movement of K^+. A similar relief of quinidine block has also been observed in a cardiac K^+ channel (Balser, Bennett, Hondeghem & Roden, 1991). The single channel data may provide a clue as to the mechanism underlying this effect. These data show that, as with Ba^{2+} (Hunter, 1991; present study), quinidine can inhibit the basolateral K^+ channel in cell-attached patches. Under this circumstance quinidine is presumably acting at the intracellular surface of the channel. Therefore, relief of block at negative potentials may be a consequence of quinidine molecules being displaced by the inward movement of K^+ ions through the channel. Such a mechanism has been postulated to occur with TEA inhibition of K^+ channels and underlies the inward rectification induced by Mg^{2+} in inward rectifiers (Armstrong, 1971; Matsuda, Saigusa & Irisawa, 1987).

The outwardly rectifying behaviour of G_{quin} could be due either to rectification of the current flow through the channels or to voltage dependence of channel open probability (with an increase in activity occurring on depolarization) or a combination of these two. The apical Ca^{2+}-activated K^+ channel observed on the apical membrane of *Necturus* proximal tubule is activated by depolarization (Filipovic & Sackin, 1991), as is a K^+ channel in primary cultures of rabbit proximal tubule cells (Merot *et al.* 1989).

Although G_{quin} was only poorly K^+ selective (about 4 times more permeable for K^+ than Na^+), its properties distinguish it from the other conductances which we have described in these cells. In symmetrical K^+-rich solutions, G_{quin} showed outward rectification; G_{out} was approximately 8 times G_{in}. It seems unlikely, therefore, that it could represent the basolateral K^+ channel previously found in these cells, as this channel demonstrated inward rectification (Hunter, 1991). Consistent with this hypothesis the basolateral K^+ channel is inhibited by Ba^{2+}, whereas G_{quin} is insensitive to Ba^{2+}. At present, no other K^+ channels have been identified in frog isolated proximal cells at the single channel level, although, as discussed previously, both mammalian and *Necturus* proximal tubule cells have at least two K^+ channel types.

Given that the electrochemical gradient for Na^+ entry is much greater than that for K^+ exit, this relative lack of discrimination begs the question as to whether G_{quin} acts to carry Na^+ into, or K^+ out of, the cell at the resting membrane potential. The reversal potential for G_{quin} was −30 mV, which lies positive to the resting membrane potential, and it thus carries a net inward current, and acts as a Na^+ leak conductance. However, given that cells may depolarize to values more positive than −30 mV during acute exposure to cotransported substrates (Lapointe *et al.* 1990), it is feasible that G_{quin} might play a role in voiding K^+ under these conditions. In this respect, it is interesting that G_{quin} showed outward rectification (G_{out} was about 8 times greater than G_{in}), such that the capacity for K^+ efflux is greater than that for Na^+ influx.

Regulation by ATP

ATP-sensitive K^+ channels (K_{ATP}) have been observed in a wide range of cell types, including pancreatic β cells (Ashcroft & Kakei, 1989), skeletal muscle (Spruce, Standen & Stanfield, 1985), cardiac muscle (Noma, 1983) and renal tubules (Hurst *et al.* 1993). K_{ATP} channels have several distinctive features. They are activated by low concentrations of ATP, via a mechanism dependent on ATP hydrolysis (Wang & Giebisch, 1991), and yet inhibited by high ATP concentrations (Wang & Giebisch, 1991; Ribalet, Ciani & Eddlestone, 1989). They also demonstrate run-down when patches containing channels are excised into nucleotide-free media (Wang & Giebisch, 1991; Ribalet *et al.* 1989).

