Mechanisms of Urinary K^+ and H^+ Excretion: Primary Structure and Functional Expression of a Novel H, K-ATPase

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Abstract. The kidney plays an essential role in regulating potassium and acid balance. A major site for these regulations is in the collecting tubule. In the present study, we report the primary sequence of a novel α subunit of the P-ATPase gene family, which we isolated from the urinary bladder epithelium of the toad *Bufo marinus*, the amphibian equivalent of the mammalian collecting tubule. The cDNA encodes a protein of 1,042 amino acids which shares ~67% identity with the α_1 subunit of the ouabain-inhibitable Na,K-ATPase and ~69% identity with the α subunit of the SCH28080-inhibitable gastric H,K-ATPase. When coexpressed in *Xenopus* oocytes with a β subunit isolated from the same cDNA library, the ATPase is able to transport rubidium (a potassium surrogate) inward, and hydrogen outward, leading to alkalization of the intracellular compartment and acidification of the external medium. The novel ATPase has a unique pharmacological profile showing intermediate sensitivity to both ouabain and SCH28080. Our findings indicate that the bladder ATPase is a member of a new ion motive P-ATPase subfamily. The bladder ATPase is expressed in the urinary tract but not in the stomach or the colon. This H,K-ATPase may be one of the molecules involved in H^+ and K^+ homeostasis, mediating the transport of these ions across urinary epithelia and therefore regulating their urinary excretion.

POTASSIUM is primarily an intracellular ion where its concentration is high (150 mM), whereas the normal concentration of the extracellular fluid is low (~4 mM) (Wright and Giebisch, 1992). This ionic gradient is generated by an ion motive P-ATPase, the ouabain-inhibitable Na,K-ATPase, present in the plasma membrane of all cells. The P-type ATPases are members of the phosphorylating class of ion transport ATPases which includes the ubiquitous Na,K-ATPase, the gastric H,K-ATPase, the sarcoplasmic and plasma membrane Ca-ATPases, plant and fungi H-ATPases, and the bacterial K-ATPase (Pedersen and Carafoli, 1988).

The potassium gradient across the cell membrane promotes the outflow of K^+ ions across K^+-selective channels, generating the intracellular negative membrane potential of most animal cells (Wright and Giebisch, 1992). The gradient is thus essential to control the excitability of nerve and muscle cells. A small change in plasma potassium concentration can have dramatic adverse effects. Hyperkalemia can be rapidly lethal by inducing membrane depolarization of heart muscle, and life-threatening arrhythmias. On the other hand hypokalemia also leads to severe clinical symptoms, including muscle weakness and paralysis, arrythmias, and renal failure (Wright and Giebisch, 1992). The extracellular potassium concentration must therefore be maintained within very narrow margins (i.e., 3.5 to 5 mM). The maintenance of a normal extracellular potassium concentration requires not only that the distribution of potassium between the extracellular and the intracellular fluid is maintained by Na,K-ATPase but also that the potassium excretion equals its intake, allowing global potassium balance. The potassium balance is mainly achieved by the control of urinary potassium excretion, and to a lesser extent by the control of K^+ absorption from the digestive tract (Wright and Giebisch, 1992). In the kidney, the major sites of controlled K^+ secretion and/or reabsorption are located in the cortical and medullary portion of the collecting tubule (CCT and MCT)[1] (Wright and Giebisch, 1992). In the secretory process, K^+ is taken up by the basolateral Na,K-ATPase and flows into the lumen across the apical membrane through K^+ selective ion channels. Under conditions of low dietary intake, potassium secretion is negligible, and net potassium reabsorption takes place (Wright and Giebisch, 1992). The molecular mechanisms of potassium reabsorption are not yet elucidated but recent biochemical and pharmacological evidences suggest that active K^+ transport is mediated by a K- or H,K-ATPase (Wingo, 1989; Wingo and Armitage, 1992; Okusa et al., 1992; Zhou and Wingo, 1992; Silver and Frindt, 1993). K- and H,K-ATPase activities were measured in the distal nephron of mammals (Doucet and Marsy, 1987;

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1. *Abbreviations used in this paper:* CCT, cortical collecting tubule; MCT, medullary collecting tubule; MBS, modified Barth's medium.

