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ATP is a coupling modulator of parallel Na,K-ATPase–K-channel activity in the renal proximal tubule

(signal transduction/kidney/epithelia/pump–leak coupling/patch clamp)

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ABSTRACT **A fundamental and essential property of nearly all salt-transporting epithelia is the tight parallel coupling between the magnitude of the K-conductive pathway at the basolateral membrane and the activity of the Na,K-dependent ATPase (Na,K-ATPase). In the present study, we demonstrate that the coupling response in the renal proximal tubule is governed, at least in part, through the interaction between ATP-sensitive K channels and Na,K-ATPase-mediated changes in intracellular ATP levels. First, we identified a K-selective channel at the basolateral membrane, which is inhibited by the cytosolic addition of ATP. Second, conventional microelectrode analysis in the isolated perfused proximal straight tubule revealed that these channels are the major determinant of the macroscopic K conductance so that ATP-mediated changes in the open probability of the K channel could alter the extent of K recycling. Indeed, the increase in the macroscopic K conductance upon stimulation of transcellular Na transport and pump activity was found to be paralleled by a decrease in intracellular ATP. Finally, a causal link between parallel Na,K-ATPase–K-channel activity and ATP was established by the finding that intracellular ATP loading uncoupled the response. With our recent observations that similar ATP-sensitive K channels are expressed abundantly in other epithelia, we postulate that ATP may act as a universal coupling modulator of parallel Na,K-ATPase–K-channel activity.**

The coordinate modulation of ion transporters permits epithelia to accommodate large fluctuations in transcellular ion flow without disturbing the delicate intracellular milieu necessary for optimum metabolic activity (1). The modification of basolateral membrane K-channel activity in parallel with changes in the turnover rate of the Na,K-dependent ATPase (Na,K-ATPase) is a fundamental property, and a classic example (2), of nearly all epithelia. For instance, in the renal proximal tubule, the prototype of sodium-reabsorbing "leaky" epithelia, the vectorial movement of Na from the tubule lumen to blood is mediated by the in-series operation of passive Na entry mechanisms at the apical membrane and an active Na-translocation step, the Na,K-ATPase, at the basolateral membrane. The obligate influx of potassium via the Na,K-ATPase is "recycled" through a dominant conductive pathway at the basolateral membrane. To maintain cell volume (3), intracellular K activity (4), and a favorable driving force for electrogenic Na entry at the apical membrane (5, 6) during physiological surges in transcellular Na transport, the basolateral K conductance increases in concert with stimulation of the Na,K-ATPase. A similar response adjusts passive cellular K efflux to active K influx in secretory epithelia (7, 8). Although the intrinsic regulatory response appears to be a ubiquitous phenomenon, relatively little is known about underlying coupling mechanisms. Alterations in intracellular pH, calcium, or both have been suggested, but the results of several recent studies indicate that these signals cannot account universally for the coupling response (5, 9). We have recently identified several ATP-sensitive K channels in renal epithelia (10, 11), like those first described in cardiac muscle (12) and the pancreatic β-islet cell (13). In this regard, the observation from our laboratory that intracellular ATP levels fall upon stimulation of Na reabsorption and an increase in the hydrolytic activity of the Na,K-ATPase (14) has made ATP an attractive coupling modulator candidate.

In the present study, patch-clamp and conventional microelectrodes were used in concert with measurements of intracellular ATP to examine this thesis. The rabbit proximal tubule, the major site of fluid and electrolyte reabsorption in the kidney, was used as a model because the regulation of basolateral K conductance is well documented (3–6) and transcellular Na transport and Na,K-ATPase activity are easily stimulated by lumenal addition of amino acids or monosaccharides, which are cotransported with Na across the apical membrane (15).

METHODS

Patch Clamp. Proximal tubules (S_1 or S_2 segments) were isolated by free-hand dissection from female New Zealand rabbit kidneys and incubated initially in a solution (bath solution; see below) containing 0.2 mg of collagenase per dl to remove the basement membrane. Tubules were washed free of collagenase, placed on a glass coverslip coated with Cell-Tak (Biopolymers, Farmington, CT), and then transferred to a chamber mounted on the stage of an inverted microscope. Single-channel currents were measured as described (10). Open probability of individual channels was determined from a data sample of 30 s with the equation $P_o = (1/N) \times \Sigma(t_1 + t_2 + \ldots t_n)$, where N is the number of channels and t is the fractional open time spent at each of the observed current levels. The pipette contained 140 mM KCl, 1.8 mM $MgCl_2$, 1.8 mM EGTA, and 10 mM Hepes (pH 7.4), and the bath contained 135 mM NaCl, 5 mM KCl, 1.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM glucose, and 10 mM Hepes (pH 7.4). ATP (Sigma) was added to the bath solution from a 10 mM stock solution suspended in the bath solution.

