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Activation of an ATP-dependent K^+ conductance in *Xenopus* oocytes by expression of adenylate kinase cloned from renal proximal tubules

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Abstract

In rabbit proximal convoluted tubules, an ATP-sensitive K^+ (K_{ATP}) channel has been shown to be involved in membrane cross-talk, i.e. the coupling (most likely mediated through intracellular ATP) between transepithelial Na^+ transport and basolateral K^+ conductance. This K^+ conductance is inhibited by taurine. We sought to isolate this K^+ channel by expression cloning in *Xenopus* oocytes. Injection of renal cortex mRNA into oocytes induced a K^+ conductance, largely inhibited by extracellular Ba^{2+} and intracellular taurine. Using this functional test, we isolated from our proximal tubule cDNA library a unique clone, which induced a large K^+ current which was Ba^{2+}-, taurine- and glibenclamide-sensitive. Surprisingly, this clone is not a K^+ channel but an adenylate kinase protein (AK3), known to convert NTP+AMP into NDP+ADP (N could be G, I or A). AK3 expression resulted in a large ATP decrease and activation of the whole-cell currents including a previously unknown, endogenous K^+ current. To verify whether ATP decrease was responsible for the current activation, we demonstrated that inhibition of glycolysis greatly reduces oocyte ATP levels and increases an inwardly rectifying K^+ current. The possible involvement of AK in the K_{ATP} channel's regulation provides a means of explaining their observed activity in cytosolic environments characterized by high ATP concentrations.

Keywords: KATP channel; Proximal tubule; Taurine; ATP content; Adenylate kinase; *Xenopus laevis* oocyte

1. Introduction

Proximal tubules reabsorb Na^+ and a variety of substrates by combining Na^+ cotransport across the apical membrane with basolateral extrusion by the Na^+/K^+-ATPase, while K^+ is recycled via K^+ channels located on the basolateral membrane. Coupling has been demonstrated between transepithelial Na^+ transport and basolateral K^+ conductance in proximal convoluted tubules (PCT) [1,2], where Na^+ transport stimulation led to an increase in macroscopic potassium conductance. This 'pump–leak' relationship, likely to be mediated via changes in intracellular ATP levels and ATP-sensitive K^+ (K_{ATP}) channels, prevents excessive cell swelling and maintains a favorable electrical gradient for sustained apical Na^+ transport. The 50 pS channel initially

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observed in the basolateral membrane of rabbit proximal tubule [3] was indeed later found to be ATP-sensitive [4,5] but its characterization remained difficult as the channel displays a rapid run-down in inside-out patches. A cloned and ubiquitously expressed K_{ATP} (uK_{ATP}-1 or Kir 6.1, [6]) was localized at the basolateral membrane of rat proximal tubules [7]. It is not yet established if this channel (70 pS after expression in oocytes) is involved in the basolateral K^+ conductance of PCT.

K_{ATP} channels were originally discovered in cardiac muscle [8], and have been subsequently identified in a variety of tissues. In spite of the numerous studies on different members of the K_{ATP} channel family, the precise regulatory mechanisms of K_{ATP} channels are still not well understood. Indeed, a paradox remains: how can K_{ATP} channels remain active in a cytoplasmic environment where the ATP concentration is often much higher than its inhibition constant (K_i) for these channels?

To determine the molecular identity of the PCT K_{ATP} channel and to study its sensitivity to intracellular ATP, we attempted expression cloning in *Xenopus laevis* oocytes of the proximal tubule K_{ATP} channel, using sensitivity to both Ba^{2+} and taurine of the whole-cell K^+ current as a functional test. In support of this, we have previously shown that the basolateral potassium conductance in PCT is inhibited by taurine [9] and that this inhibition is specifically related to the presence of the K_{ATP} previously identified [10].

Expression of total mRNAs from rabbit renal cortex in oocytes enhanced whole-cell currents which included a K^+ current that was Ba^+- and taurine-sensitive. This functional test allowed us to isolate a unique clone from our renal cortex cDNA library, which induced a large Ba^{2+}-, taurine- and glibenclamide-sensitive K^+ current. cDNA sequencing determined that this clone was not a K^+ channel but a truncated adenylate kinase (AK) with high similarity with AK3. We then isolated the full-length AK3 clone from proximal tubule cDNA. Further experiments showed that expression of the AK3 clone in oocytes resulted in a diminution of intracellular ATP concentration as well as an increase in the whole-cell currents including a previously unknown, endogenous inwardly rectifying K^+ current which is Ba^+-, glibenclamide- and taurine-sensitive, properties suggesting the presence of a K_{ATP} channel in *X. laevis* oocytes.

2. Materials and methods

2.1. Molecular biology

2.1.1. Expression cloning of truncated AK3

Renal cortex tissue was dissected from rabbit kidneys which had been rapidly perfused with ice-cold phosphate-buffered saline. RNA was extracted from the tissue using Trizol reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. Polyadenylated RNA was then isolated using oligo-dT cellulose chromatography (Collaborative Biomedical Research, Bedford, MA, USA).

First-strand synthesis of cDNA was performed using Superscript II (Life Technologies) in accordance with the manufacturer's instructions with the modifications that methylated dCTP and an *Xho*I linker-primer were employed. Double-stranded cDNA was then synthesized using the ZAP cDNA synthesis kit (Stratagene, San Diego, CA, USA). The cDNA was ligated into the vector pMT21 [11] which had been cleaved with *Eco*RI and *Xho*I. The ligated DNA product was used to electroporate DH10B *Escherichia coli* bacteria (Life Technologies) and a library of 300 000 colonies was produced. The bacteria were grown in an agarose suspension [12] and the plasmids were purified by an alkaline lysis technique followed by binding to glassmilk.

Five microgram of the recombinant plasmids were relaxed with topoisomerase, purified and 1 μg of the relaxed DNA was separated on a preparative 0.5% low melting agarose gel. After staining with ethidium bromide, 50 slices of size-separated plasmids were removed from the gel and digested with agarase (New England Biolabs, Beverly, MA, USA). Each one of the 50 DNA samples was used to transform competent Xl-2 Blue *E. coli* bacteria (Stratagene). Colonies were grown in suspension and plasmids purified. When one sample was shown to induce a significant K^+ current, the plasmid preparation was used again to transform competent bacteria, which were plated out onto Petri dishes. Copies were made of each Petri dish onto nitrocellulose filters and plas-

mids were purified from the copies. When one Petri dish containing bacteria was shown to harbor the plasmid which induced K^+ current, the colonies on the plate were grown individually. Then the plasmid responsible was isolated by sib selection (aliquots of individual plasmid preparations were combined into pools and then tested to determine which group contained the desired DNA). The process was reiterated to eventually produce a single clone.