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Garg and Narang, 1988; Cheval et al., 1991) and amphibia (Planelles et al., 1991). Two different inhibitors of the gastric H,K-ATPase, SCH28080, and omeprazole, drastically reduce proton secretion and net K^+/Rb^+ absorption in collecting duct (Wingo, 1989; Gifford et al., 1992; Okusa et al., 1992; Wingo and Armitage, 1992) without affecting the transepithelial potential difference (Okusa et al., 1992; Wingo and Armitage, 1992). This suggested the presence of an electroneutral H,K-ATPase playing a significant role in urinary acidification and K^+ excretion (Wingo and Cain, 1993). Recently, compelling evidence for the presence of an H,K-ATPase in the intercalated cells of the CCT has been presented (Silver and Frindt, 1993). The lack of sensitivity to ouabain (the specific inhibitor of Na,K-ATPase), the sensitivity to omeprazole and SCH28080 (the specific inhibitors of the gastric H,K-ATPase) (Garg and Narang, 1988; Wingo, 1989; Cheval et al., 1991; Planelles et al., 1991; Wingo and Armitage, 1992; Silver and Frindt, 1993), and the partial immunoreactivity between the gastric and the renal H,K-ATPase (Wingo et al., 1990) suggested the presence of a yet unidentified ion motive P-ATPase. Recently, a new P-ATPase has been cloned from rat colon (Crowson and Shull, 1992; Jaisser et al., 1993*a*). Sequence similarity with gastric H,K- and Na,K-ATPase led to the speculation that it corresponded to the K- or H,K-ATPase activity present in this tissue. No functional expression of the rat colon putative H,K-ATPase α subunit has been reported.

In the case of both the Na,K- and H,K-ATPase, it is known that a second subunit, the β subunit, is essential for functional activity (Geering, 1991). We have recently cloned a novel β subunit from the toad bladder epithelium (β_{bl}) (Jaisser et al., 1993*b*) and we speculated that it could associate with a H,K-ATPase α subunit to form a functional pump. We report here the primary structure of another member of the P-ATPase α subunit gene family, that was found in the urinary bladder of *Bufo marinus* but not in the colon. When coexpressed with β_{bl} in *Xenopus* oocyte, the ATPase is able to transport Rb^+ inward and H^+ outward, leading to alkalization of the intracellular compartment and acidification of the external medium. Hence, this new member of the Na,K-/H,K-ATPase subfamily is able to mediate K^+ reabsorption and H^+ secretion which are measured physiologically in the distal tubule of the mammalian kidney.

Materials and Methods

Cloning Strategy and DNA Sequencing

3 μg of fractionated poly A+ RNA from epithelial toad bladder cells were used to prepare single strand cDNA, using a conventional protocol (Sambrook et al., 1989). Two degenerate oligomers (5′ sense EIEHF:GAA/GATIGAA/GCAC/TTT and 3′ antisense MGKAPE:TCIGGIGGICCT/CTTCAT) were designed to PCR amplify a conserved region of P type ATPases, as previously described (Jaisser et al., 1993*a*). PCR products (680 bp) were then digested with Cla I, Xba I (both restriction sites encoded by the oligomers) and Dra III, an enzyme known to cut within this particular region of the *B. marinus* Na,K-ATPase α_1 subunit cDNA (Jaisser et al., 1992). The PCR products that were not digested were subcloned in the Bluescript vector (Stratagene Corp., La Jolla, CA). Only one cDNA subtype was identified. This cDNA encodes part of a protein highly related to the P-ATPase family. The cDNA was then used as a probe to screen a cDNA library (100,000 independent colonies). Only one positive clone containing a 5′ 2-kb insert was isolated. A second probe was prepared after subcloning a new cDNA fragment of 1.6 kb generated by PCR amplification of the single strand cDNA, with one specific sense oligomer encoding part of the new sequence (5′ sense CCAGCTATCAATACATG) and one degenerate oligomer designed against a P-ATPase conserved region located near the COOH-terminal end (3′ antisense YGQQ/EWT:GTCCAITC/GT/CTGCCG/ATA) (Jaisser et al., 1993*a*). This 1.6-kb fragment, including part of the previously characterized cDNA, was subcloned in Bluescript and used to rescreen the cDNA library. A new clone was isolated but the 1.1-kb insert did not overlap the former 5′ 2-kb cDNA, missing 450 bp. Since we were unable to find any overlapping clone, a full-length 3.7-kb clone was reconstructed using PCR and classical DNA recombinant technology (Sambrook et al., 1989). The full-length cDNA was sequenced in both directions, as well as the initial cDNAs cloned from the library. The nucleotide sequence of the 450-bp region obtained by PCR was confirmed by sequencing three clones obtained from three independent PCR reactions.

