

Cross-talk between ATP-regulated K⁺ channels and Na⁺ transport via cellular metabolism in frog skin principal cells

Valérie Urbach, Emmy Van Kerkhove*, Donal Maguire and Brian J. Harvey

Wellcome Trust Cellular Physiology Research Unit, Department of Physiology, University College, Cork, Ireland and *Department of Physiology, Limburgs Universitair Centrum, B-3590 Diepenbeek, Belgium

1. Isolated frog skin epithelium, mounted in an Ussing chamber and bathed in standard NaCl Ringer solution, recycles K⁺ across the basolateral membrane of principal cells through an inward-rectifier K⁺ channel (K_{ir}) operating in parallel with a Na⁺-K⁺-ATPase pump. Here we report on the metabolic control of the K_{ir} channel using patch clamping, short-circuit current measurement and enzymatic determination of cellular ATP (ATP_i).
2. The constitutively active K_{ir} channel in the basolateral membrane has the characteristics of an ATP-regulated K⁺ channel and is now classed as a K_{ATP} channel. In excised inside-out patches the open probability (P_o) of K_{ATP} channels was reduced by ATP_i with half-maximum inhibition at an ATP_i concentration of 50 μM.
3. ATP_i measured (under normal Na⁺ transport conditions) with luciferin-luciferase was 1.50 ± 0.23 mM (mean ± s.e.m.; range, 0.4–3.3 mM, n = 11). Thus the K_{ATP} channel would be expected to be inactive in intact cells if ATP_i was the sole regulator of channel activity. K_{ATP} channels which were inactivated by 1 mM ATP_i in excised patches could be reactivated by addition of 100 μM ADP on the cytosolic side. When added alone, ADP blocks this channel with half-maximal inhibition at [ADP_i] > 5 mM.
4. Sulphonylureas inhibit single K_{ATP} channels in cell-attached patches as well as the total basolateral K⁺ current measured in frog skin epithelia perforated with nystatin on the apical side.
5. Na⁺-K⁺-ATPase activity is a major determinant of cytosolic ATP. Blocking the pump activity with ouabain produced a time-dependent increase in ATP_i and reduced the open probability of K_{ATP} channels in cell-attached membranes.
6. We conclude that the ratio of ATP/ADP is an important metabolic coupling factor between the rate of Na⁺-K⁺ pumping and K⁺ recycling.

The reabsorption of Na⁺ across principal cells of frog skin epithelium is mediated by passive entry through channels in the apical membrane and active secretion via an Na⁺-K⁺-ATPase localized in the basolateral membrane. In parallel with the Na⁺-K⁺ pump activity, K⁺ is recycled through a channel which was recently identified as an inward-rectifier K⁺ channel (K_{ir}) localized in the basolateral membrane of principal cells (Urbach, Van Kerkhove & Harvey, 1994).

The maintenance of equilibrium between transepithelial Na⁺ absorption and K⁺ recycling involves a concerted action of cellular signals (cross-talk). Coupling between the apical Na⁺ conductance and the basolateral K⁺ conductance has been described in frog skin and turtle and toad urinary

bladder (Davis & Finn, 1982; Harvey, Thomas & Ehrenfeld, 1988; Dawson & Richards, 1990). These studies have demonstrated a role for membrane potential, pH and Ca²⁺ in mediating cross-talk. In tight epithelia, pH_i has been shown to be a potent regulator of net sodium absorption via the pH sensitivity of apical Na⁺ and basolateral K⁺ channels (Harvey *et al.* 1988; Oberleithner, Kersting & Gassner, 1988; Willumsen & Boucher, 1992; Rick, 1994). Recent studies in rat collecting duct, however, failed to demonstrate significant changes in pH_i following ouabain inhibition of the Na⁺-K⁺-ATPase pump. It has been shown that the latter effect produces cross-talk by inhibition of apical Na⁺ absorption (Silver, Frindt, Windhager & Palmer, 1993) and basolateral K⁺ conductance (Messner, Wang

Paulmichl, Oberleithner & Lang, 1985). The lack of effect of ouabain on pH_i in renal CCT (cortical collecting tubule) may indicate that other factors, such as ATP, which are influenced by pump activity can mediate cross-talk under these conditions. Intracellular ATP has been described as a putative modulator of cross-talk between the Na^+ pump and K^+ channels in renal proximal tubule (Tsuchiya, Wang, Giebisch & Welling, 1992) and cortical collecting tubule (Wang & Giebisch, 1991). In the present study in frog skin principal cells, we have identified a sulphonylurea-inhibitable, ATP-regulated K^+ channel which is identical in electrophysiological terms to the K_{ir} channel. We have investigated K_{ir} channel sensitivity to changes in the ATP/ADP ratio using a combination of patch clamping, short-circuit current (SCC) measurement and enzymatic determination of cellular ATP. We demonstrate the control of the K_{ir} channel by the cytosolic ATP/ADP ratio. The important influence of the Na^+ - K^+ pump on the ATP/ADP ratio suggests a metabolic coupling between pump and channel. We have tested this hypothesis by determining the effect of inhibition of Na^+ - K^+ -ATPase by ouabain on the intracellular ATP concentration and on single K_{ATP} channel activity.

METHODS

Short-circuit current and basolateral membrane K^+ conductance

The ventral skin was dissected off doubly pithed frogs (*Rana esculenta* and *Rana temporaria*) and mounted in an Ussing-type chamber and bathed in standard amphibian Ringer solution containing (mM): 110 NaCl, 3.7 KOH, 6 Hepes, 2 CaCl_2 and 1 MgCl_2 , pH 7.4, 260 mosmol l^{-1} . The spontaneous transepithelial voltage, measured with calomel electrodes, was clamped to 0 mV by applying a SCC using an automatic voltage-current clamp (model DVC-100, World Precision Instruments, Sarasota, FL, USA). The transepithelial Na^+ transport rate was estimated as equivalent to the amiloride (10 μM)-sensitive SCC. To examine the basolateral membrane conductance in isolation, the apical membrane was permeabilized using the ionophore nystatin (Kirk & Dawson, 1983; Garty, 1994). In these experiments, the apical side was bathed in a high- K^+ solution (potassium bathing solution, KBS) to mimic the intracellular ionic composition. This contained (mM): 20 NaCl, 90 potassium gluconate, 3.2 MgSO_4 , 1.2 KH_2PO_4 , 2.9 K_2HPO_4 , 11 glucose, 10 Hepes, adjusted to pH 7.2 with KOH, 260 mosmol l^{-1} . Ca^{2+} was buffered to 100 nM using EGTA (0.2 mM CaCl_2 + 5 mM EGTA). Nystatin (500 i.u. ml^{-1}) was added to the apical side after equilibration of SCC. The basolateral membrane was bathed with standard amphibian Ringer solution. Under these conditions, the transepithelial SCC was generated essentially by K^+ diffusion across the basolateral membrane.

Patch-clamp recording

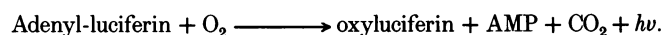
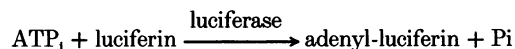
The methods used for tissue isolation and patch-clamp recording from basolateral membranes in intact epithelia mounted in Ussing chambers have been described in detail (Urbach *et al.* 1994). Epithelial sheets were isolated from the ventral skin of *Rana esculenta* and *Rana temporaria* using collagenase to separate the

corium. The isolated epithelium was cut into four discs (1.6 cm^2) and mounted basolateral side upwards in a miniature Ussing chamber on the stage of an inverted microscope (Nikon TMD). The basement membrane was detached by flushing the epithelium in Ca^{2+} -free Ringer solution buffered with 2 mM EGTA for 3–10 min. Epithelia isolated by this method had Na^+ transport rates and transepithelial resistances comparable to whole skins. Micro-electrode access to the basolateral membranes of the first apical cell layer (principal cells) was achieved by clearing a small area (100 μm^2) of the underlying germinal cells using a suction micropipette while locally flushing the area in Ca^{2+} -free EGTA Ringer through a second micropipette. The apical and basolateral sides of the epithelium were normally bathed in standard amphibian Ringer solution. In patch-clamp experiments on excised inside-out membranes the basolateral bath Ringer solution was replaced by a solution designed to mimic the intracellular ionic composition (K_{int}), which contained (mM): 15 NaCl, 150 KCl, 5 EGTA, 0.2 CaCl_2 , 10 Hepes, 3 MgCl_2 , pH adjusted to 7.2 with KOH. The free concentration of calcium was 10 nM, calculated using a computer program (Chang, Hsieh & Dawson, 1988). All chemicals were purchased from Sigma.