2.1.2. Cloning of the full-length AK3

A suspension of cortical renal tubules of New Zealand White rabbits was prepared as described previously [13]. The tubule fragments were separated in a 50% Percoll solution by the method of Vinay et al. [14]. The F4 layer, 98% of which is proximal tubules, was then removed from the Percoll gradient and washed three times by centrifugation. Total RNA was extracted from the PCT using Trizol reagent (Life Technologies) according to the manufacturer's instructions. Double-stranded cDNA was obtained from PCT RNA using the SMART PCR cDNA Synthesis kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. This method presents the advantage of a significant enrichment in full-length cDNAs.

A degenerate PCR primer corresponding to the first nucleotides of rat, bovine and human AK3s (5′-CCACCATGGGGGCRTCKGGGCGGC-3′, containing a start codon and a Kozak consensus sequence) and a primer directly designed from the 3′ end of our rabbit truncated AK3 (5′-GCTCCTGCTCTCTTCCTGGTGATGTGCG-3′) were used to amplify a 681 bp product from proximal tubule cDNA corresponding to the full-length rabbit AK3. The product was ligated into the vector pMT21.

The nucleotide sequence of the truncated and full-length clone was obtained via the dideoxy chain-termination method using the T7 Sequenase DNA sequencing kit (Amersham, Life Science, Cleveland, OH, USA).

2.2. Oocyte preparation and injection

Stage V and VI oocytes were removed from *X. laevis* frogs anaesthetized with 3-aminobenzoic acid ethyl ester and placed into Barth's solution (88 mM NaCl, 3 mM KCl, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.60). The follicular layer was removed by incubation in a Ca^{2+}-free Barth's solution containing 1.6 mg/ml collagenase (Sigma, St Louis, MO, USA) for 45 min with gentle stirring, followed by washing of the oocytes in Barth's solution containing 1% albumin. Defolliculated oocytes were stored at 18°C in Barth's solution supplemented with 5% horse serum, 2.5 mM Na^+ pyruvate, 100 U/ml penicillin and 0.1 mg/ml streptomycin for 48 h. Then, healthy oocytes were selected and injected, either into the cytoplasm with 46 nl of water (46 ng mRNA) or into the nucleus with 4.6 nl of water containing 300 pg of a recombinant expression vector encoding green fluorescent protein (rB-GFP) plus (where required) 300 pg of recombinant pMT21 containing other cDNA inserts [15]. The oocytes were incubated in antibiotic Barth's solution for 4–7 days and a simple fluorescent screening was used to select oocytes that were expressing the injected plasmids.

2.3. Two-microelectrode voltage clamp technique

Oocyte currents were measured with the two microelectrode voltage-clamp technique using a commercial amplifier (Oocyte Clamp model OC-725, Warner Instrument Co., Hamden, CT, USA) as described earlier [16]. Current–voltage curves were obtained from 11 pulses (500 ms duration) separated by 600 ms periods at the resting potential of −40 mV. The voltage range covered was from −165 to +85 mV. The voltage pulse protocol was first performed while oocytes were perfused (at approximately 1.5 ml/min) with a modified saline Barth's solution (M-Barth: 40 mM NaCl, 3 mM KCl, 50 mM *n*-methyl-D-glucamine-Cl (NMDG-Cl), 0.82 mM $MgCl_2$, 0.74 mM $CaCl_2$ and 5 mM HEPES–Tris, pH 7.60) and then with a medium K^+ saline Barth's solution (K-Barth: 40 mM NaCl, 20 mM KCl, 33 mM NMDG-Cl, 0.82 mM $MgCl_2$, 0.74 mM $CaCl_2$ and 5 mM HEPES–Tris, pH 7.60), with a K^+-free saline Barth's solution (K-free Barth: 40 mM NaCl, 53 mM NMDG-Cl, 0.82 mM $MgCl_2$, 0.74 mM $CaCl_2$ and 5 mM HEPES–Tris, pH 7.60) or with a high K^+ saline Barth's solution (HK-Barth: 93 mM KCl, 0.82 mM $MgCl_2$, 0.74 mM $CaCl_2$ and 5 mM HEPES–Tris, pH 7.60). Current and voltage traces were analyzed by averaging the signal in a window of

25 ms positioned after the decay of all capacitative transients.

2.4. ATP concentration measurements

For each oocyte in which ATP concentration was determined, whole-cell currents were first measured using the two-electrode voltage clamp technique. Each oocyte was then separately homogenized in 25 μl of ice-cold antibiotic Barth's solution supplemented with perchloric acid (3.5 μl of a 40% solution) using a vortex mixer for 10 min. The tubes were microcentrifuged at 4°C for 10 min at $15\,000 \times g$ to pellet cell debris. To neutralize the supernatant, 6.6 μl of 20% KOH were mixed in and the tubes were microcentrifuged at 4°C. The supernatant's ATP concentration was quantified by the addition of 75 μl luciferin–luciferase at 20 mg/ml (in glycine buffer, Sigma), followed 15 s later by the measurement of luminescence at 520 nm; the values obtained were compared with a standard [ATP] curve.

2.5. Statistics

Where statistical analysis was performed, all tests done were paired or unpaired *t*-tests as required; where average values are given, they represent the mean ± S.E.M. and '*n*' represents the number of oocytes from at least three different animals.

3. Results

3.1. Potassium currents expressed from total rabbit renal cortex mRNAs

In order to define a functional test which would allow expression cloning of the K_{ATP} channel of rabbit PCT, we first characterized the properties of an induced K^+ current when total rabbit renal cortex mRNAs were injected into *X. laevis* oocytes. We compared whole-cell currents measured in a two-microelectrode voltage-clamp setup at 4–7 days after the injection of water (Fig. 1A) or mRNA (Fig. 1B) into oocytes. Water-injected oocytes developed a current of 128 ± 12 nA at +85 mV, and −33 ± 3 nA at −110 mV in M-Barth (Fig. 1C, $n = 8$). Conductance was higher in mRNA-injected oocytes:

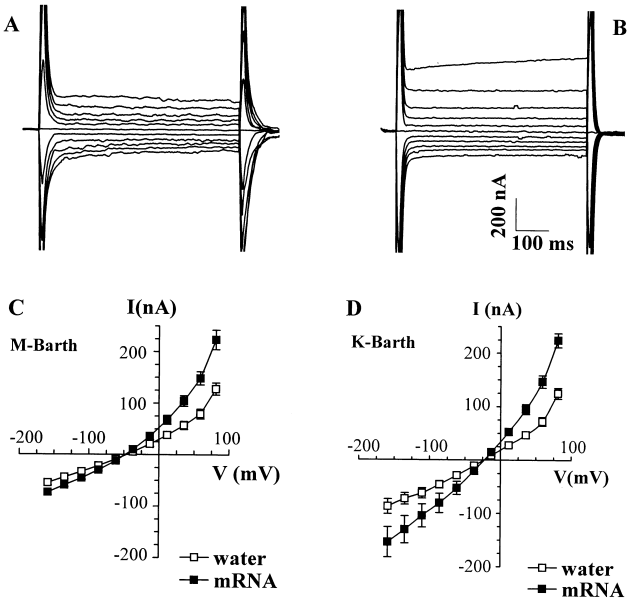

Fig. 1. Membrane currents of oocytes expressing rabbit renal cortex mRNA. Test pulse potentials from +85 mV to −165 mV were applied in steps of 25 mV and currents were recorded from oocytes injected with either water (control situation, A) or rabbit renal cortex mRNA (B) in M-Barth. *I–V* relationships of water- (□) or mRNA- (■) injected oocytes in M-Barth (3 mM K^+, C) or K-Barth (20 mM K^+, D).

whole-cell currents are 223 ± 19 nA at +85 mV ($P < 0.001$ vs. water-injected oocyte), and −46 ± 3 nA at −110 mV in M-Barth ($n = 10$). As extracellular K^+ was increased (from 3 mM in M-Barth to 20 mM in K-Barth, Fig. 1D), the reversal potential for whole-cell currents shifted to a more positive value (−27 ± 2 mV, $n = 39$) and the inward currents activated by external addition of K^+ were significantly higher in RNA-injected oocytes (−56 ± 13 nA at −110 mV) than in control oocytes (−29 ± 7 nA at −110 mV, compare Fig. 1C and D, $n = 10$). This suggests the presence of a new K^+ current among whole-cell currents measured in total mRNA expressing oocytes (a mRNA-induced current of 100 nA and −43 nA; respectively at +85 mV and −110 mV could be deduced by subtracting the control current from the current measured following mRNA injection in K-Barth).

To confirm the presence of a K^+ current, the inhibitory effect of 2 mM Ba^{2+} was tested. Fig. 2A shows the Ba^{2+}-sensitive currents, representing total currents subtracted with the currents measured after barium exposure, measured in K-Barth for water-

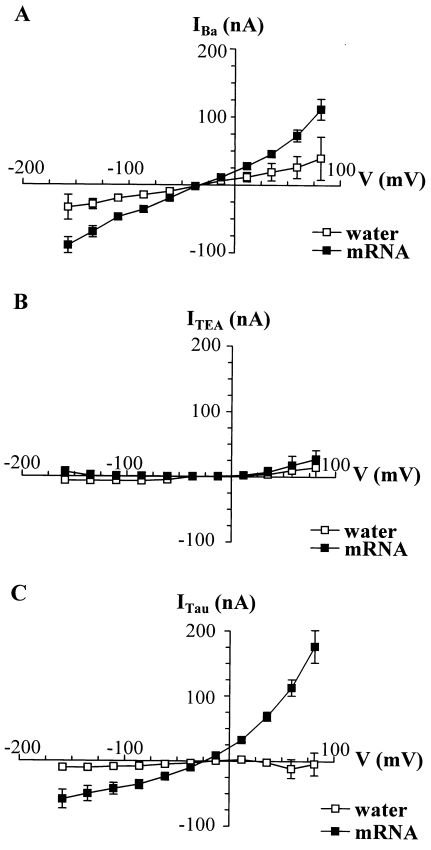

Fig. 2. Effect of inhibitors on membrane currents of renal cortex mRNA expressing oocytes. Ba^{2+}- (2 mM, A), TEA- (10 mM, B) or taurine- (15 mM, C) sensitive currents of water- (□) or mRNA- (■) injected oocytes were measured in K-Barth.

and mRNA-injected oocytes. We observed that Ba^{2+}-sensitive currents were significantly higher in mRNA-injected oocytes (112 ± 15 nA and -48 ± 4 nA at +85 and −110 mV, $n = 6$) than in water-injected oocytes (40 ± 32 nA and -20 ± 5 nA respectively, $n = 6$). The reversal potential of the Ba^{2+}-sensitive current for both control and mRNA-injected oocytes was found to be -28 ± 2 mV in K-Barth solution. We also applied tetraethylammonium (TEA), another K^+ channel blocker, but found that 10 mM of this compound had no effect on the currents of either water- or mRNA-injected oocytes. Fig. 2B shows the TEA-sensitive currents for water- or mRNA-injected oocytes, obtained by subtracting currents measured after TEA exposure from the one observed before TEA application ($n = 5$).

As the ATP-sensitive basolateral potassium conductance in PCT is sensitive to intracellular taurine [9,10], the taurine sensitivity of oocyte currents was measured (Fig. 2C). Control and mRNA-expressing oocytes were injected 1–2 h prior to current measurement, with a bolus of either mannitol or taurine (to produce 15 mM intracellular concentrations, assuming a cytoplasmic volume of 0.5 μl). Taurine-sensitive current was obtained by subtracting the current measured in presence of taurine from that observed after injection of mannitol. Intracellular taurine had no significant effect on water-injected oocytes but mRNA-expressing oocytes exhibited a taurine-sensitive current. In mRNA-injected oocytes, intracellular taurine diminished conductance to the level seen in water-injected oocytes (i.e. 182 ± 9 nA and 194 ± 7 nA, respectively in mRNA- and water-injected oocytes at +85 mV in presence of taurine). The reversal potential of the taurine-sensitive current for mRNA-injected oocytes was found to be −24 mV. A lower concentration of taurine (5 mM) had no detectable effect (data not shown), nor did mannitol injection (5 or 15 mM) in either water- or mRNA-injected oocytes.

From the above findings, we concluded that the oocyte current-sensitivities to Ba^{2+} and taurine after mRNA injection can constitute a functional test to allow expression cloning of the channel responsible for the K_{ATP} current of PCT.

3.2. K^+ currents induced by an isolated rabbit renal cDNA

From the total renal cortex mRNAs, a cDNA library was prepared, composed of 3×10^5 colonies, among which we set up to isolate a unique clone responsible for the barium- and taurine-sensitive K^+ current. Plasmids were purified and the DNA was size-separated on a preparative gel that was later cut in 50 slices. The plasmid group 22, containing inserts of approximately 2.0–2.2 kb, was shown to induce in oocytes a significant K^+ current, higher than the one obtained with total mRNAs. This plasmid preparation was used again to transform competent bacteria, which were plated out onto seven different Petri dishes. Copies were made onto nitrocellulose filters and plasmids purified. The Petri dish B was shown to harbor the plasmid which induced a K^+ current higher than the one of the plasmid group 22 or total mRNAs. The 400 different colonies of

this plate were grown individually and a sib selection was used to identify a single cloned (#288-cDNA) which was positive in our functional test.