Northern Blot Analysis

Northern blot analysis were performed using 1.5 μg poly A(+) RNA obtained from various tissues from the toad *B. marinus* and from the TBM 18-23 clonal cell line, which derives from the urinary bladder (Asher et al., 1988). Hybridizations were done with a 500-bp *B. marinus* H,K-ATPase α subunit probe (covering 250 bp of the 5′ untranslated region and 250 bp of the divergent NH_2-terminal coding region) and a 400-bp *B. marinus* Na,K-ATPase α_1 subunit probe (covering the 3′ untranslated region). Probes were labeled with ^{32}P-dCTP. Final washes of the blot were done with 0.2× SSC, 0.1% SDS at 65°C for 30 min (Sambrook et al., 1989). Autoradiograms were exposed for 3 d (Na,K-ATPase α_1 subunit) or 10 d (H,K-ATPase α subunit). Similar results were obtained using a 3′ untranslated probe of the H,K-ATPase α subunit.

cRNA Synthesis and Expression in Xenopus Oocytes

The 3.7-full-length cDNA was subcloned in the pSD 3 vector containing a 110-bp poly A(+) tail before the HindIII linearization site (Horisberger et al., 1991*a*). Preliminary experiments showed that cRNA obtained from SP6 in vitro transcription of the full-length cDNA was poorly translated in a reticulocyte lysate. We deleted 170 bp of the 5′ untranslated region, removing a GC-rich region. This improved dramatically the translatability in vitro and in the *Xenopus* oocytes. *Xenopus* oocytes were injected either with 7 ng H,K-ATPase α_{bl} subunit, 7 ng *B. marinus* Na,K-ATPase α_1 subunit or 2 ng of the recently described *B. marinus* P-ATPase β subunit (β_{bl}) cRNAs, alone or in combination.

Rubidium Uptake

2 to 3 d after injection, oocytes were used to measure ^{86}Rb uptake as a marker of K^+ transport (Jaunin et al., 1992). Depending on the experimental protocol, oocytes were first preincubated with or without potassium in order to increase intracellular Na^+, a maneuver required to insure full activation of the Na,K-ATPase (Horisberger et al., 1991*a*). Then, they were incubated in modified Barth's medium (MBS) containing 10 μM ouabain for 10 min in the presence of 1 mM K to inhibit the endogenous Na,K-ATPase, as previously described (Jaisser et al., 1992). Thereafter, ouabain was washed in MBS without KCl with or without the inhibitors or the diluent (DMSO) at appropriate concentrations, in the presence of 1 to 5 mM $BaCl_2$. Barium can be omitted without affecting significantly the results. ^{86}Rb uptake was done as described (Jaunin et al., 1992). Results were expressed after correction for the specific activities of ^{86}Rb. The activation ($K_{1/2}$) and inhibition K_i were obtained by fitting the ^{86}Rb uptake data to a single site model (inserts).

Intracellular pH (pH_i) Measurements

pH-sensitive microelectrodes were manufactured using the liquid ion exchanger H-ionophore II–Cocktail A (Fluka) as described earlier (Horisberger and Giebisch, 1988). These electrodes had a resistance of 2 to 10 Gohms. They were calibrated in pH 6.5 and pH 7.5 solution immediately before and after each intracellular measurement, as described later in Fig. 6. Only electrodes with a response of >54 mV/decade were used. A conventional microelectrode filled with 3 M KCl was used to measure the oocyte membrane potential (Vm) and pH_i was calculated from the voltage read with the pH electrode minus Vm.

Extracellular pH Measurements

A qualitative assay of external medium acidification was designed to demonstrate H^+ secretion by *Xenopus* oocyte. The assay was performed 2 d af-

ter coinjection of α_{bl}/β_{bl}, β_{bl} alone, or α_{NaK}/β_{bl} cRNAs. Oocytes were transferred for 5 min in a weakly buffered solution (NaCl 110 mM, KCl 1 mM, $CaCl_2$ 0.5 mM, $MgCl_2$ 0.5 mM, MOPS 500 μM, adjusted to pH 7.8 with NaOH), containing the pH indicator Red phenol (200 mg/l). In some experiments, ouabain (1 mM) was added to the solution. Oocytes were placed individually in a small drop of the same solution under oil. The volume of the droplet was then reduced to 0.5 to 1.0 μl by gently removing the solution with a thin glass pipette (the final volume of the droplet was checked after the experiment by aspirating all the extracellular volume in a calibrated constriction pipette). After a 15-min incubation, at room temperature, a color picture was taken.

Quantitative estimates of external medium acidification were obtained by measuring the pH using ion-sensitive microelectrodes. Double-barreled liquid ion-exchanger microelectrodes (H-Ionophore II-Cocktail A; Fluka) were manufactured as described previously (Horisberger and Giebisch, 1988) and calibrated in pH 6.5 and 7.5 solutions before and after each measurement. Oocytes were prepared in the same way as for the pH-sensitive dye experiments. After a 15-20-min incubation time in the 0.5-1.0-μl droplet of weakly buffered solution, the double-barreled electrode was introduced in the droplet to measure the pH of the external solution.