The cell-attached and inside-out configurations of the patch-clamp technique were used for single-channel studies (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) and their application to epithelial sheets has been previously described in detail (Urbach *et al.* 1994). Briefly, membrane current was recorded under voltage-clamp conditions and amplified using a Biologic RK300 patch-clamp amplifier (Biologic, Claix, France). Current signals were low-pass filtered (–3 dB) at 5 kHz (8-pole Bessel filter, model 902; Frequency Devices, Inc., Haverhill, MA, USA) and recorded on digital audio tape (DTR model 1202; Biologic). Current and voltage signals were digitized using a 16-bit A/D converter (CED 1401; Cambridge Electronic Design, Cambridge, UK). Current–event histograms, current–voltage relations, mean channel open and closed times, and open probability were calculated from 60 s current record segments using PAT software (supplied by J. Dempster, Strathclyde Electrophysiology Software, V6.1, University of Stathclyde, Scotland). Cation movement across the membrane from the external to the cytoplasmic side is defined as inward current and shown as downward deflections in single-channel records.

[ATP]_i measurements

Intracellular adenosine triphosphate concentration, $[\text{ATP}]_i$, was measured on 7 cm^2 pieces of isolated epithelium, in response to experimentally induced changes in cellular metabolism. ATP_i was extracted from the isolated epithelium composed mainly of principal cells (gland-free preparation) after destruction of the membranes by boiling (2 min). Subsequently the sample was maintained at 0 °C to prevent hydrolysis of ATP. The $[\text{ATP}]_i$ was determined in each sample by a bioluminescence technique (Luciferin/Luciferase; Sigma) which uses the reaction:



The intensity of emitted light ($h\nu$) is proportional to the quantity of ATP in the sample and was detected with a photometer (Chem-Glow, American Instrument Company, Silverspring, MD, USA). The $[\text{ATP}]_i$ of each sample was quantified against standard ATP

solutions (0.5, 1, 2 and 3 mM) and is given as mmoles per litre of cytosol (corrected for extracellular space).

Data are shown as mean values \pm s.e.m., and n is the number of experiments.

RESULTS

Inward-rectifier K_{ir} channel

As in a previous study (Urbach *et al.* 1994) the predominant channel recorded in cell-attached basolateral membranes was an inward-rectifier K^+ selective channel (K_{ir}). The voltage dependence of single-channel current and open probability for this channel is shown in typical experiments presented in Fig. 1. In cell-attached patches exposed to physiological ionic gradients (Na^+ Ringer solution in bath and patch pipette; Fig. 1A) the single-channel conductance (γ) was 3 ± 1 pS for outward current and 15 ± 2 pS for inward current ($n = 8$). In excised inside-out patches exposed to a standard Ringer solution in pipette and K_{int} solution in the bath (Fig. 1A) the K_{ir} channel $I-V$ relationship displayed similar inward rectification to that in cell-attached patches ($\gamma = 5 \pm 1$ pS for outward current and $\gamma = 17 \pm 2$ pS for inward current, $n = 8$). From the reversal potential and the known transmembrane Na^+ and K^+ gradients, a Na^+-K^+ selectivity coefficient of 0.013 was calculated from the Goldman-Hodgkin-Katz (GHK) equation. Under physiological ionic gradients the open probability of this channel was maximal at the resting membrane potential and decreased when the membrane was hyperpolarized (Fig. 1B). When the channel was exposed to a high external K^+ concentration (K_{int} solution in the pipette; Fig. 1A) the channel was transformed to a pure inward-rectifier channel ($\gamma = 35$ pS for inward current).

Effect of ATP/ADP on K_{ir} channel activity

ATP has been described as an inhibitor (Bleich, Schlatter & Greger, 1990; Wang & Giebisch, 1991) or an activator (Friedrich, Weiss, Paulmichl & Lang, 1989; Ohno-Shoshaku, Kubota, Yamaguchi & Fujimoto, 1990) of inward-rectifier K^+ channels in epithelia. We examined the effects of ATP over a range of probable cellular concentrations. Mg-ATP (0.1–5 mM) was added to the cytosolic side of spontaneously active excised inside-out patches bathed in standard Na^+ Ringer or symmetrical K^+ solutions.

The single K_{ir} channel recordings show that ATP_i (1 mM) reversibly blocked inward currents (Fig. 2A) and outward currents. Inward current amplitude histograms for the three situations of control, ATP and washout are given in Fig. 2B. ATP_i reversibly decreased open channel probability (P_o) as demonstrated by the decreased area under the Gaussian curve fit to open channel current events (Fig. 2B). Open channel probability was reduced from 0.23 in control to 0.06 in the presence of ATP (1 mM) and recovered to 0.17 with washout of ATP. The reduction in open probability resulted from a 50% decrease in mean open time (control, 3.62 ms; ATP, 1.77 ms) and a 30% increase in mean closed time (from 16.6 to 22.1 ms). Open-time dwell histograms were best fitted by a single-exponential decay function with mean open half-times, $\tau_{1,open}$, of 2.71 ± 0.05 ms in control and 1.22 ± 0.01 ms ($n = 8$) in ATP. Closed-time dwell histograms were best fitted by a double-exponential decay function with mean closed half-times, $\tau_{1,closed}$ and $\tau_{2,closed}$, of, respectively, 1.49 ± 0.05 and 21.4 ± 9.4 ms in control and 1.30 ± 0.03 and 18.7 ± 8.5 ms in ATP ($n = 8$).

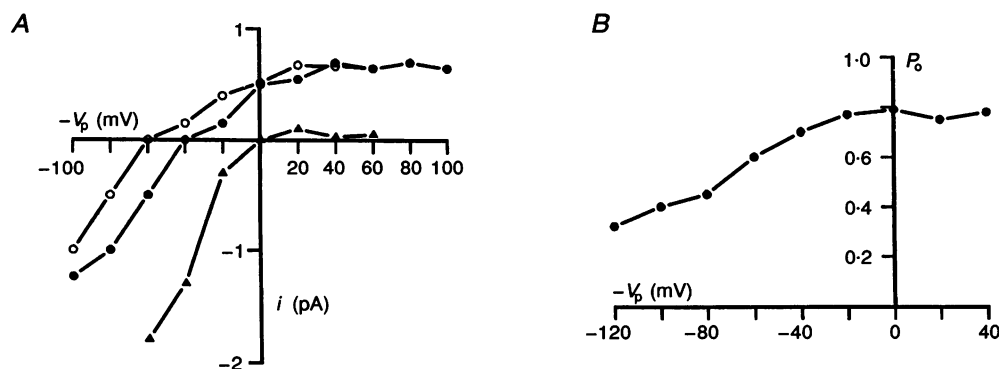


Figure 1. Identification of the K_{ir} channel

A, current-voltage relations of single K_{ir} channels in cell-attached configuration with normal Ringer solution in patch pipette and bath (●), after excision into inside-out configuration with normal Ringer solution in the pipette and K_{int} solution in the bath (○), and in an inside-out patch exposed to K_{int} solution in the external and cytosolic side (▲). Data are from different cells in each case. B, open probability of the K_{ir} channel as a function of membrane patch potential ($-V_p$ is indicative of changes produced in V_m , the membrane potential) obtained in cell-attached configuration with normal Ringer solution in the patch pipette and bath.