Typical examples of currents recorded in K-Barth from rB-GFP (control, Fig. 3A) and #288-cDNA expressing oocytes (Fig. 3B) showed that oocytes which expressed the #288-cDNA demonstrated significantly greater conductance at all membrane potentials measured. Indeed #288-cDNA expressing oocytes exhibited 606 ± 100 nA and -347 ± 55 nA at +85 mV and −110 mV respectively, vs. 139 ± 8 nA and -81 ± 8 nA for control ($P < 0.0001$, Fig. 3C, $n = 11$). The reversal potential of whole-cell currents measured in K-Barth on #288-cDNA-injected oocytes was found to be -22.3 ± 1.7 mV.

To confirm that a large portion of the #288-cDNA-induced currents was carried by potassium, we measured the effects of extracellular K^+ on the amplitude of the inward currents (at −160 mV;) in control and #288-cDNA expressing oocytes (Fig. 4, $n = 6$). In control oocytes, we observed that changing from 0 to 20 mM external K^+ induced an inward current of 107 ± 39 nA which was completely sensitive to the presence of 2 mM Ba^{2+}. In #288-cDNA-injected oocytes the inward currents induced by exposure to 20 mM external K^+ were 275 ± 31 nA (i.e. a 2.6 fold increase, $P < 0.005$, vs. control oocytes) and were also completely sensitive to 2 mM Ba^{2+}. This is consistent with the hypothesis that the #288-cDNA clone induces a conductance that involves K^+ influx. It should be noted (Fig. 4) that, in the absence of K^+ (0 K) or in presence of 2 mM Ba^{2+}, the currents measured in #288-cDNA expressing oocytes are slightly higher than in control oocytes, suggesting that a portion of the #288-cDNA-induced currents involves other ions than K^+.

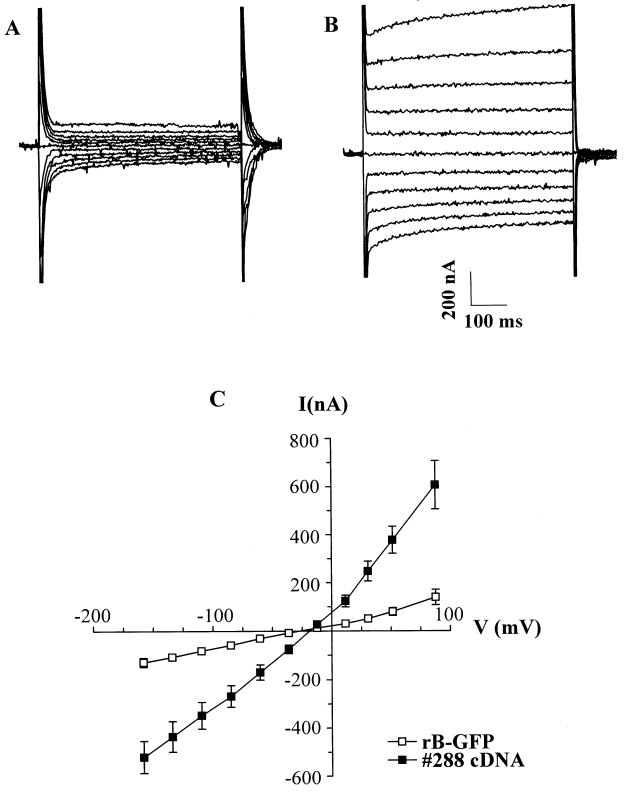

Fig. 3. Membrane currents for #288 cDNA expressing oocytes. Typical example of currents recorded from rB-GFP- (A) or #288 cDNA- (B) expressing oocytes bathed in K-Barth. *I–V* relationship of rB-GFP-(□) or #288 cDNA-(■) injected oocytes in K-Barth (C).

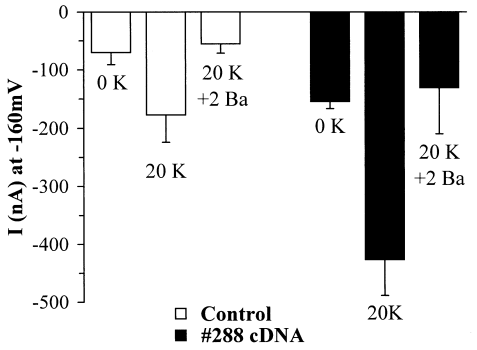

Fig. 4. Effect of extracellular K^+ and Ba^{2+} on inward currents. Inward currents from control (open boxes) or #288 cDNA-injected (black boxes) oocytes measured at −160 mV, in Barth's solution which either 0 mM K^+ (0 K, K-free Barth), 20 mM K^+ (20 K, K-Barth) or 20 mM K^++2 mM Ba^{2+}.

Fig. 5A shows the *I–V* curve of the Ba^{2+}-sensitive current measured in K-Barth solution for rB-GFP and 288-cDNA-injected oocytes. In this series of experiments, the Ba^{2+}-sensitive current was about five-fold higher for 288-cDNA-injected oocytes than for control oocytes. Ba^{2+} was able to block currents in both directions; the Ba^{2+}-sensitive current reverses at -26 ± 2 mV for both control and #288-cDNA-injected oocytes ($n = 4$).

The inhibitory effects of taurine were also examined on #288-cDNA expressing oocytes. As shown in Fig. 5B, these oocytes develop a large taurine-sensitive current (274 ± 19 nA and -98 ± 6 nA respectively at +85 mV and −110 mV), which reverses at -30 ± 2mV ($n = 5$). As seen in Fig. 2, control oocytes

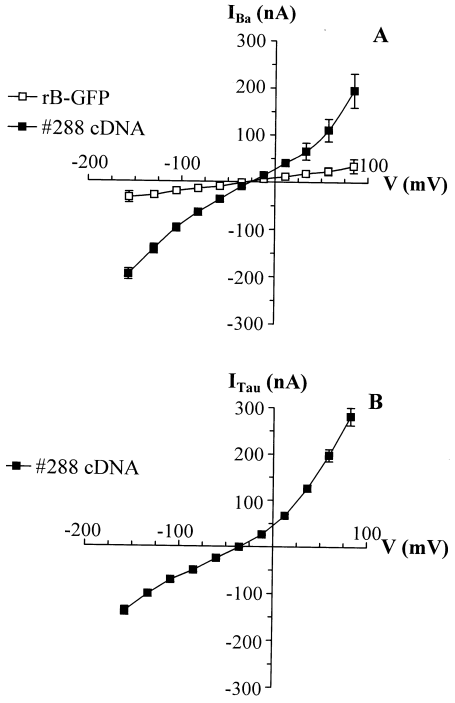

Fig. 5. Inhibitor-sensitive currents from #288 cDNA expressing oocytes. (A) 2 mM Ba^{2+}-sensitive currents were measured after 5 min of external exposure for control oocytes (rB-GFP injected, □) or #288 cDNA expressing oocytes (■). (B) 15 mM taurine-sensitive currents were measured 1–2 h following taurine injection into #288 cDNA expressing oocytes (■).

do not present any significant taurine-sensitive current.

