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

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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 (Kᵢᵣ) operating in parallel with a Na⁺–K⁺-ATPase pump. Here we report on the metabolic control of the Kᵢᵣ channel using patch clamping, short-circuit current measurement and enzymatic determination of cellular ATP (ATP₁).

2. The constitutively active Kᵢᵣ channel in the basolateral membrane has the characteristics of an ATP-regulated K⁺ channel and is now classed as a Kᵢ ATP channel. In excised inside-out patches the open probability (Pₒ) of Kᵢ ATP channels was reduced by ATP₁ with half-maximum inhibition at an ATP₁ concentration of 50 μM.

3. ATP₁ 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 Kᵢ ATP channel would be expected to be inactive in intact cells if ATP₁ was the sole regulator of channel activity. Kᵢ ATP channels which were inactivated by 1 mM ATP₁ 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] > 5 mM.

4. Sulphonylureas inhibit single Kᵢ 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 ATP₁ and reduced the open probability of Kᵢ 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 (Kᵢᵣ) 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, pH₁ 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 pH₁ 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, Prindt, Windhager & Palmer, 1993) and basolateral K⁺ conductance (Messner, Wang
Paulmichl, Oberleithner & Lang, 1985). The lack of effect of ouabain on pH$_{1}$ 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, Giebish & Wellung, 1992) and cortical collecting tubule (Wang & Giebish, 1991). In the present study in frog skin principal cells, we have identified a sulphureylurea-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 pitted frogs (*Rana esculenta* and *Rana temporaria*) and mounted in a Ussing-type chamber and bathed in standard amphibian Ringer solution containing (mM): 110 NaCl, 3-7 KOH, 6 Heps, 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$_2$PO$_4$, 2·9 KH$_2$PO$_4$, 11 glucose, 10 Heps, 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. Microelectrode 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$_{m}$), which contained (mM): 15 NaCl, 150 KCl, 5 EGTA, 0·2 CaCl$_2$, 10 Heps, 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, Clai, 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 Strathclyde, 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, [ATP], 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 [ATP]$_{i}$ was determined in each sample by a bioluminescence technique (Luciferin/Luciferase; Sigma) which uses the reaction:

\[
\text{ATP} + \text{luciferin} \xrightarrow{\text{luciferase}} \text{adenyl-luciferin} + \text{Pi}
\]

\[
\text{Adenyl-luciferin} + \text{O}_2 \rightarrow \text{oxy-luciferin} + \text{AMP} + \text{CO}_2 + h\nu.
\]

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, Silver Springs, MD, USA). The [ATP]$_{i}$ of each sample was quantified against standard ATP.
solutions (0·5, 1, 2 and 3 mm) and is given as moles per litre of cytosol (corrected for extracellular space).

Data are shown as mean values ± 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 ($\gamma$) was $3 \pm 1 \, \mu S$ for outward current and $15 \pm 2 \, \mu S$ 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 ($\gamma = 5 \pm 1 \, \mu S$ for outward current and $\gamma = 17 \pm 2 \, \mu S$ 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 ($\gamma = 35 \, \mu S$ 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-Shosha & 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$_1$ (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$_1$ 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, $\tau_{1,\text{open}}$, of $2·71 \pm 0·05$ ms in control and $1·22 \pm 0·01$ ms ($n = 8$) in ATP. Closed-time dwell histograms were best fitted by a double-exponential decay function with mean closed half-times, $\tau_{1,\text{closed}}$ and $\tau_{2,\text{closed}}$, of, respectively, $1·49 \pm 0·05$ and $21·4 \pm 9·4$ ms in control and $1·30 \pm 0·03$ and $18·7 \pm 8·5$ 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 ($-V_m$ 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 Kir channel
A, effects of ATP on Kir channel inward currents recorded from excised inside-out membranes exposed to K_int solutions on both sides; \(V_p = 40\,\text{mV}\); 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 Kir channel
A, single-channel recording (\(V_p = 40\,\text{mV}\)) from an excised membrane exposed to symmetrical K_int solutions. Upper trace, control conditions. Middle trace, effect of 1 mM ATP concentration on Kir inward currents. Lower trace, partial reversal of ATP block by 0.1 mM ADP. B, dependence of open probability (Po) 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:

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

where [nucl]_{min}, [nucl]_{max} and [nucl]_{50} are the concentrations of nucleotide at which \(P_o/P_{\text{max}}\) 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_i 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_i concentration of 152 ± 44 µM (n = 8). The best-fit ATP_i-binding coefficient (k = 0·80 ± 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). Adenyllyl 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 affect 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_i 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_i, 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_i was shifted to 670 ± 30 µM ATP_i (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

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**Figure 4. Glibenclamide effect on \( K_{ATP} \) channel activity**

_A._ single-channel recording (\( V_p = 80 \text{ mV} \)) from a cell-attached patch exposed to K_int solution. Upper trace, inward current in control condition. Middle trace, partial inhibition by 10 µM (10⁻² 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].
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<sub>ATP</sub> channel activity is necessary for Na⁺ absorption. A direct effect of sulphonylureas on the basolateral K⁺

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.