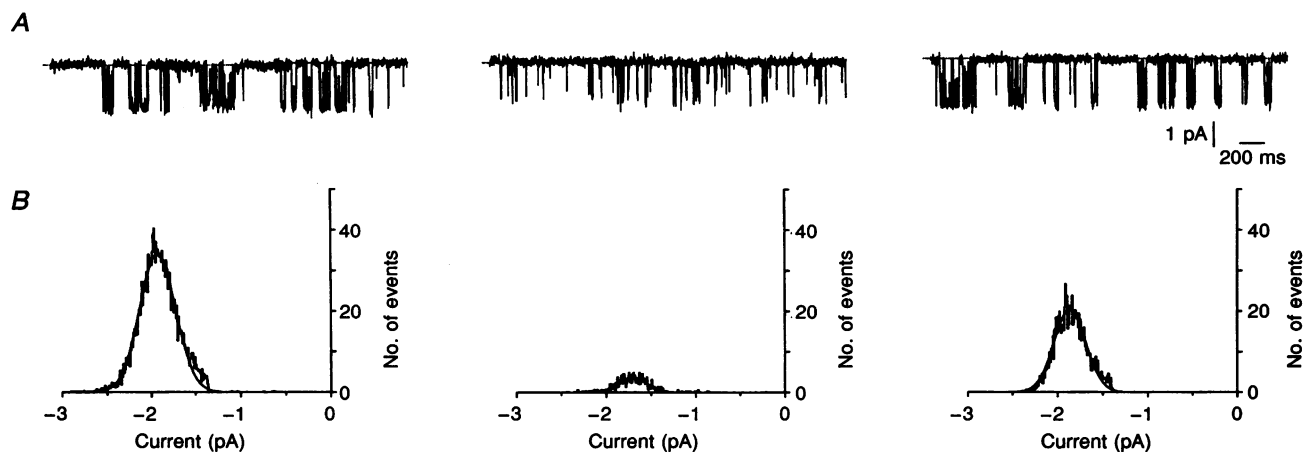


Figure 2. ATP effect on inward currents through the K_{Ir} channel

A, effects of ATP on K_{Ir} channel inward currents recorded from excised inside-out membranes exposed to K_{int} solutions on both sides; $V_p = 40$ mV; cut-off frequency 1 kHz. Left trace, control conditions. Middle trace, 60 s after addition of 1 mM ATP to the bath. Right trace, 60 s after wash-out of ATP. *B*, current amplitude for control, and during inhibition by ATP and washout conditions from 60 s records. ATP reduced the single-channel current and mean open time.

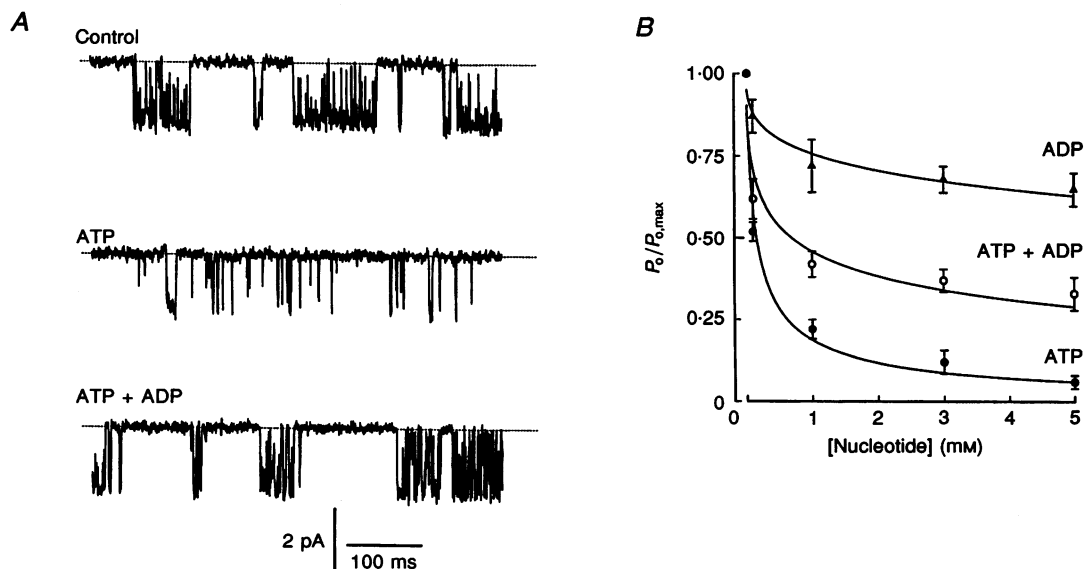


Figure 3. ATP and ADP effects on inward currents through the K_{Ir} channel

A, single-channel recording ($V_p = 40$ mV) from an excised membrane exposed to symmetrical K_{int} solutions. Upper trace, control conditions. Middle trace, effect of 1 mM ATP concentration on K_{Ir} inward currents. Lower trace, partial reversal of ATP block by 0.1 mM ADP. *B*, dependence of open probability (P_o) for inward current, on internal ATP/ADP concentration: ●, ATP only; ▲, ADP only; ○, variable [ATP] and 0.1 mM ADP. Data are shown as means of 8 cells from 4 different epithelia for control and 0.1 mM ATP, and 8 cells from 3 epithelia for 1 mM ATP and ADP. Data points fitted by the Hill equation:

$$P_o/P_{max} = \frac{[nucl]_{min} - ([nucl]_{max} - [nucl]_{min})}{(1 + ([nucl]/[nucl]_{50})^k)}$$

where $[nucl]_{min}$, $[nucl]_{max}$ and $[nucl]_{50}$ are the concentrations of nucleotide at which P_o/P_{max} is minimal, maximal and 50%, respectively, and k is the nucleotide-binding coefficient.

The single-channel record presented in Fig. 3A shows that lower concentrations of ATP_1 corresponding to approximately one tenth of the expected normal cytosolic level of ATP caused a marked reduction in K_{ir} channel activity. The dependence of open probability on the cytosolic ATP concentration is presented in Fig. 3B. Channel activity was reduced by 50% at an ATP_1 concentration of $152 \pm 44 \mu M$ ($n = 8$). The best-fit ATP_1 -binding coefficient ($k = 0.80 \pm 0.15$; $n = 8$) is consistent with a single ATP-binding site model. $[ATP]_i$ measured in epithelial cells under normal Na^+ transport conditions was 1.50 ± 0.23 mM (range, 0.4–3.3 mM; $n = 11$). Adenylyl imidophosphate (AMP-PNP; 1 mM) a non-hydrolysable analogue of ATP, had a similar effect to Mg-ATP (1 mM) in reducing the open probability of K_{ir} channels ($n = 8$; data not shown). Thus it is likely that ATP does not effect channel closure by phosphorylation. If ATP is the sole cytosolic regulator, the P_o/P_{max} value of a single K_{ir} channel at this $[ATP]_i$ is predicted to be 0.14 in the intact cell. Since this open channel probability value is 6-fold lower than that normally observed, other cytosolic factors besides ATP_1 may be involved in modulating K_{ir} channel activity. The decrease of open channel probability for both inward and outward currents was the same at 1 mM ATP_1 and could be largely reversed by addition of 100 μM ADP to the cytosolic side. In the presence of a constant background of ADP (100 μM), the half-maximal inhibition

by ATP_1 was shifted to $670 \pm 30 \mu M$ ATP_1 ($n = 8$). These results indicate that the ratio ATP/ADP can provide an important regulation of K^+ recycling. We therefore classify the K_{ir} channel as a K_{ATP} channel (Ashcroft, 1988).