Microelectrode Techniques in the Isolated Perfused Tubule. Proximal tubules (S_1 and S_2) were isolated by free-hand dissection without the use of enzymes, perfused *in vitro* in a rapid-exchange chamber, and impaled with conventional microelectrodes across the basolateral membrane as described (9, 16). The relative basolateral membrane macroscopic K conductance (G_K/G_{cell}) was estimated by monitoring the abrupt change in basolateral membrane potential, ΔV_{bl}, on a 10-fold increase in the peritubular K concentration. Provided that all the ionic conductances are both concentra-

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tion and voltage independent and the paracellular pathway is at least 1 order of magnitude higher than the apical membrane (5), the relative K conductance can be approximated by the relation $G_K/G_{cell} = \Delta V_{bl}/[(RT/zF) \times \ln([K]'/[K]'')]$, where K′ is 5 mM and K″ is 50 mM, z is valence, and F is the Faraday constant.

In several studies, G_{cell} was estimated (I/V) by the shift in potential, ΔV_{bl}, sensed by one barrel of a double-barrel electrode upon current delivery (I = 2–3 nA) through the other barrel of the electrode. Since the proximal tubule cells are electrically coupled, the measured G_{cell} is an overestimate of the cellular membrane resistance. Changes in G_{cell} therefore underestimate changes in apical and basolateral membrane conductance.

The tubules were perfused in symmetrical solutions containing 146.2 mM Na, 134 mM Cl, 5 mM K, 1 mM Mg, 1 mM Ca, 1.2 mM H_2PO_4, 25 mM H_2CO_3, and 5% CO_2 (pH 7.4). Since the relative Na conductance at the basolateral membrane is negligible, Na was replaced with K when bath K was raised to 50 mM. In the experiments in which transcellular Na transport and pump activity were stimulated (see Figs. 3 and 5), proximal tubules were perfused with identical solutions as indicated above, except the lumenal perfusate contained either 11 mM mannitol or equimolar glucose (5 mM) and alanine (6 mM).

Intracellular ATP Levels. The intracellular ATP content was measured from tubules that were isolated, perfused *in vitro*, and subjected to the experimental perturbations indicated below by a method that has been described (14). All comparisons were made in a paired fashion with tubules isolated from the same kidney. At the end of the experimental period, ATP metabolism was stopped by flushing the perfusion chamber with a cold bath solution (5°C). ATP was extracted by incubation in 50 μl of 2% perchloric acid. After the tubule was removed, the solution was neutralized with an equivalent volume of 1 M KOH and diluted to 360 μl with 10 mM Tris buffer (pH 7.7). ATP content of the solution was measured with a luminometer using a firefly luciferase assay kit (Sigma) according to the manufacturer's specifications. The measured ATP content was standardized to tubule volume as assessed by an optical-imaging method as described (17) and is therefore expressed as the apparent intracellular concentration. In this regard, the apparent intracellular ATP concentration $[ATP]_i$ was taken to be equal to the total intracellular ATP pool extracted by perchloric acid divided by the tubule volume. Since ATP is likely to be compartmentalized (18), the measurement is apt to be an overestimate of the actual ATP concentration. Subsequently, measured changes in the total ATP pool underestimate actual changes in the cytosolic ATP concentration.

To accurately assess the actual intracellular ATP content in the series of studies in which ATP was added to the tubular fluid (see Fig. 5), we determined the amount of residual ATP carried with the tubule after it was washed with 2-ml vol of cold bath solution devoid of ATP. In these studies, [^{3}H]inulin (1 μCi; 1 Ci = 37 GBq) was added to the chamber and followed to determine the residual volume carried with the tubule. The amount of residual ATP carried with the tubule was considered to be the product of the ATP concentration remaining in the chamber and the trapped volume with the tubule. This value was therefore subtracted from the total ATP measured to give the actual intracellular ATP content.