Thus, the properties of the protein expressed by the cDNA clone are similar to those seen when renal cortex mRNA is expressed in oocytes, as well as to the known characteristics of the K_{ATP} conductance of PCT and cardiac cells.

3.3. Sequencing the isolated clone

The #288 clone contained an insert of approximately 2.1 kb, the first 920 bases of which were sequenced (the coding region of the cDNA insert was totally sequenced and the 3′ untranslated region was partially sequenced). The cDNA was predicted to encode a 126 amino acid polypeptide where alignment through GenBank scored the highest similarity with the family of AK. The coding region of the cDNA was 40% identical with chicken and yeast AK1 or bovine and human AK2 and 90% identical with bovine and rat AK3. AK3 is a mitochondrial protein involved in interconverting NTP+AMP into NDP+ADP (where N could be G, I or A). The insert does not contain the entire coding region; it starts at a position equivalent to one nucleotide prior to the codon for amino acid 89 of bovine AK3 [17]. Surprisingly, we observed that the insert did not contain any ATG, though there was a CTG codon located within a region that bears a striking similarity to a Kozak consensus sequence [18]. There have been some reports of CTG occasionally acting as an alternative initiation codon for translation [19,20]. Thus, the coding region started at a position equivalent to amino acid #102 of bovine AK3, suggesting that the #288 clone encoded a truncated rabbit AK3.

3.4. Cloning, sequencing and expression of the full-length AK3

As the #288 clone appeared truncated and did not contain a conventional codon for initiation of translation, we isolated from proximal tubule cDNA, the full-length rabbit AK3, taking advantage of the high degree of similarity in the 5′ ends of bovine, rat and human AK3 (see Section 2). The full-length cDNA encodes a 227 amino acid protein, which is 90% identical to the bovine and rat AK3 proteins [21] and 55% identical to human AK3 (see Fig. 6). Fig. 7 shows the average currents obtained using oocytes from the same donors that were injected with rGFP alone ($n=4$) or with 300 pg of either the truncated ($n=13$) or the full-length ($n=13$) AK3 cDNAs. Expression of full-length AK3 cDNA in oocytes induces currents that are comparable to those generated by the truncated AK3 (612 ± 77 nA and 937 ± 142 nA at +85 mV, -186 ± 31 nA and -249 ± 42 nA at −110 mV respectively in truncated- or full-length-AK3 expressing oocytes). The reverse potential of currents measured in truncated or full-length AK3 expressing oocytes were not significantly different (-22.5 ± 2.1 mV and -27.5 ± 3.9 mV respectively). Oocytes expressing the full-length AK3 exhibited a slightly higher current than those expressing truncated AK3. This difference could be due to a higher translation efficiency and/or a higher activity of the full-length protein. Nevertheless, the truncated and full-length AK3 proteins induced a similar effect suggesting that the #288 clone is functional despite encoding a truncated AK3.

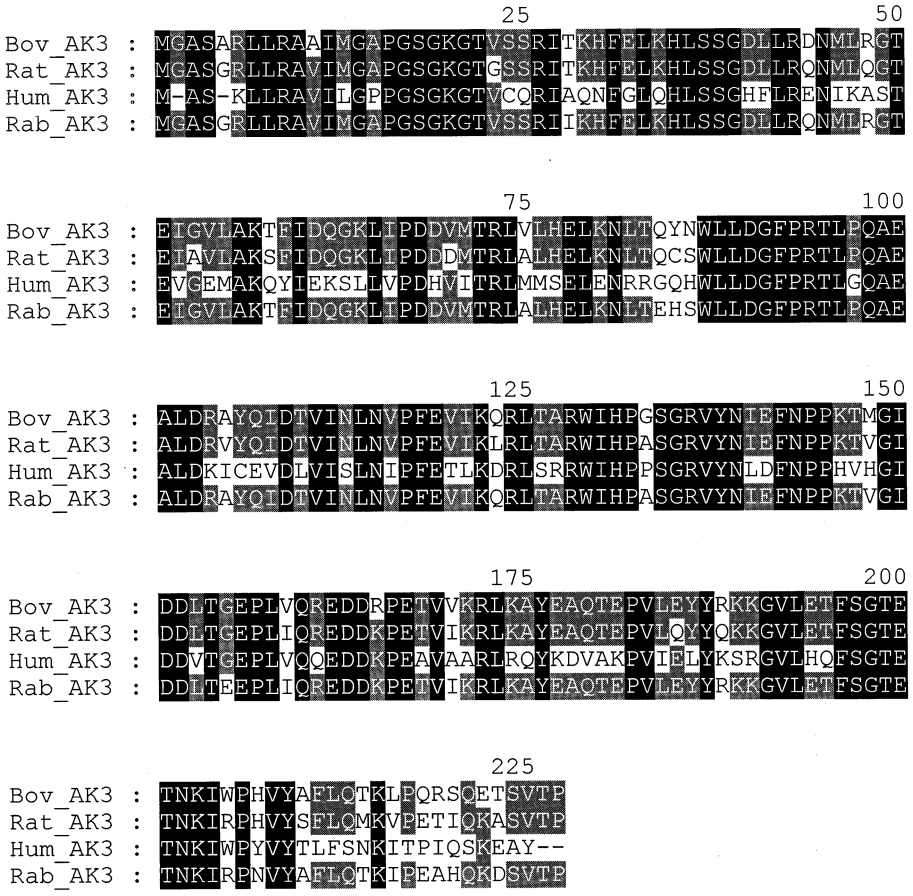

Fig. 6. Comparison of AK3 primary structures. The full-length rabbit AK3 sequence (Rab_AK3) was aligned with the bovine (Bov_AK3), rat (Rat_AK3) and human (Hum_AK3) AK3 sequences using BLAST [51]. Dark and gray regions indicate perfect identity amongst either the four or three of the four sequences, respectively.

3.5. Relation between AK3 expression and potassium current generation

Since the isolated clone does not encode a K^+ channel, it seems reasonable that expression of the AK3 protein must activate some pathways that affect endogenous K^+ currents. In light of the capacity of AK to hydrolyze triphosphate nucleotides, we hypothesized that AK3 could decrease the ATP concentration and activate a putative endogenous K_{ATP} channel.