Inhibition of single K_{ATP} channels and SCC by sulphonylureas

The effect of glibenclamide was tested on K_{ATP} channel activity in cell-attached patches of principal cells (K_{int} solution in the pipette, normal Ringer solution in the bath). The records of the channel activity presented in Fig. 4A, show an inhibition of the K_{ATP} channel openings by glibenclamide applied in the bath solution. Glibenclamide affected the open probability of this channel without modifying conductance. The dose–response relationship between glibenclamide concentration and K_{ATP} channel activity is shown in Fig. 4B. Channel activity was decreased by glibenclamide exposure of the cells with a half-maximal inhibition at 0.8 μM .

Potassium recycling can affect the rate of Na^+ absorption by its influence on Na^+ – K^+ pump activity and membrane potential. Inhibition of basolateral K^+ channel activity would be expected to reduce Na^+ absorption. The effect of sulphonylureas was tested on transepithelial reabsorption of Na^+ measured by the SCC in whole skins (Fig. 5). Tolbutamide and glibenclamide, added to the basolateral side produced a decrease in SCC. Glibenclamide had no

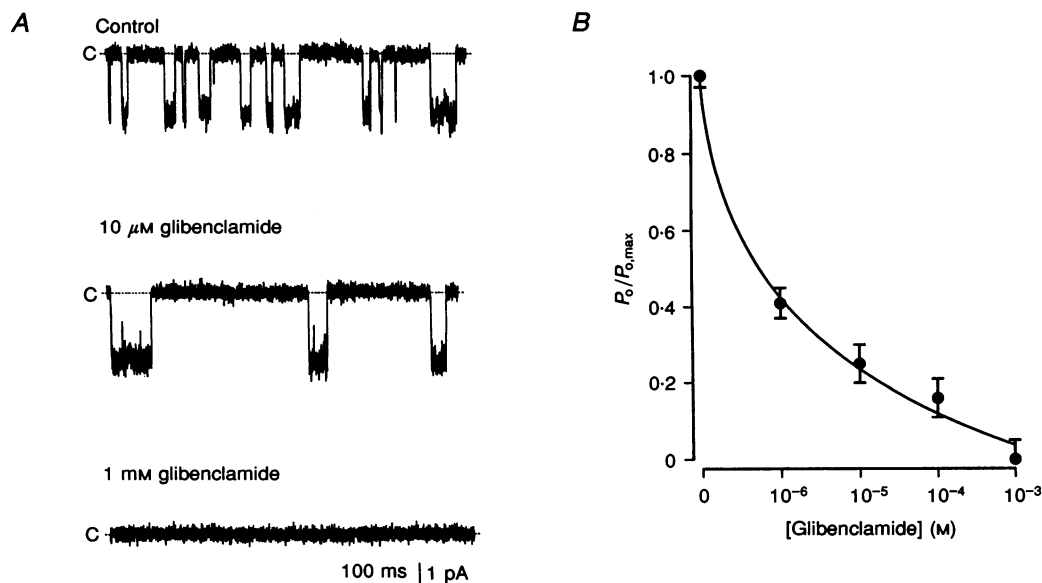


Figure 4. Glibenclamide effect on K_{ATP} channel activity

A, single-channel recording ($V_p = 80$ mV) from a cell-attached patch exposed to K_{int} solution. Upper trace, inward current in control condition. Middle trace, partial inhibition by 10 μM (10^{-5} M) glibenclamide. Lower trace, total block by 1 mM (10^{-3} M) glibenclamide. B, dependence of open probability on glibenclamide concentration. Line through data point corresponds to the best fit of the Hill equation in Fig. 3, substituting [glibenclamide] for [nucl].

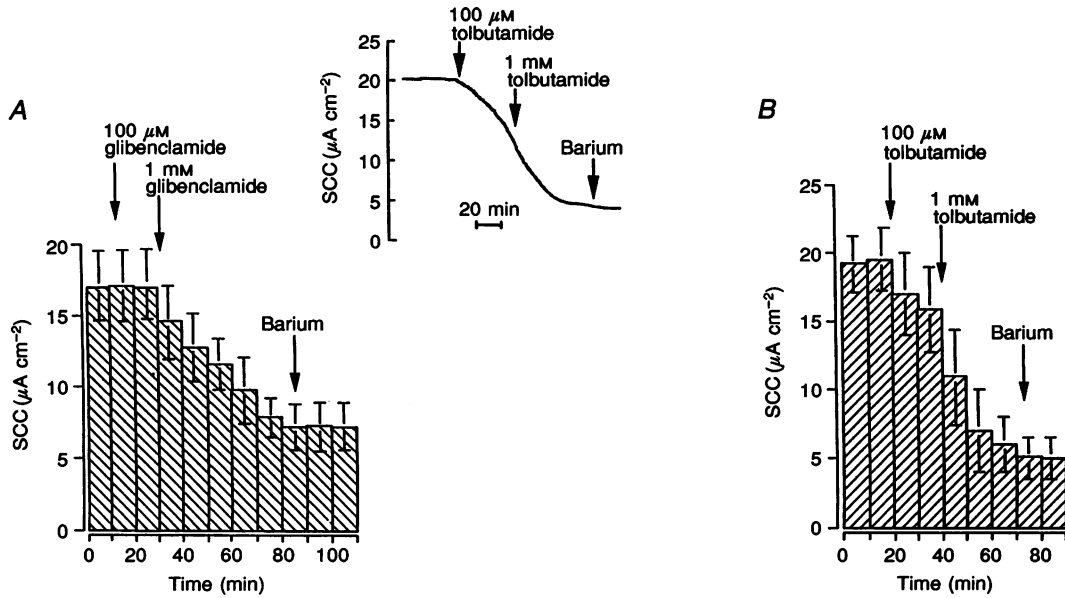


Figure 5. Sulphonylurea effect on the transepithelial Na^+ current

Short-circuit current (SCC) measured across an intact frog skin epithelium bathed in standard Ringer solution. Inhibition of transepithelial Na^+ transport by 100 μM glibenclamide (A) and 100 μM tolbutamide (B) added on the basolateral side. The columns represent the SCC measured at 10 min intervals (means values for 6 epithelia). Inset shows a representative experiment.

effect at concentrations lower than or equal to 100 μM , but tolbutamide at 100 μM decreased the SCC by 10% after 20 min. Glibenclamide (1 mM) decreased the SCC by 60% after 50 min and tolbutamide at the same concentration decreased the current by 75% after 30 min. Following sulphonylurea inhibition of SCC, the addition of barium (1 mM) had little effect (< 5%) on the SCC. Tolbutamide

had no effect on SCC when added from the apical side. Taken together these results indicate that sulphonylurea inhibition of sodium absorption is indirect and due to effects on basolateral membrane K^+ conductance.