RESULTS AND DISCUSSION

Previous patch-clamp studies identified a Ca-independent, K-selective ($P_{Na/K}$ = 0.06), intermediate conductance (50 pS) channel as the element responsible for K recycling across the proximal tubule basolateral membrane (19, 20). Similarly, in the present study under nearly identical conditions, a channel

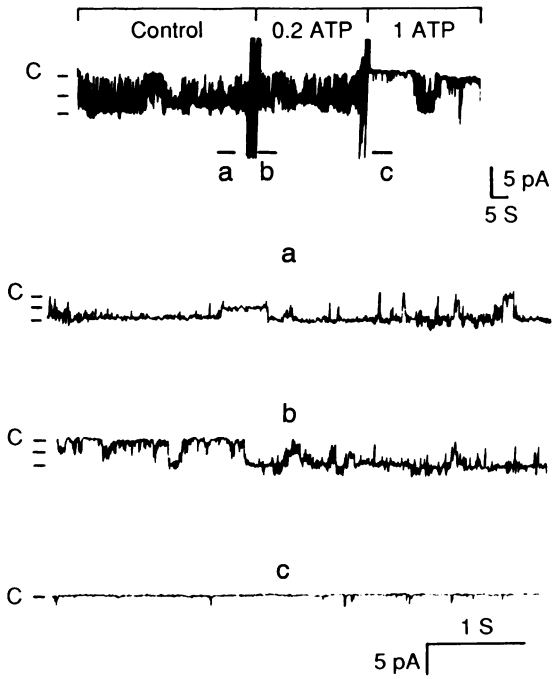

FIG. 1. Representative single-channel current record from an inside-out patch of the proximal tubule basolateral membrane, demonstrating that the channel is directly gated by ATP. (*a*) Control. Two channels are evident; P_o = 0.82. (*b*) Upon superperfusion of 0.2 mM ATP, P_o = 0.75. (*c*) ATP (1 mM) caused P_o to diminish to 0.2 and returned to control levels upon ATP washout (data not shown). Holding potential was 0 mV. Closed state is indicated by C.

with an inward slope conductance of 56 pS (Fig. 1) and an extrapolated reversal potential near the estimated E_K was the sole channel observed in basolateral membrane patches of the late proximal convoluted (S_1) or early proximal straight (S_2) tubule. The observation that the channel was active at the spontaneous membrane potential (V_p = 0), exhibiting an open probability, P_o, of 0.72 ± 0.02 in the cell-attached configuration, is consistent with physiological operations. As shown in Fig. 1, in the excised, inside-out mode, superperfusion of ATP (1 mM) to the cytoplasmic face of the patch caused P_o to diminish from 0.70 to 0.16. The response is not due to spontaneous channel "rundown." In all patches in which the gigaseal was maintained (four of five patches), we were able to demonstrate that the response was fully reversible; P_o was restored to 0.66 (94.3% of control) upon ATP removal. This observation demonstrates that ATP directly gates an epithelial basolateral membrane K channel in an inhibitory fashion. The response is consistent with the role of intracellular ATP as a modulator of K-channel activity.

To specifically address the involvement of these channels and ATP as a coupling modulator of parallel Na,K-ATPase–K leak activity in the intact epithelium, the basolateral membrane potential, relative basolateral K conductance (G_K/G_{cell}), and cell-input conductance (G_{cell}) were monitored with conventional microelectrodes in the isolated perfused proximal tubule as described (9, 16). Because the apical and basolateral membranes are independently perfused in this preparation, transcellular Na transport can be stimulated in a physiological manner. Moreover, the conventional electrophysiological approach in intact tubules was favored; since the proximal tubule cells are electrically coupled, whole-cell or perforated patch-clamp techniques necessitate the use of dissociated cells that have lost functional polarity and the coupling response (21).

ATP-mediated changes in the P_o of the K channel observed by the patch-clamp technique would be expected to alter the