K_{ATP} channels are known to be made from the combination of inward rectifier K^+ channels and sulfonylurea receptors (high-affinity SUR1 or low-affinity SUR2A or SUR2B). If this is true for the putative K_{ATP} channels of oocytes, the K^+ currents stimulated by expression of AK3 might be sensitive to glibenclamide, a antidiabetic sulfonylurea and a specific blocker of K_{ATP} channels [22]. Glibenclamide sensitivities of native K_{ATP} channels vary over a range from nM to mM depending on the tissues studied [23–25] which probably reflects the specific subtype of sulfonylurea receptor present. On the other hand, in reconstitution experiments, an IC_{50} for channel inhibition by glibenclamide in the nM range was found for the SUR1 subunit [26], while SUR2A and SUR2B receptor required 100–1000 fold higher concentrations to inhibit the channel [27,28]. Fig. 8 shows the currents of AK3 expressing oocytes in the presence of 0, 1, 10, 50, 100 or 250 μM glibenclamide. An inhibitory effect could be observed at 1 μM (glibenclamide-sensitive current at +85 mV: 185 ± 37 nA) and increasing the glibenclamide concentration to 250 μM enhanced the inhibition (glibenclamide-

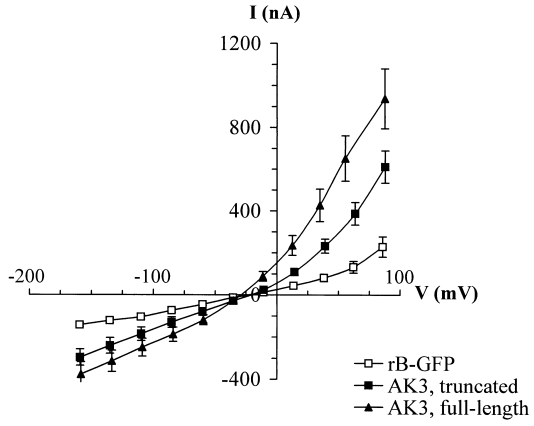

Fig. 7. Comparison of truncated and full-length AK3-induced currents. *I–V* relationship of rB-GFP- (control situation, $n=4$, □), truncated AK3 cDNA- (■, $n=13$) or full-length AK3 cDNA (▲) expressing oocytes ($n=13$).

sensitive currents at +85 mV: 369 ± 41 nA, $n=7$). It can be seen that the inhibition is not complete suggesting that expression of AK3 may stimulate more than one type of conductances. The insert in Fig. 8 shows the glibenclamide (50 μM)-sensitive currents in cDNA-injected oocytes (298 ± 39 nA at +85 mV) and in rB-GFP-injected oocytes where no significant inhibition could be observed, even at 250 μM. Interestingly, in non-paired experiments, a significant glibenclamide-sensitive current (at +85 mV: 49 ± 20 nA, $n=5$) could be observed at a concentration as low as 1 nM.

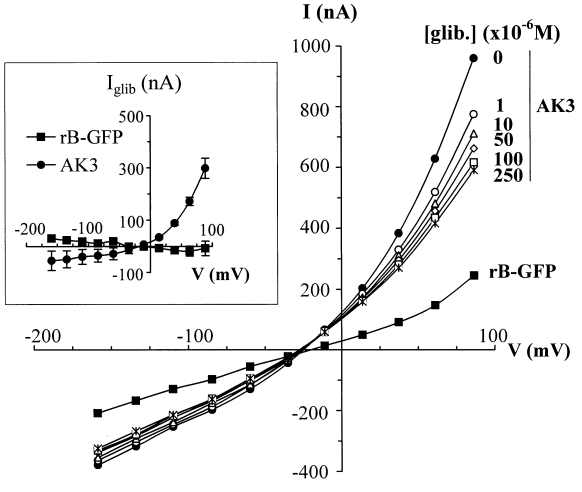

Fig. 8. Effect of glibenclamide. *I–V* relationship of rB-GFP (■) injected oocytes compared with the *I–V* relationship of AK3 expressing oocytes after 5 min of external exposure to 0 (●), 1 (○), 10 (△), 50 (◇), 100 (□), 250 (*) μM glibenclamide. The insert represents the glibenclamide (50 μM)-sensitive currents in rB-GFP- (■) or AK3 (●) expressing-oocytes.

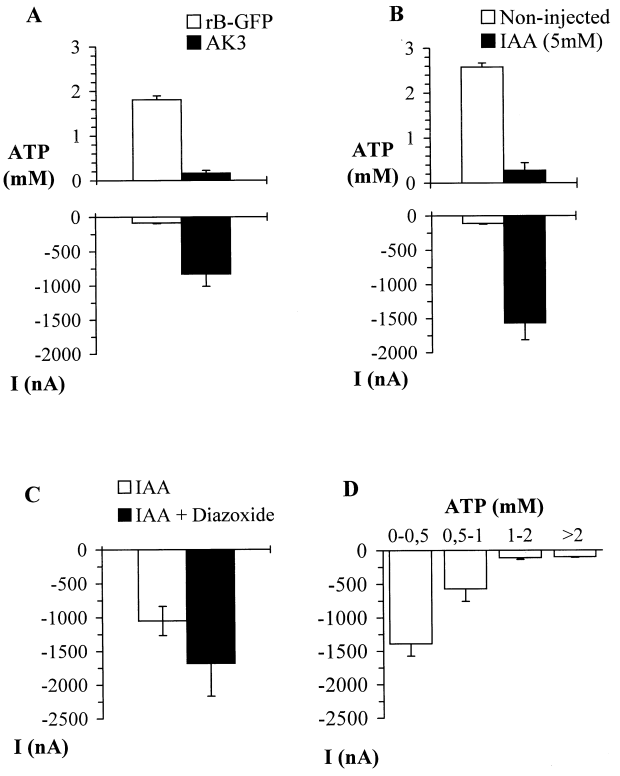

Fig. 9. Relationship between ATP concentration and whole-cell currents in *Xenopus* oocytes. ATP concentration and whole-cell currents at −110 mV were measured in rB-GFP- (control, open boxes) and AK3-injected (black boxes) oocytes (A); in non injected oocytes (control, open boxes) and IAA- (5 mM) treated oocytes (for 5 h, black boxes) (B); or in IAA- (5 mM, 5 h) treated oocytes before (open boxes) and after (black boxes) a 5 min application of 300 μM diazoxide (C). Current and ATP content where determined for each individual oocyte tested. A correlation of membrane current at −100 mV vs. ATP concentration is shown in (D) for the 73 oocytes tested in this portion of the study.

The current remaining in the presence of high glibenclamide concentration probably represents the control current measured in non-injected oocytes combined with AK3-induced conductances other than K_{ATP}. Assuming that the inhibitory effect measured in presence of 250 μM glibenclamide is maximal, we calculated that 10 μM glibenclamide was sufficient to produce 75% of the maximal inhibitory effect, while 56% and 10% of this effect were measured respectively with 1 μM and 1 nM. These results are consistent with an IC_{50} around 1 μM which

suggests that low-affinity SUR receptors (SUR2A or SUR2B) could be implicated.