The above experiments in whole skins demonstrate that K_{ATP} channel activity is necessary for Na^+ absorption. A direct effect of sulphonylureas on the basolateral K^+

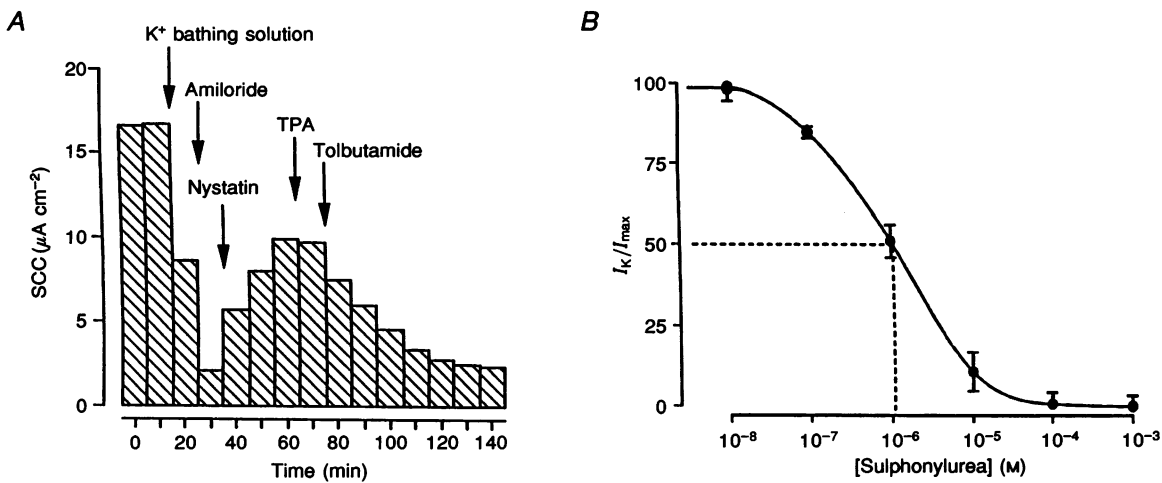


Figure 6. Sulphonylurea effect on the basolateral K^+ current

A, basolateral membrane current measured when the apical membrane of frog skin was permeabilised by nystatin. Tetrapentyl ammonium (TPA; 100 μM) did not affect significantly the SCC (inhibition < 5%), but tolbutamide (100 μM) decreased the SCC by more than 75%. B, dose-response relationship for tolbutamide (●) inhibition of the normalized basolateral membrane current ($I_{\text{K}}/I_{\text{max}}$) ($n = 8$). Amiloride was added at 10 μM and nystatin at 500 i.u. ml^{-1} .

conductance was tested in isolated frog skin epithelia after perforation of the apical membrane with nystatin (Fig. 6). When the SCC had stabilized in standard Ringer solution, the apical bathing solution was changed to a K^+ bathing solution (KBS). Under these conditions, the SCC declined immediately from a mean of 16.6 ± 1.8 to $8.57 \pm 2.1 \mu A cm^{-2}$ with a decrease in transepithelial conductance from 0.31 ± 0.02 to $0.20 \pm 0.009 mS cm^{-2}$ ($n = 8$). Addition of amiloride ($10 \mu M$) to the KBS apical bath resulted in a decrease in transepithelial conductance to a mean value of $0.136 \pm 0.01 mS cm^{-2}$ ($n = 8$). Nystatin was then added at a final concentration of $500 i.u. ml^{-1}$ to the apical KBS bath and caused an increase in SCC from 2.1 ± 1.2 to $9.68 \pm 1.4 \mu A cm^{-2}$ after 30 min. Transepithelial conductance increased from 0.13 ± 0.01 to $0.57 \pm 0.02 mS cm^{-2}$. Subsequent basolateral addition of tetrapentylammonium (TPA; $100 \mu M$), an inhibitor of calcium-dependent K^+ channels (Harvey & Urbach, 1992; Andersen, Urbach, Van Kerkhove, Prosser & Harvey, 1995) had a small effect on SCC, resulting in a 10% decline in SCC (which stabilized within 10 min) to $8.76 \pm 1.6 \mu A cm^{-2}$, with a decrease in conductance to $0.54 \pm 0.008 mS cm^{-2}$. Subsequent addition of tolbutamide ($100 \mu M$) resulted in a decrease in SCC to $2.4 \pm 0.6 \mu A cm^{-2}$, with conductance falling to $0.28 \pm 0.008 mS cm^{-2}$ ($n = 8$) over 40 min. Addition of 1 mM barium did not affect SCC after treatment with tolbutamide, indicating complete inhibition of basolateral membrane K^+ conductance by the sulphonylurea. A dose-response relationship for tolbutamide inhibition of basolateral K^+ current was established in nystatin-perforated isolated frog skin epithelium mounted in Ussing chambers (Fig. 6B). Tolbutamide was added at 30 min intervals in increasing concentrations to the basolateral bathing solution. The basolateral K^+ current was half-maximally inhibited at $1 \mu M$ tolbutamide. Glibenclamide produced a similar inhibition of basolateral current with half-maximal inhibition produced at $6 \mu M$. These results show that the spontaneous basolateral K^+ conductance is generated by the activity of sulphonylurea-sensitive K_{ATP} channels.

Activation of basolateral K^+ conductance by 2-deoxy-glucose

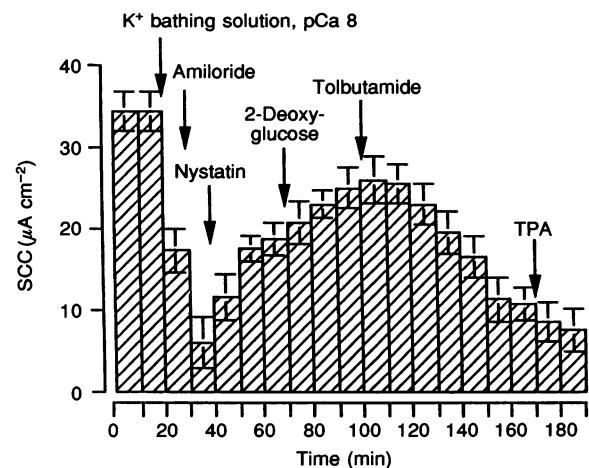
If ATP_1 is a physiological regulator of K_{ATP} channels then manoeuvres designed to lower ATP_1 should increase basolateral membrane K^+ conductance. We have tested this hypothesis by using non-metabolizable 2-deoxy-glucose to reduce the cellular ATP levels and have recorded the effect on basolateral membrane K^+ conductance in nystatin-perforated frog skin (Fig. 7). When the SCC had stabilized following addition of nystatin on the apical side of the frog skin, 2-deoxy-glucose (2 mM) was added to the apical solution. This produced an increase in SCC (from 20.8 ± 1.9 to $26.0 \pm 1.8 \mu A cm^{-2}$, $n = 8$) after 30 min incubation. 2-Deoxy-glucose caused an upregulation of K_{ATP} channels since 95% of the K^+ -dependent SCC was inhibited by tolbutamide under these conditions. The stimulation of the basolateral K^+ current could, therefore, have resulted from activation of K_{ATP} channels as a consequence of a decrease in the ATP/ADP ratio.

Ouabain effect

The sensitivity of the K_{ATP} channel to the ATP/ADP ratio suggests that a metabolic coupling is possible between the recycling of potassium through the channel and its uptake via the Na^+-K^+ pump. A similar mechanism has been proposed to couple apical Na^+ absorption and K^+ secretion with the Na^+-K^+ pump in the renal proximal tubule (Beck, Breton, Mairbäurl, Laprade & Giebisch, 1991; Tsuchiya *et al.* 1992; Beck, Laprade & Lapointe, 1994). We therefore tested the effect of Na^+-K^+ -ATPase inhibition by ouabain on single K_{ATP} channel activity in intact epithelia. The transepithelial Na^+ transport rate was decreased to zero after 30 min exposure to ouabain. This inhibition is associated with an increased intracellular Na^+ activity (Harvey & Kernan, 1984) and a depolarization of the basolateral membrane potential (Messner *et al.* 1985; Horisberger & Giebisch, 1988). Membrane depolarization could be due not only to the arrest of the electrogenic pump activity (Nagel, 1979) but also to the inhibition of K_{ATP} channel activity. We observed that after 15 min exposure

Figure 7. Deoxy-glucose effect on the basolateral K^+ current

Short-circuit current measured in frog skin treated with nystatin. 2-Deoxy-glucose (2 mM) increased the tolbutamide-inhibitable ($100 \mu M$) basolateral current ($n = 8$). Amiloride was added at $10 \mu M$ and nystatin at $500 i.u. ml^{-1}$.