extent of K recycling across the basolateral membrane if these channels comprise a significant fraction of the total macroscopic K conductance. Accordingly, the contribution of the ATP-sensitive K channels to the spontaneous basolateral membrane potential and total basolateral membrane conductance was determined by pharmacological inhibition. The sulfonylurea agents glibenclamide and tolbutamide are considered to be specific ATP-channel antagonists (see ref. 22 for review) and were therefore used. As shown in Fig. 2, addition of glibenclamide depolarized the V_{bl} and reduced the relative basolateral membrane K conductance in a dose-dependent manner with a K_i of ≈250 μM. As assessed from the magnitude of the shift in the basolateral membrane potential on a 10-fold increase in basolateral K activity, glibenclamide maximally (1 mM) reduced the relative K conductance from 0.56 ± 0.04 to 0.03 ± 0.01 and the G_{cell} by 53%. The response represents a 95% ± 2% inhibition of the total basolateral membrane K conductance. As an index of K-channel specificity, barium (5 mM $BaCl_2$) inhibited the response by 84% ± 2% (n = 6). The response of tolbutamide was qualitatively similar. Addition of this agent reduced the relative K conductance with $K_i \approx 1$ mM. Although the K_i values of the two drugs are considerably greater than those observed in pancreatic β-islet cells, they are consistent with the lower sulfonylurea affinity ATP-sensitive K channels expressed in other tissues (22). This finding is in keeping with the existence of a large family of ATP-sensitive K channels proposed by Ashcroft, S. J. H. and Ashcroft, F. M. (22), which have, among other properties, different sensitivities to sulfonylureas. Furthermore, the observation that glibenclamide nearly completely abolished the macroscopic, basolateral K conductance is consistent with the notion that the ATP-sensitive channels identified by the patch-clamp technique are the major, if not exclusive, determinant of the basolateral K conductance. The finding is consistent with the observation that the ATP-sensitive K channel was the sole

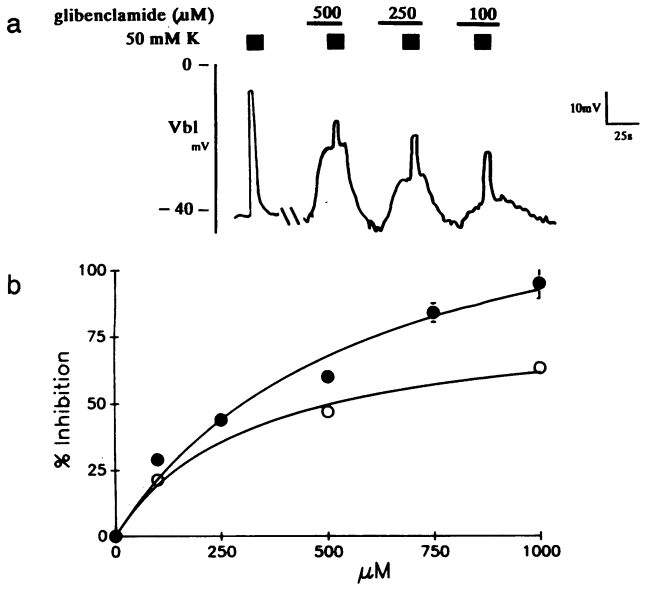

FIG. 2. Effect of sulfonylurea agents on basolateral membrane potential, V_{bl}, and relative basolateral membrane K conductance, G_K/G_{cell}. (*a*) Representative tracing of V_{bl} demonstrating that application of glibenclamide depolarized V_{bl} in a dose-dependent manner and as assessed from the shift of V_{bl} on a 5–50 mM increase in K (bar), glibenclamide reduced relative K conductance. (*b*) Summary of sulfonylurea-mediated inhibition of relative K conductance. Percentage inhibition is calculated as $1 - t_K'/t_K^0$ where t_K' and t_K^0 are relative K conductance in the presence and absence of the drug, respectively. Solid circles, glibenclamide (n = 4); open circles, tolbutamine (n = 5).

channel observed by the patch-clamp technique and corroborates previous comparisons of the intermediate conductance channel density with the macroscopic conductance (19).

To determine the extent to which the ATP-gated K channels are coupled with the activity of the Na,K-ATPase and physiological variations in intracellular ATP levels, the relative K conductance and the apparent $[ATP]_i$ were monitored while stimulating transcellular Na transport. Luminal addition of glucose (5 mM) and alanine (6 mM) enhances transcellular Na transport by stimulating apical Na entry via electrogenic, Na-dependent substrate cotransporters (15, 23). As shown in Fig. 3, the increase in apical membrane conductance after substrate addition causes the relative K conductance, G_K/G_{cell}, to decrease initially from 0.42 ± 0.02 to 0.25 ± 0.02 (n = 6; $P \leq 0.01$). Subsequently, basolateral Na,K-ATPase activity is enhanced to match apical Na entry (24, 25). The increase in the hydrolytic activity of the Na,K-ATPase causes the apparent intracellular ATP concentration to decrease (Fig. 3), as observed previously (14). Indeed, the 57% decrease in $[ATP]_i$ observed in the present and in previous studies can be accounted for solely by the increased turnover of the pump.† In contrast to pancreatic β-islet cells, in which glucose stimulates glycolysis, enhances intracellular ATP levels, and inhibits ATP-sensitive channels (26, 27), the proximal tubule lacks hexokinase activity (28) and exhibits no glycolytic capacity (29). Hence, the response to glucose and alanine addition in the proximal tubule is governed by energy-consuming substrate transport rather than substrate metabolism. Indeed, basolateral addition of the substrates and subsequent facilitated diffusion-mediated entry across the basolateral membrane has no effect on intracellular ATP levels (29) or on the electrophysiologic parameters (data not shown).