As glibenclamide sensitivity is a strong evidence of the presence of K_{ATP} channels, we tested the hypothesis that AK3 expression could stimulate these channels through a decrease in ATP concentration. Fig. 9A shows measurements of the oocyte's inward currents at −110 mV and average ATP concentration for both control oocytes (rB-GFP injected oocytes) and AK3-injected oocytes. The mean ATP concentration in rB-GFP-injected oocytes was 1.81 ± 0.09 mM ($n = 11$) and the inward current was -86 ± 10 nA ($n = 10$). When AK3 cDNA was injected, we observed two apparent groups of oocytes: one group ($n = 11$, i.e. 37% of the tested oocytes) did not seem to express the clone as is often seen with intranuclear cDNA injection and consequently the ATP concentration and the current (1.90 ± 0.10 mM and -102 ± 42 nA respectively) were not different from those of oocytes injected solely with rB-GFP. In the other group of oocytes (Fig. 9A, $n = 19$, 63% of the tested oocytes), AK3 expression induced a greatly reduced ATP concentration (0.16 ± 0.06 mM) and a large increase in oocyte current (-830 ± 180 nA), suggesting a link between these phenomena.

To test the hypothesis that decreases in cytoplasmic ATP can induce an increase in oocyte conductance, we examined changes in cytoplasmic ATP concentration and whole-cell membrane currents in native oocytes subjected to a 5 h treatment with iodoacetic acid (IAA, 5 mM), which inhibits glycolysis. The ATP concentration of untreated oocytes was 2.58 ± 0.16 mM and the mean current -112 ± 11 nA ($n = 15$). The ATP concentration was decreased to 0.27 ± 0.11 mM by treatment with IAA and oocyte conductance was largely increased yielding currents of -1570 ± 250 nA at −110 mV ($n = 19$, Fig. 9B). Interestingly, after IAA treatment, a 5 min application of the K_{ATP} channel opener diazoxide (300 μM) induced an additional increase in K^+ current by $+52 \pm 12\%$ at −110 mV ($n = 6$, $P < 0.01$, i.e. -1050 ± 216 nA and -1685 ± 485 nA before and after diazoxide treatment respectively, Fig. 9C). Diazoxide was able to increase currents in both directions ($+45 \pm 3\%$ increase at +85 mV, $P < 0.001$). In contrast, diazoxide had no measurable effect on control oocytes.

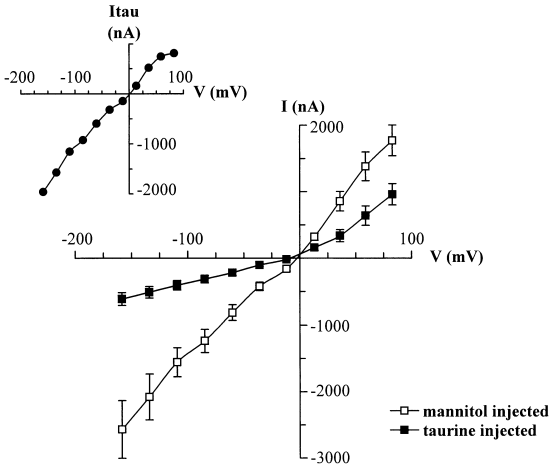

Fig. 10. Membrane currents of mannitol- or taurine-injected oocytes after lowering ATP with 5 mM IAA. *I–V* relationships of IAA-treated oocytes 1 h after injection of mannitol (□) or taurine (■). Insert represents the taurine-sensitive current obtained by subtracting the current measured in presence of taurine from that measured in the presence of mannitol.

We concluded that directly decreasing intracellular ATP levels was sufficient to stimulate a current that was similar to the current associated with AK3 expression (comparable *I–V* curves, sensitivity to Ba^{2+} and glibenclamide, data not shown). In Fig. 9D, the results of all oocytes tested ($n = 73$) were pooled into four categories independent of the experimental protocol used. It is observed that currents are activated by a reduction in intracellular ATP levels below a threshold concentration of approximately 1 mM.

K_{ATP} channels (Kir6.x), members of the Kir family, are weak inwardly rectifying channels. In contrast, as shown in Figs. 4, 5, 7 and 8, whole-cell current–voltage relationships of AK3-expressing or IAA-treated oocytes were outwardly rectifying. To test the hypothesis that this rectification is in reality coming from the fact that these currents were measured with low external K^+ concentration, current–voltage relationship of the pure taurine-sensitive K^+ current of IAA-treated oocytes were then determined (Fig. 10) in the presence of high K^+ concentrations (in HK-Barth). As expected from a K^+ current, we observed a reversal potential of 0 mV and the outward rectification (compare to Figs. 2C and 5) was changed into a weak inward rectification (taurine-sensitive current of 812 nA and −923 nA; respectively at +85 mV and −85 mV, see Fig. 10, insert).

4. Discussion

The original purpose of this project was to identify, by expression cloning in *Xenopus* oocytes, the K_{ATP} channel of rabbit PCTs. Inhibition of this channel by taurine and Ba^{2+} was used as a functional test during the expression cloning procedure. From a rabbit renal cortex cDNA library, we isolated a unique clone which induced in oocytes a large current (4–5 fold larger than the endogenous current in control oocytes). This current was sensitive to extracellular K^+ concentration and largely inhibited by (i) Ba^{2+}, (ii) glibenclamide and (iii) intracellular taurine. The cDNA sequence showed that the isolated clone was not a K^+ channel but a truncated AK (AK3). We, then isolated from proximal tubule cDNA, the full-length rabbit AK3 cDNA, which developed a current similar to the one associated with the truncated AK3. We demonstrated a relationship between AK3 expression, diminution of cell ATP concentration and an increase in an endogenous K^+ current. This indicates that ATP is a key factor in the activation of a hitherto unknown endogenous K^+ current of *Xenopus* oocytes.

4.1. Evidence for an endogenous ATP-sensitive K^+ current

To date, six different K^+ channels have been reported in *Xenopus* oocytes: a slowly inactivating delayed rectifier K^+ channel [29,30], a 170 pS Na^+-activated K^+ channel [31], two types of non-selective cationic channels [32,33], a large conductance Ca^{2+}-activated K^+ channel [34] and a TEA-sensitive (40 mM TEA) K^+ channel [35]. Supported by the following results, we now add to this list a previously unknown endogenous ATP-sensitive K^+ current.