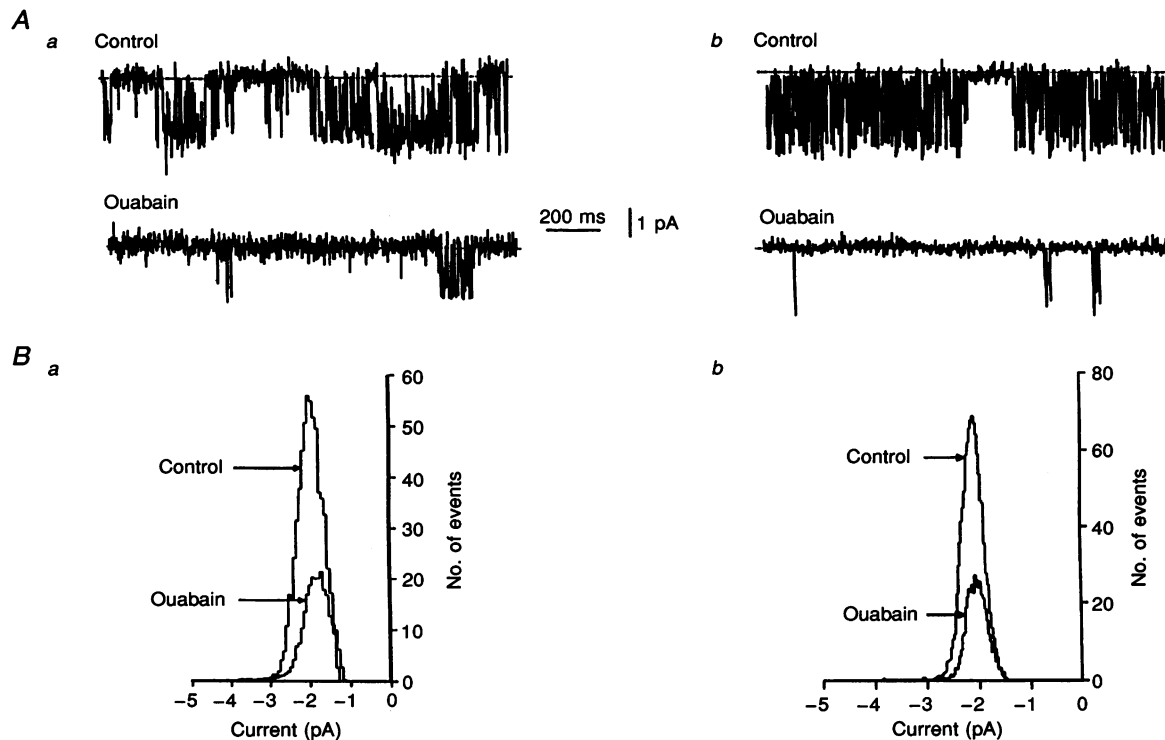


Figure 8. Ouabain effect on the K_{ATP} channel activity

A, single-channel recording ($V_p = 40$ mV) from a cell-attached patch exposed to K_{int} solution in the pipette and standard Ringer buffered with HEPES (*a*) or CO_2 - HCO_3^- (*b*) in the bath, before (upper traces) and 15 min after (lower traces) addition of ouabain ($100 \mu M$) in the bath solution. *B* shows the corresponding current amplitude histograms.

to ouabain ($100 \mu M$), the K_{ATP} channel activity decreased in cell-attached patches (Fig. 8*A*). The current event histograms indicate that the K_{ATP} channel open probability was decreased by 50%, but the current amplitude was not significantly changed (control, 2.01 ± 0.12 pA; ouabain, 1.82 ± 0.12 pA; $n = 6$). The increase in intracellular Na^+ following Na^+ - K^+ pump inhibition could decrease the activity of a basolateral Na^+ - H^+ exchange to produce a fall in pH_i (Harvey *et al.* 1988; Silver, Frindt & Palmer, 1992) which may inhibit K^+ conductance. To test this hypothesis, the effect of ouabain on K_{ATP} channel activity was recorded under conditions where pH_i variations would be limited by raising the intracellular buffering power in a CO_2 - HCO_3^- -buffered Ringer solution on the basolateral side. This condition, compared with a HEPES-buffered Ringer solution, has been shown to produce a doubling in the intracellular buffering power (Harvey *et al.* 1988). Ouabain produced the same inhibitory effect on the K_{ATP} channel activity when the epithelium was bathed in either HEPES- or CO_2 - HCO_3^- -buffered Ringer solution (Fig. 8*B*). Therefore, a cross-talk signal other than changes to pH_i may be implicated in coupling K_{ATP} channel activity to the Na^+ - K^+ pump rate.

One possibility is that a change in ATP_i concentration following pump inhibition produces downregulation of the

K_{ATP} channel by ouabain treatment. The Na^+ - K^+ pump accounts for over 60% of O_2 consumption (Leaf & Renshaw, 1957) and we have verified that pump activity has an important influence in determining the intracellular level of ATP. The total ATP content in epithelial cells was measured by enzymatic reaction before and after addition of ouabain ($100 \mu M$). $[ATP]_i$ was significantly increased after pump inhibition by ouabain (control, 1.5 ± 0.3 mM; ouabain for 10 min, 1.7 ± 0.3 mM; ouabain for 20 min, 1.8 ± 0.4 mM; ouabain for 30 min, 2.5 ± 0.3 mM; $n = 17$). Taken together these results support the hypothesis that Na^+ - K^+ pump activity determines the rate of K^+ recycling (pump-leak coupling) via effects on cellular metabolism.

DISCUSSION

In this study we have identified an ATP-regulated K^+ channel, in the basolateral membrane of principal cells in frog skin epithelium. The K_{ATP} channel has similar biophysical properties to an inward-rectifier K^+ channel (K_{ir}) which we described in a recent paper (Urbach *et al.* 1994). The inward conductance of the K_{ATP} channel was 15 pS in cell-attached patches bathed in physiological solution. At the resting membrane potential (at $V_p = 0$ mV), the slope conductance of this channel was 3 pS with a high open

probability of 0.8. The K_{ATP} was transformed to a pure inward rectifier when the patch pipette contained 120 mM KCl Ringer solution. These properties are identical to those previously described for the inward-rectifier K^+ channel (K_{ir}) in frog skin principal cells (Urbach *et al.* 1994). In the latter study, we showed that the K_{ir} channel is inhibited by Ba^{2+} and H^+ and is insensitive to quaternary ammonium ions (tetraethylammonium, tetrapentylammonium). The macroscopic and single-channel K^+ currents were inhibited by sulphonylureas, demonstrating that K^+ recycling across the basolateral membrane occurs via a K_{ATP} channel. We conclude therefore that K_{ATP} and K_{ir} channels are identical. The sensitivity of the K_{ATP} channel to sulphonylureas is similar for K_{ATP} channels in pancreatic β -cells (Zunkler, Lenzen & Manner, 1988), and excitable cells (Spruce, Standen & Stanfield, 1987). The basolateral conductance of A6 cultured cells (Broillet & Horisberger, 1993) and frog skin have the same pharmacology as the single K_{ATP} channels described here. Tolbutamide is not completely selective for K^+ channels and has recently been shown to inhibit the CFTR (cystic fibrosis transmembrane conductance regulator) Cl^- channel (Sheppard & Welsh, 1992). $BaCl_2$ had no effects on basolateral membrane K^+ conductance after tolbutamide treatment. Thus, the sulphonylurea inhibition of SCC is due solely to the effect of the drug on K^+ channels. We have shown that K_{ir} (K_{ATP}) channels generate the basolateral membrane conductance in principal cells. These channels are therefore the pathway for K^+ recycling which is necessary to drive Na^+ absorption by effects on membrane potential and Na^+ - K^+ pump activity. Inhibition of K_{ATP} channels would therefore be expected to reduce Na^+ transport. This was shown by the sensitivity of SCC to sulphonylureas. The SCC was apparently more sensitive to tolbutamide than glibenclamide. Previous binding studies to β -cell membranes show that the affinity of the sulphonylurea receptor for glibenclamide is 1000-fold higher than for tolbutamide (Kramer, Oekonomopulos, Punter & Summ, 1988). The apparent similar sensitivity of transepithelial Na^+ transport and basolateral K^+ current to glibenclamide and tolbutamide may be dependent more on the permeability of these drugs, which were dissolved in different solvents, across the serosal connective tissue layer.