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<sub>K</sub>/I<sub>max</sub>) (n = 8). Amiloride was added at 10 μM and nystatin at 500 i.u. ml<sup>-1</sup>.
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±2±1 μA cm⁻² with a decrease in transepithelial conductance from 0±31±0±02 to 0±20±0±009 mS cm⁻² (n = 8). Addition of amiloride (10 μM) to the KBS apical bath resulted in a decrease in transepithelial conductance to a mean value of 0±136±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±1±4 μA cm⁻² after 30 min. Transepithelial conductance increased from 0±13±0±01 to 0±57±0±02 mS cm⁻². Subsequent basolateral addition of tetrabutylammonium (TPA; 100 μM), an inhibitor of calcium-dependent K⁺ channels (Harvey & Urbach, 1992; Andersen, Urbach, Van Kerckhove, Prosser & Harvey, 1995) had a small effect on SCC, resulting in a 10% decline in SCC (which stabilized within 10 min) to 8±76±1±6 μA cm⁻², with a decrease in conductance to 0±54±0±008 mS cm⁻². Subsequent addition of tolbutamide (100 μM) resulted in a decrease in SCC to 2±4±0±6 μA cm⁻², with conductance falling to 0±28±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 μM tolbutamide. Glibenclamide produced a similar inhibition of basolateral current with half-maximal inhibition produced at 6 μM. These results show that the spontaneous basolateral K⁺ conductance is generated by the activity of sulphonylurea-sensitive Kₐtp channels.

**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 μM) basolateral current (n = 8). Amiloride was added at 10 μM and nystatin at 500 i.u. ml⁻¹.

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

If ATP is a physiological regulator of Kₐtp channels then manoeuvres designed to lower ATP 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±1±8 μA cm⁻², n = 8) after 30 min incubation. 2-Deoxy-glucose caused an upregulation of Kₐtp 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 Kₐtp channels as a consequence of a decrease in the ATP/ADP ratio.

**Ouabain effect**

The sensitivity of the Kₐtp 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, Mainz, 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 Kₐtp 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 Kₐtp channel activity. We observed that after 15 min exposure
to ouabain (100 μM), the $K_{ATP}$ channel activity decreased in cell-attached patches (Fig. 8A). 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. 8B). 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 $ATP_1$ 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 μM). $[ATP]_i$ 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 bioelectrical properties to an inward-rectifier $K^+$ channel ($K_K$) 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 $K_{\text{ATP}}$ 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 ($K_n$) in frog skin principal cells (Urbach et al. 1994). In the latter study, we showed that the $K_n$ 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 $K_{\text{ATP}}$ channel. We conclude therefore that $K_{\text{ATP}}$ and $K_n$ channels are identical. 

The sensitivity of the $K_{\text{ATP}}$ channel to sulphonylureas is similar for $K_{\text{ATP}}$ channels in pancreatic $\beta$-cells (Zunkler, Lenzen & Manner, 1988), and excitable cells (Spruce, Standen & Stanfield, 1987). The basolateral conductance of A6 cultured cells (Brollet & Horisberger, 1993) and frog skin have the same pharmacology as the single $K_{\text{ATP}}$ 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 $K_p$ ($K_{\text{ATP}}$) 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 $K_{\text{ATP}}$ 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 glibencamide. Previous binding studies to $\beta$-cell membranes show that the affinity of the sulphonylurea receptor for glibenclamide is 1000-fold higher than for tolbutamide (Kramer, Oekonomopoulos, 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 concentration of 30–80 $\mu$m. This is in the range of ATP inhibition found for the ATP-regulated $K^+$ channels of pancreatic $\beta$-cells (Cook & Hales, 1984; Korsman & Trube, 1985; Ashcroft, 1988) and rat TAL (Bleich et al. 1990). Much higher concentrations (millimolar) of ATP are required to inhibit apical $K_{\text{ATP}}$ 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 $K_{\text{ATP}}$ channel activity with a sensitivity similar to that of Mg-ATP. In fact, cytosolic Mg$^{2+}$ blocks outward currents through the $K_{\text{ATP}}$ channel (Urbach et al. 1994). Therefore, inhibition of $K_{\text{ATP}}$ channels by ATP, is not mediated by phosphorylation of the channel protein and does not require hydrolysis of ATP.

The acute sensitivity of the $K_{\text{ATP}}$ 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 $K_{\text{ATP}}$ channels. From the $P_0$ versus cytosolic ATP concentration relationship and the change in ATP$_1$ concentration produced by ouabain, we can predict the expected reduction in $P_0$ if ATP is the sole mediator of the ouabain effect on $K_{\text{ATP}}$ channels. According to the relationship $P_0$ versus ATP/ADP in Fig. 3B the measured increase in ATP$_1$, from 1.5 to 2.5 $\text{mm}$ after ouabain, cannot account for the 50% reduction in $P_0$. Unless there is also a substantial fall in [ADP], this may indicate the concomitant involvement of other $K_{\text{ATP}}$ 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 $K_{\text{ATP}}$ 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 $K_{\text{ATP}}$ 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 Heps-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], close to the channel pore may be underestimated. In addition we have shown that ATP inhibition of $K_{\text{ATP}}$ 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 $K_{\text{ATP}}$ activity in the same membrane. Similar evidence for
the role of ATP in coupling the basolateral $\text{Na}^+-\text{K}^+$ pump and apical $\text{K}^+$ secretion (Tsuchiya et al. 1992) and $\text{Na}^+$ amino acid/glucose absorption (Beck et al. 1991) has been described in renal proximal tubule. Recent studies have shown that ATP inhibits the apical $\text{Na}^+$ 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 $\text{Na}^+$ absorption and the pump rate is determined primarily by the supply of intracellular $\text{Na}^+$. Thus the $\text{Na}^+-\text{K}^+$ pump rate and ATP/ADP ratio will be determined by the activity of the amiloride-sensitive $\text{Na}^+$ channel. We therefore propose that the ATP/ADP ratio can couple $\text{Na}^+$ and $\text{K}^+$ transport at opposite cell membranes in principal cells (Fig. 9).


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