As shown in Fig. 3*b*, the response, pump stimulation and decrease in intracellular ATP, is paralleled by a gradual increase in the relative conductance to 0.48 ± 0.02 (n = 6). Since the response occurs with a concomitant increase in the total cellular conductance (5, 23) and reduction of $[ATP]_i$, the observation is consistent with an ATP-dependent increase in the macroscopic basolateral membrane K conductance. Indeed, the involvement of ATP-gated K channels is demonstrated by the finding that prior addition of glibenclamide (500 μM) nearly completely inhibits (78% ± 9% inhibition) the increase in basolateral K conductance after stimulation of transcellular Na transport and Na,K-ATPase activity (26).

The increase in glibenclamide-sensitive K conductance during the Na,K-ATPase-mediated decrease in intracellular ATP levels suggests a significant role of $[ATP]_i$ as a coupling modulator. To establish a causal relationship and extend our single-channel observations to the macroscopic level in the

†The transport of sodium from lumen to blood is an active process that is dependent on the action of the basolaterally oriented Na,K-ATPase. Upon addition of lumenal substrates, electrogenic Na transport is stimulated. In the proximal straight tubule (S_2), the response is accompanied by a −2-mV hyperpolarization of the transepithelial potential. Using a value of the transepithelial resistance of 1050 Ω·cm tubule length (23) and Ohm's law, this accounts for 1.9 $\mu A \cdot cm^{-1}$ of Na current requiring 5.7 $J \cdot s^{-1} \cdot cm^{-1}$ of energy. Accordingly, to maintain active Na transport at this level, the Na,K-ATPase must hydrolyze an additional 6.5×10^{-12} $mol \cdot cm^{-1} \cdot min^{-1}$ of ATP (52,200 J per mol of ATP). If ATP production remains constant and considering that tubule volume is ≈10 $nl \cdot cm^{-1}$, we would expect to observe a 2.6 mM decrease in ATP within 4 min of substrate addition. Thus, the 1.65 mM decrease in the apparent $[ATP]_i$ observed in the present study can be fully accounted for by increased Na,K-ATPase-mediated ATP hydrolysis. That the observed change was less than the theoretical change is compatible with observations that ATP production is enhanced during stimulation of transcellular Na transport (24) and intracellular ATP is compartmentalized (18).

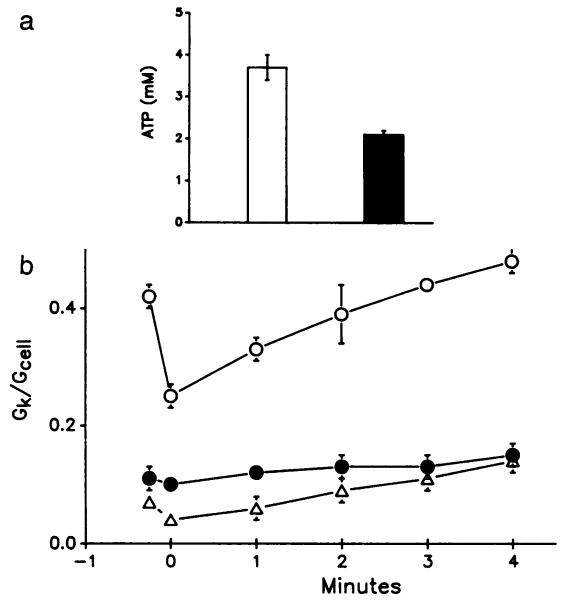

FIG. 3. Intracellular ATP decline upon transcellular sodium transport stimulation coincides with an increase in glibenclamide-sensitive K conductance. (*a*) ATP levels decreased 57% upon stimulation of transcellular Na transport and Na,K-ATPase activity by lumenal addition of 5 mM glucose and 6 mM alanine. Apparent intracellular ATP concentration in the absence (open bar) (3.7 ± 0.3 mM; $n = 5$) and presence (solid bar) (2.1 ± 0.1 mM; $n = 5$) of lumenal substrates. (*b*) Relative K conductance in the presence of 500 μM glibenclamide (solid circles) ($n = 6$) or 2 mM $BaCl_2$ (open triangles) ($n = 5$) relative to control (open circles) ($n = 8$) after addition of lumenal substrates at time 0.