Several lines of evidence support our contention that the current associated with AK3 expression is in large part mediated by K^+, through a K_{ATP} channel. First, in AK3 expressing oocytes, the inward currents specifically induced by external K^+ addition (20 mM) were 2.6 fold higher than those measured in control oocytes and were totally Ba^{2+}-sensitive. In addition, Ba^{2+}-sensitive currents are up to fivefold higher for cDNA injected than for rB-GFP injected oocytes over the whole voltage range. Second, in K-Barth, the glibenclamide-sensitive current reverses at -29 ± 3 mV, like the Ba^{2+}-sensitive current measured under the same conditions (-26 ± 2 mV). These inversion potentials are in the range expected for E_K when intracellular K^+ concentration is around 57 to 63 mM. In addition, in HK-Barth, the taurine-sensitive IAA-induced current reverses at 0 mV and a weak inward rectification, characteristic of the Kir6 channel, was observed. Third, the same type of currents can be stimulated by a reduction in intracellular ATP concentration. Interestingly, the current stimulated by a reduction in intracellular ATP concentration can be further stimulated by diazoxide, a K_{ATP} channel opener. The glibenclamide effect on AK3- and IAA-induced currents gives additional support to the presence of K_{ATP} channels in *Xenopus* oocytes. Finally, the AK3 induced current was largely taurine-sensitive which is a property recently described for the K_{ATP} channels from cardiac cells [36,37] and from a mammalian proximal tubule [9,10]. Although a number of different channels are modulated by taurine, including Ca^{2+}, Na^+, Cl^-, and K^+ channels [36–40], the only K^+ channel type known to be directly inhibited by taurine is the ATP-sensitive potassium channel.

Recently, Gribbles et al. [24], expressing exogenous K_{ATP} channels in *Xenopus* oocytes, did not observe endogenous K_{ATP} currents when they lowered [ATP] from 2.3 to 1.7 mM. The present study confirms the finding that [ATP] can be lowered without activating an endogenous ATP-sensitive K^+ current provided the ATP level remains above 1 mM (see Fig. 9). It is also quite clear that lowering the ATP concentration further will produce a very significant K^+ current which is Ba^{2+}-, taurine- and glibenclamide-sensitive.

4.2. Molecular identity of the oocyte putative K_{ATP} channel

K_{ATP} channels are formed from the combination of inwardly rectifying K^+ channels (Kir6.1 or Kir6.2) with sulfonylurea receptors (SUR1, SUR2A or SUR2B).

Kir6.x channels are weakly rectifying channels compared with some others members of the Kir family like IRK1 (Kir2.1) or ROMK (Kir1.1) channels. In the present case, the molecular identity of the putative K_{ATP} channel in *Xenopus* oocytes is

unknown but the weak inward rectification found in the presence of high external K^+ concentration, is consistent with the presence of Kir6.1 or Kir6.2.

If it is assumed that other conductances are also changed by AK3 expression and that 250 μM glibenclamide produces a maximal effect on the portion of the current carried by K_{ATP} channels, the experiment series presented in Fig. 8 would suggest a IC_{50} of about 1 μM under our experimental conditions. With respect to other K_{ATP} channels, this is considered to be a low affinity that would be consistent with the presence of SUR2A or SUR2B in oocytes. More RT-PCR is needed using *X. laevis* oocytes to clearly identify the subunits responsible for the ATP-sensitive current.

4.3. Possible role of AK in regulating K_{ATP} channels

In vertebrates, four AK isoenzymes are present which catalyze the reaction: MgNTP+AMP→MgNDP+ADP (where N could be A, G or I). AK1 has been localized to the cytosol of skeletal muscle, brain and erythrocytes [41,42]; AK2 is present in the mitochondrial intermembrane space of liver, kidney, spleen and heart cells [42] and AK3 exists in the mitochondrial matrix of liver, kidney and heart cells [43]. Recently, a novel subtype (AK4) has been identified in mouse and rat brain [44]. It is 53% homologous with rat AK3 and 89% homologous with the human AK3 suggesting that the reported human AK3 would actually belong to the AK4 subfamily. The rabbit kidney cDNA cloned in the present study shows such a high degree of sequence similarity to bovine or rat AK3 (90% identical) that it must represent the rabbit homologue of this protein. The fact that human AK3 probably belongs to the AK4 subfamily [44] explains the lower degree of homology with rabbit AK3 (only 55%). AK3, also named GTP:AMP phosphotransferase, prefers GTP over ATP as a substrate. However, bovine liver AK3, for example, is active with ATP as a phosphate donor, even though its activity is smaller than in the presence of GTP [17]. In oocytes, we observed that AK3 expression induced a large decrease in ATP content suggesting that AK3 can effectively use ATP. Heldt and Schwalbach [45] reported that GTP-AMP phosphotransferase indirectly participates in the ATP-ADP mitochondrial metabolism of rat liver, and thus AK3 expression in oocytes may be affecting the ATP level indirectly. According to Dzeja and Terzic [46], the spatial arrangement of the different isoforms of AKs (in mitochondria, cytosol or bound to membrane) provides a efficient phosphotransfer system which could be instrumental in K_{ATP} channel regulation. This capacity of AK to modulate K_{ATP} channels activity has been suggested by different studies. For example, Elvir-Mairena et al. [47] showed that in guinea pig cardiomyocytes the up- and down-regulation of AK1 activity correlated with the open and closed status of K_{ATP} channels, presumably through a modulation of the ATP/ADP ratio in the microenvironment of K_{ATP} channels. In addition, intracellular diadenosine polyphosphates (ApnA), potent blockers of AK, may prevent the opening of K_{ATP} channels in pancreatic cells and cardiac myocytes (for review see [48]).

It would be interesting to ascertain whether AKs (AK3 or others) play a physiological role in the regulation of the proximal tubule K_{ATP} channels. In PCT, we have previously determined that the ATP level was 4.4 mM [4]. Tsuchiya et al. [5] also measured an intracellular ATP level of 3–4 mM in the proximal tubule, whereas the K_{ATP} channel was found to be 80% inhibited by 1 mM ATP in excised patch-clamp experiments. This paradox implies a regulatory mechanism which permits transition from a closed- (ATP-ligated) status to an open- (ADP-ligated) status of the K_{ATP} channel in the presence of high cytosolic ATP concentrations. It has been suggested that the microenvironment in the vicinity of K_{ATP} channels may be associated with the regulation of these channels [49,50]. Intracellular localization of AK3 and other AK proteins would be needed to determine if these enzymes could participate in the control of the ATP/ADP ratio in the vicinity of K_{ATP} channel within the proximal tubule cells.

In summary, while attempting to isolate the proximal tubule taurine-sensitive K_{ATP} channel, we cloned a cDNA for AK, a protein that lowers ATP levels in expressing oocytes and activates an previously unknown, endogenous taurine- and glibenclamide-sensitive K^+ current. It is not readily apparent whether our cloned renal AK can play a role in activating K_{ATP} channels in proximal tubules, but the

possible involvement of Aks in the regulation of these channels could provide a new way to explain the paradox of active K_{ATP} channels in an environment of high intracellular ATP levels.

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