ATP-sensitive K^+ channels were first discovered in heart muscle (Noma, 1983). K^+ channels of varying sensitivity to ATP have been described in apical membranes of thick ascending limb of Henle's loop (TAL) in rat (Bleich *et al.* 1990; Ho *et al.* 1993) and in rabbit (Wang, White, Geibel & Giebisch, 1990), and ATP blockade is relieved by ADP in these tissues. In frog skin, the half-maximal inhibition of the K^+ channel was obtained at an ATP_1 concentration of 30–80 μM . This is in the range of ATP inhibition found for the ATP-regulated K^+ channels of pancreatic β -cells (Cook & Hales, 1984; Rorsman & Trube, 1985; Ashcroft, 1988) and rat TAL (Bleich *et al.* 1990). Much higher concentrations (millimolar) of ATP are required to inhibit apical K_{ATP}

channels in rat cortical collecting duct (Wang & Giebisch, 1991) and rabbit TAL (Wang *et al.* 1990). We found that non-hydrolysable analogues of ATP and Na-ATP in Mg^{2+} -free solutions inhibited K_{ATP} channel activity with a sensitivity similar to that of Mg-ATP. In fact, cytosolic Mg^{2+} blocks outward currents through the K_{ATP} channel (Urbach *et al.* 1994). Therefore, inhibition of K_{ATP} channels by ATP_1 is not mediated by phosphorylation of the channel protein and does not require hydrolysis of ATP.

The acute sensitivity of the K_{ATP} channel to ATP_1 levels in principal cells and its modulation by ADP suggests a metabolic control of K^+ recycling. Cross-talk between pump and leak pathways could be mediated via changes in the ATP/ADP ratio. Inhibition of the Na^+ - K^+ pump by ouabain was shown to reduce the open probability of single K_{ATP} channels. From the P_o versus cytosolic ATP concentration relationship and the change in ATP_1 concentration produced by ouabain, we can predict the expected reduction in P_o , if ATP_1 is the sole mediator of the ouabain effect on K_{ATP} channels. According to the relationship P_o versus ATP/ADP in Fig. 3B the measured increase in ATP_1 , from 1.5 to 2.5 mM after ouabain, cannot account for the 50% reduction in P_o . Unless there is also a substantial fall in [ADP], this may indicate the concomitant involvement of other K_{ATP} channel regulators (pH_i , Ca^{2+}) in reducing channel activity. An increase in intracellular Na^+ after pump inhibition could slow efflux of H^+ or Ca^{2+} via Na^+ - H^+ or Na^+ - Ca^{2+} exchangers. Basolateral membrane K^+ conductance and K_{ATP} channel activity are decreased by a fall in pH_i or a rise in Ca^{2+} (Harvey *et al.* 1988; Urbach & Harvey, 1993). Although ouabain produces an intracellular acidification in principal cells (Harvey & Ehrenfeld, 1988; Silver *et al.* 1993) we found no difference in ouabain effects on K_{ATP} channels when the intracellular buffering power was doubled. It was previously shown that experimentally induced pH_i variations in principal cells are much reduced when the epithelium is bathed in CO_2 - HCO_3^- -buffered solutions compared with a Hepes-buffered solution (Harvey *et al.* 1988). Thus ATP rather than pH_i may be the primary pump-leak coupling signal. Using the luciferase method to measure total intracellular ATP, it is likely that localized submembrane changes in ATP are not detected and the effect of ouabain on $[ATP]_i$ close to the channel pore may be underestimated. In addition we have shown that ATP inhibition of K_{ATP} channels is modulated by ADP. A much greater inhibitory effect for a given change in ATP would be predicted if ADP levels were also decreased following ouabain treatment. Similar arguments can, however, be proposed for localized submembrane Ca^{2+} or pH_i variations following inhibition of Na^+ - Ca^{2+} or Na^+ - H^+ exchange. We are currently examining these possibilities using spatial Ca^{2+} and H^+ imaging spectrofluorescence. The energy potential of the cell depends largely on Na^+ - K^+ pump activity and could couple the Na^+ - K^+ -ATPase and the K_{ATP} activity in the same membrane. Similar evidence for

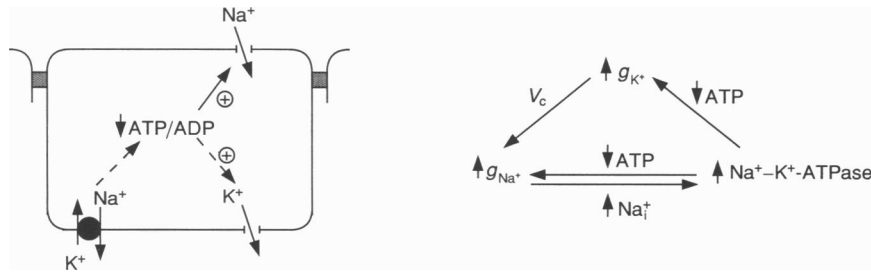


Figure 9. Model proposed for the cross-talk between the transepithelial Na^+ transport and the K^+ recycling in principal cells

The ATP/ADP ratio controlled by the Na^+ - K^+ pump simultaneously regulates the activity of apical Na^+ channels and the basolateral K^+ channels. The membrane hyperpolarization (V_c) due to basolateral K^+ conductance facilitates the Na^+ entry. The intracellular Na^+ activity regulated by the apical Na^+ conductance controls the Na^+ - K^+ pump activity.

the role of ATP in coupling the basolateral Na^+ - K^+ pump and apical K^+ secretion (Tsuchiya *et al.* 1992) and Na^+ amino acid/glucose absorption (Beck *et al.* 1991) has been described in renal proximal tubule. Recent studies have shown that ATP_i inhibits the apical Na^+ channel in primary cultures of human sweat duct and human distal lung (Prosser, Urbach, Thomas & Harvey, 1994). Sodium entry across the apical membrane is the rate-limiting step in transepithelial Na^+ absorption and the pump rate is determined primarily by the supply of intracellular Na^+ . Thus the Na^+ - K^+ pump rate and ATP/ADP ratio will be determined by the activity of the amiloride-sensitive Na^+ channel. We therefore propose that the ATP/ADP ratio can couple Na^+ and K^+ transport at opposite cell membranes in principal cells (Fig. 9).

ANDERSEN, H. K., URBACH, V., VAN KERKHOVE, E., PROSSER, E. & HARVEY, B. J. (1995). Maxi K^+ channels in the basolateral membrane of the exocrine frog skin gland regulated by intracellular calcium and pH. *Pflügers Archiv* **431**, 52–65.

ASHCROFT, F. M. (1988). Adenosine-triphosphate sensitive potassium channels. *Annual Review of Neuroscience* **11**, 97–118.

BECK, J. S., BRETON, H., MAIRBÄURL, H., LAPRADE, R. & GIEBISCH, G. (1991). Relationship between sodium transport and intracellular ATP in isolated perfused rabbit proximal convoluted tubule. *American Journal of Physiology* **261**, F634–639.

BECK, J. S., LAPRADE, R. & LAPOINTE, J.-Y. (1994). Coupling between transepithelial Na transport and basolateral conductance in renal proximal tubule. *American Journal of Physiology* **266**, F517–527.

BLEICH, M., SCHLATTER, E. & GREGER, R. (1990). The luminal K^+ channel of the thick ascending limb of Henle's loop. *Pflügers Archiv* **415**, 449–460.