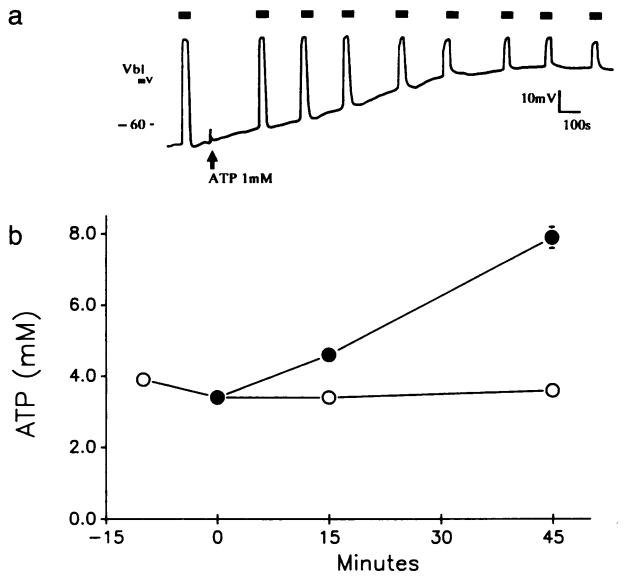

FIG. 4. Intracellular ATP loading blocks the macroscopic, basolateral K conductance and uncouples parallel Na,K-ATPase–K-channel activity. (*a*) Addition of 1 mM ATP to the bathing medium causes a depolarization of V_{bl} and, as assessed by a 10-fold increase in extracellular K (bar), a decrease in relative K conductance. (*b*) Intracellular ATP levels increase 2.6-fold (solid circles) above the control level (open circles) in a time-dependent manner during external ATP incubation.

intact epithelium, the influence of directly modulating $[ATP]_i$ levels on the basolateral K conductance was examined. Weinberg *et al.* (30, 31) demonstrated that proximal tubule intracellular ATP levels increase dramatically upon addition of millimolar concentrations of exogenous ATP via cellular uptake of adenosine and adenosine kinase-mediated conversion to AMP. Accordingly, the effects of intracellular ATP loading by basolateral ATP incubation were examined. As shown in Fig. 4, addition of 1 mM ATP to the bathing medium caused intracellular ATP to increase 2.6-fold in a time-dependent fashion. The response was paralleled by a gradual depolarization of V_{bl}, a decrease in the relative basolateral membrane K conductance, and an increase in cellular input resistance (16% ± 5.5% increase; $n = 3$), which was abolished by prior addition of 2 mM barium. These observations are consistent with an effect on the K channel rather than ATP-induced formation of a nonspecific leak pathway. Indeed, G_K was inversely and linearly related to $[ATP]_i$ over the range tested ($r = 0.94$). Moreover, the observation that the response was largely irreversible and the finding that intracellular ATP levels and relative K conductance were unaffected by exogenous concentrations of ATP of <1 mM are consistent with a direct effect on the channel rather than through purinoceptors, which either have a threshold for ATP action in submicromolar concentrations (P_2) or do not recognize ATP (P_1) (32). Indeed, application of the nucleotide uptake blocker, dipyridamole (10 μM) partially blocks both intracellular ATP loading (31) and the electrophysiological response to exogenous ATP (47% ± 9% inhibition; $n = 6$). In these regards, the macroscopic K conductance appears to be sensitive to variations in $[ATP]_i$, consistent with our observations at the single-channel level.