In the kidney, K_{ATP} channels are present on the basolateral membrane of rabbit proximal tubule (Hurst *et al.* 1993). In the renal proximal tubule it has been suggested that K_{ATP} is coupled to the activity of the basolateral Na^+,K^+-ATPase, with a rise in pump activity leading to a fall in intracellular ATP and relief of ATP inhibition of K_{ATP} (Hurst *et al.* 1993). This is thought to prevent K^+ accumulation by the cell and therefore maintain intracellular ion composition and cell volume. Additionally, loss of K^+ by K_{ATP} may also help to maintain the electrical driving force for the movement of Na^+ into the cell across the apical membrane, thus

sustaining Na^+–substrate reabsorption via amino acid and sugar cotransporters (Lapointe *et al.* 1990).

In the present study, G_{Ba}, G_{quin} and the basolateral K^+ channel ran down in the absence of ATP and maintenance of channel activity was dependent upon the presence of ATP. Thus in the absence of ATP, cells lost their K^+ selectivity, single channel activity disappeared and G_{Ba} and G_{quin} both decreased. In contrast they were stimulated or maintained in the presence of ATP. The activation of G_{Ba} by ATP was dependent on ATP hydrolysis. However, in addition, G_{Ba} was maintained via an allosteric action of ATP, since the non-hydolysable analogue of ATP, AMP-PNP, maintained, yet failed to activate, the conductance. In contrast, the maintenance of G_{quin} by ATP was dependent solely on ATP hydrolysis. Both G_{Ba} and G_{quin} therefore have characteristics in common with the classic K_{ATP} channels, e.g. run-down and activation by ATP via a mechanism dependent on ATP hydrolysis (Ribalet *et al.* 1989; Wang & Giebisch, 1991). However, the concentration of ATP used in the present study, 2 mM, would typically be expected to inhibit K_{ATP}, as has been shown in a number of studies including those on rabbit proximal tubule (Ribalet *et al.* 1989; Wang & Giebisch, 1991; Hurst *et al.* 1993). Therefore, although these conductances are sensitive to ATP, they do not seem to be attributable to K_{ATP}. There are three reports of renal K^+ channels which are similarly activated by millimolar concentrations of ATP. These are K^+ channels of opossum kidney cells and medullary collecting duct cells, and the cloned K^+ channel, ROMK1 (Ohno-Shosaku, Kubota, Yamaguchi, Fukase, Fujita & Fujimoto, 1989; Ho *et al.* 1993; Sansom, Mougouris, Ono & DuBose, 1994). Thus, there seems to be a population of K^+ channels in the kidney which are dependent upon ATP, but which are different from K_{ATP} in that they are not inhibited by millimolar concentrations of ATP.

Comparison of single channel and whole-cell data

The single channel data suggest that G_{Ba} may represent the basolateral K^+ channel, as they share many properties. A previous study demonstrated that the basolateral K^+ channel shows inward rectification, with inward conductance approximately 5 times larger than outward conductance (Hunter, 1991). G_{Ba} also shows inward rectification. Under symmetrical K^+ conditions, G_{in} was approximately 8 times larger than G_{out}. Both G_{Ba} and the basolateral K^+ channel are inhibited by the typical K^+ channel blockers Ba^{2+} and quinidine. Although the effect of quinidine on whole-cell currents was reversible, its effect on the single channel was not. This difference in reversibility could reflect the different techniques used; in cell-attached recordings quinidine might become trapped within the cell, whereas in the whole-cell experiments quinidine may be washed out into the (relatively) vast reservoir of the pipette solution. Finally, G_{Ba} and the basolateral K^+ channel are activated by ATP. Therefore it seems likely that G_{Ba} represents the basolateral K^+ channel.

In summary, frog proximal tubule cells contain two ATP-sensitive K^+-selective conductances which may be separated on the basis of their blocker sensitivity. G_{Ba} seems to be attributable to the basolateral K^+ channel identified previously in these cells and may represent the resting K^+ conductance. Indeed, at the resting membrane potential (approximately −70 mV) this conductance seems to provide the sole K^+ leak pathway. On the other hand, G_{quin} acts as a Na^+ leak pathway at the resting potential. However, G_{quin} may act as an additional K^+ leak pathway when the cells are in a depolarized state, e.g. during acute exposure to cotransported substrates.

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