BROILLET, M.-C. & HORISBERGER, J.-D. (1993). Tolbutamide-sensitive potassium conductance in the basolateral membrane of A6 cells. *Journal of Membrane Biology* **134**, 181–188.

CHANG, D., HSIEH, P. S. & DAWSON, D. C. (1988). Calcium: A program in BASIC for calculating the composition of solution with specified concentrations of calcium, magnesium and other divalent cations. *Computers in Biology and Medicine* **18**, 353–366.

COOK, D. L. & HALES, C. N. (1984). Intracellular ATP directly blocks K^+ channels in pancreatic b cells. *Nature* **311**, 271–273.

DAVIS, C. W. & FINN, A. (1982). Sodium transport inhibition by amiloride reduces basolateral K conductance in tight epithelia. *Science* **26**, 525–527.

DAWSON, D. C. & RICHARDS, N. W. (1990). Basolateral K conductance: role in regulation of NaCl absorption and secretion. *American Journal of Physiology* **28**, C181–195.

FRIEDRICH, F., WEISS, H., PAULMICH, M. & LANG, F. (1989). Activation of potassium channels in renal epithelioid cells (MDCK) by extracellular ATP. *American Journal of Physiology* **256**, C1016–1021.

GARTY, H. (1994). Current-voltage relations of the basolateral membrane in tight amphibian epithelia: use of nystatin to depolarise the apical membrane. *Journal of Membrane Biology* **77**, 213–222.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. (1981). Improved patch-clamp techniques for high-resolution recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

HARVEY, B. J. & EHRENFELD, J. (1988). Role of Na/H exchange in the control of intracellular pH and cell membrane conductances in frog skin epithelium. *Journal of General Physiology* **92**, 793–810.

HARVEY, B. J. & KERNAN, R. P. (1984). Intracellular ion activities in frog skin in relation to external sodium and effects of amiloride and/or ouabain. *Journal of Physiology* **349**, 501–517.

HARVEY, B. J., THOMAS, S. R. & EHRENFELD, J. (1988). Intracellular pH controls cell membrane Na^+ and K^+ conductances and transport in frog skin epithelium. *Journal of General Physiology* **92**, 767–791.

HARVEY, B. J. & URBACH, V. (1992). Cellular regulation of ATP and calcium-sensitive K channels in frog skin and distal renal cell culture (A6) epithelia. *Journal of General Physiology* **100**, 19a.

HO, K., NICHOLS, C. G., LEDERER, W. J., LYTTON, J., VASSILEV, P. M., KANAZIRSKA, M. V. & HEBERT, S. C. (1993). Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. *Nature* **362**, 31–38.

HORISBERGER, J.-D. & GIEBISCH, G. (1988). Voltage dependence of basolateral membrane in the Amphiuma collecting tubule. *Journal of Membrane Biology* **105**, 257–263.

KIRK, K. L. & DAWSON, D. C. (1983). Basolateral potassium channel in turtle colon. Evidence for single-file ion flow. *Journal of General Physiology* **82**, 297–313.

- KRAMER, W., OEKONOMOPULOS, R., PUNTER, J. & SUMM, H.-D. (1988). Direct photoaffinity labelling of the putative sulfonylurea receptor in rat b-cell tumor membranes by [3 H]glibenclamide. *FEBS Letters* **229**, 355–359.
- LEAF, A. & RENSHAW, A. (1957). Ion transport and respiration of isolated frog skin. *Biochemical Journal* **65**, 82–90.
- MESSNER, G., WANG, W., PAULMICHL, M., OBERLEITHNER, H. & LANG, F. (1985). Ouabain decreases apparent potassium-conductance in proximal tubules of the amphibian kidney. *Pflügers Archiv* **404**, 131–137.
- NAGEL, W. (1979). Inhibition of potassium conductance by barium in frog skin epithelium. *Biochimica et Biophysica Acta* **552**, 346–357.
- NOMA, A. (1983). ATP-regulated K^+ channel in cardiac muscle. *Nature* **305**, 147–148.
- OBERLEITHNER, H., KERSTING, U. & GASSNER, B. (1988). Aldosterone-controlled linkage between Na/H exchange and K channels in fused renal epithelial cells. *Ciba Foundation Symposium* **139**, 201–219.
- OHNO-SHOSAKU, T., KUBOTA, T., YAMAGUCHI, J. & FUJIMOTO, M. (1990). Regulation of inwardly rectifying K^+ channels by intracellular pH in opossum kidney cells. *Pflügers Archiv* **416**, 138–143.
- PROSSER, E., URBACH, V., THOMAS, S. & HARVEY, B. J. (1994). ATP inhibits sodium channels in human sweat duct and lung epithelia of human airway and sweat duct epithelia. *Journal of General Physiology* **104**, 42a.
- RICK, R. (1994). P_{Hi} determines rate of sodium transport in frog skin: results of a new method to determine pH_i . *American Journal of Physiology* **266**, F367–376.
- RORSMAN, P. & TRUBE, G. (1985). Glucose-dependent K^+ channels in pancreatic β cells are regulated by intracellular ATP. *Pflügers Archiv* **405**, 305–309.
- SHEPPARD, D. N. & WELSH, M. J. (1992). Effect of ATP-sensitive K^+ channel regulator on cystic fibrosis transmembrane conductance regulator chloride currents. *Journal of General Physiology* **100**, 573–591.
- SILVER, R. B., FRINDT, G. & PALMER, L. G. (1992). Regulation of principal cell pH by Na/H exchange in rabbit cortical collecting tubule. *Journal of Membrane Biology* **125**, 13–24.
- SILVER, R. B., FRINDT, G., WINDHAGER, E. E. & PALMER, L. G. (1993). Feedback regulation of Na channels in rat CCT I: Effects of inhibition of Na pump. *American Journal of Physiology* **264**, F557–564.
- SPRUCE, A. E., STANDEN, N. B. & STANFIELD, P. R. (1987). Studies of the unitary properties of adenosine-5'-triphosphate-regulated potassium channels of frog skeletal muscle. *Journal of Physiology* **382**, 213–236.
- TSUCHIYA, K., WANG, W., GIEBISCH, G. & WELLING, P. A. (1992). ATP is a coupling modulator of parallel Na/K-ATPase K channel activity in the renal proximal tubule. *Proceedings of the National Academy of Sciences of the USA* **89**, 6418–6422.
- URBACH, V. & HARVEY, B. J. (1993). Potassium channels and ATP in epithelia cells of frog skin. *Journal of Physiology* **459**, 283P.
- URBACH, V., VAN KERKHOVE, E. & HARVEY, B. J. (1994). Inward rectifier K^+ channels in basolateral membranes of frog skin epithelium. *Journal of General Physiology* **103**, 583–604.
- WANG, W. & GIEBISCH, G. (1991). Dual effect of ATP on the apical small conductance potassium channel of the rat cortical collecting duct. *Journal of General Physiology* **98**, 35–62.
- WANG, W., WHITE, S., GEIBEL, J. & GIEBISCH, G. (1990). A potassium channel in the apical membrane of rabbit thick ascending limb of Henle's loop. *American Journal of Physiology* **258**, F244–253.
- WILLUMSEN, N. J. & BOUCHER, R. C. (1992). Intracellular pH and its relationship to regulation of ion transport in normal and cystic fibrosis human nasal epithelia. *Journal of Physiology* **455**, 247–269.
- ZUNKLER, B. J., LENZEN, S. & MANNER, K. (1988). Concentration dependent effects of tolbutamide, meglitinide, glipizidine, glibenclamide and diazoxide on ATP-regulated K^+ currents in pancreatic b-cells. *Naunyn-Schmiedeberg's Archives of Pharmacology* **337**, 225–230.

Acknowledgements

This work was supported by The Wellcome Trust UK (programme grant 040067/Z/93) and the Health Research Board of Ireland (project grant 24-93).

Received 7 April 1995; accepted 1 September 1995.