Using the ATP-loading model, the thesis that the increase in basolateral membrane conductance following Na transport stimulation is dependent on a decrease in $[ATP]_i$ can be tested directly. In an additional series of experiments shown in Fig. 5, the relative K conductance and intracellular ATP levels were monitored as described above upon stimulation of

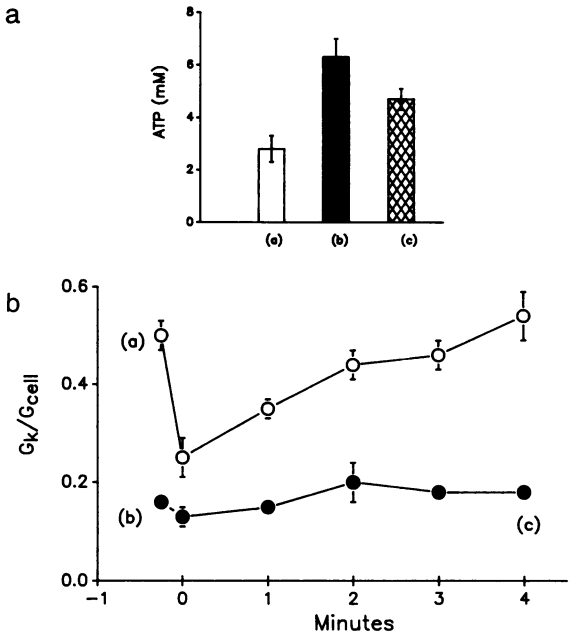

FIG. 5. Increased intracellular ATP uncouples parallel Na,K-ATPase–K conductance. (*a*) Steady-state $[ATP]_i$ after ATP incubation (solid bar) and stimulation of transcellular Na transport by lumenal addition of glucose and alanine (hatched bar) relative to the absence of extracellular ATP and lumenal substrate (open bar). (*b*) Relative basolateral K conductance upon addition of lumenal glucose and alanine after ATP loading (solid circles) relative to control (open circles). After addition of glibenclamide, barium, or ATP, basolateral K conductance approaches the limit of zero. Thus, under these conditions, activation of electrogenic apical Na entry by lumenal addition of glucose and alanine and a subsequent increase in G_{cell} are not reflected by a decrease in the G_K/G_{cell} ratio.

transcellular Na transport and Na,K-ATPase activity after intracellular ATP loading. Although the lumenal addition of the substrates and subsequent stimulation of Na,K-ATPase activity caused $[ATP]_i$ to fall nearly to the same extent as the control, $[ATP]_i$ remained 1.7-fold above the basal levels (Fig. 5*a*) and the basolateral K conductance failed to increase (Fig. 5*b*). Indeed, intracellular ATP loading inhibited the increase in the relative K conductance after Na,K-ATPase stimulation by 80% ± 3% relative to control. We conclude from these observations that parallel Na,K-ATPase–basolateral K recycling can be uncoupled by preventing a critical $[ATP]_i$ decline.

Collectively, these data demonstrate directly that ATP acts as a coupling modulator of parallel Na,K-ATPase–basolateral K-channel activity. Patch-clamp and conventional microelectrode studies revealed that physiological concentrations of ATP reduce the activity of the major determinants of the macroscopic basolateral membrane K conductance, presumably by binding ATP to an allosteric epitope on the channel or closely associated protein as described for other ATP-sensitive K channels (22, 32). As evidenced by the ability of intracellular ATP loading to uncouple the response, increased hydrolysis of ATP during physiological surges in transcellular Na transport causes intracellular ATP concentration to drop so that the K channel is released from ATP-mediated inhibition. The subsequent increase in the macroscopic K conductance allows passive cellular K efflux to match active uptake without a deleterious increase in the intracellular K activity.

We cannot, however, exclude the possibility that additional mechanisms regulate the channel and ATP sensitivity. This prospect is supported by the finding that the channel was more sensitive to ATP in inside-out patches than in the intact cell. The ability of physiological concentrations of ADP to reduce the affinity of ATP for the channel as observed in other ATP-sensitive channels (22, 32) may explain this observation. In this regard, increases in intracellular ADP during stimulation of Na,K-ATPase hydrolytic activity (33) would also enhance the basolateral K-channel activity. Moreover, we have recently shown that the ATP-sensitive K channel expressed in the renal cortical collecting duct apical membrane is regulated in a dual fashion by cAMP-dependent protein kinase A and protein kinase C (34). Accordingly, such mechanisms may also serve as avenues for basolateral K-channel regulation during changes in transcellular Na transport. In any regard, our data demonstrate that ATP is a critical coupling modulator of parallel Na,K-ATPase–basolateral K-channel activity. With the observation that similar ATP-sensitive K channels are the major determinants of the macroscopic K conductance in other renal epithelia (10, 11), we postulate that this underlying coupling mechanism may be as universal as the response.

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