Results

Primary Structure and Tissue Distribution of a P-ATPase α Subunit from Bladder Epithelium

Using a set of degenerate oligonucleotides designed to amplify a PCR product common to all Na,K- and H,K-ATPases α subunits from different species (Jaisser et al., 1993*a*), we have cloned, characterized and sequenced a 3.7-kb cDNA from a toad bladder epithelium library. The full cDNA sequence and the predicted amino acid sequence are shown in Fig. 1. The novel α subunit encodes a protein of 1,042-amino acids long with many features characteristic of the ion-motive P-ATPase gene family, including a highly conserved phosphorylation site (Cys-Ser-Asp399-Lys) and the FITC-binding site (Met-Lys531-Gly-Ala) (Horisberger et al., 1991*b*). The overall identity (similarity) is 67% (82%) with α_1 Na,K-ATPase from the same species (Jaisser et al., 1992), 69% (79%) with the rat gastric H,K-ATPase (Shull and Lingrel, 1987) and 75% (87%) with the rat colonic putative H,K-ATPase (Crowson and Shull, 1992; Jaisser et al., 1993*a*).

Because of the high degree of identity with the gastric H,K-ATPase and the colonic putative H,K-ATPase, we have compared the tissue distribution of the novel α subunit in epithelia of the gut, the kidney and the bladder of *B. marinus*. As shown in Fig. 2, a transcript of 3.8 kb is detected in large amounts in the bladder and small amounts in the kidney. Within the bladder, the level of expression is about sixfold lower than that of the Na,K-ATPase α_1 subunit. The stomach and the colon are clearly negative as well as the TBM cells, a cell line derived from the granular cells of the urinary bladder of the toad.

Functional Expression of α_{bl} and β_{bl} in Xenopus oocytes

To examine the possibility that the α_{bl} subunit could function as a K-ATPase to reabsorb potassium from the extracellular fluid or as a H,K-ATPase to secrete protons in exchange for potassium reabsorption, we have used the *Xenopus* oocyte expression system, which has been shown to be a convenient system to study the function of Na,K-ATPase (Horisberger et al., 1991*a*).

The measurement of radioactive rubidium uptake (^{86}Rb) allows to probe for K- pump function when the other K^+ pathways are blocked (Jaunin et al., 1992). We designed an experimental protocol (Fig. 3 *A*) which allows to minimize the contribution of the endogenous oocyte Na,K- pump by a short exposure to 10 μM ouabain. This maneuver has been previously shown to inhibit the *Xenopus laevis* Na,K- pump by more than 98% for at least 20 min (Jaisser et al., 1992). The Na,K-/H,K-ATPase family requires α/β subunit assembly for functional expression at the plasma membrane (Geering, 1991). In the present study, we selected a novel β subunit isoform (β_{bl}) which we have characterized from the same toad bladder epithelium cDNA library (Jaisser et al., 1993*b*). We have postulated that the β_{bl} could be functionally associated with an α subunit of either Na,K- or H,K-ATPase. As shown in Fig. 3 *B*, the injection of α_{bl} cRNA

Figure 1. Nucleotide and deduced amino acid sequence of the bladder H,K-ATPase α subunit of *B. marinus*. The deduced amino acid sequence starts with the presumed initial methionine. The full-length cDNA includes 222 bp of 5′ untranslated region, 3,126 bp of coding region, 310 bp of 3′ untranslated region, and a poly A tail. Putative transmembrane domains are underlined. The consensus phosphorylation site (D399) and FITC-binding site (K531) are indicated in bold. These sequence data are available from EMBL/GenBank under accession number Z25809.

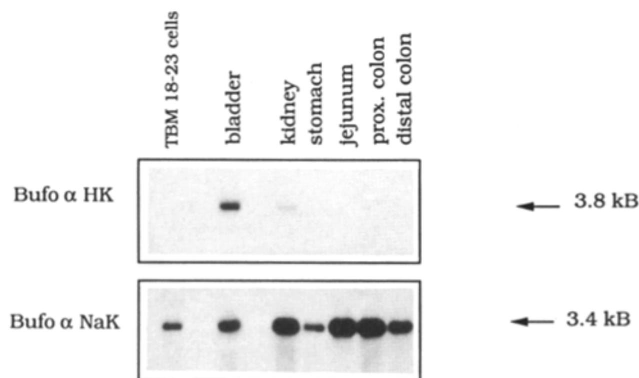

Figure 2. Tissue distribution of the H,K-ATPase α_{bl} subunit and the Na,K-ATPase α_1 subunit. Northern blot analysis were performed using 1.5 μg poly A(+) RNA obtained from various tissues from the toad *B. marinus* and from the TBM 18-23 clonal cell line, which derives from the urinary bladder. Autoradiograms were exposed for 3 d (Na, K-ATPase α_1 subunit) or 10 d (H,K-ATPase α subunit).

alone (lane *2*) or β_{bl} cRNA alone (lane *3*) did not induce any significant increase of rubidium uptake compared to the water-injected oocytes (lane *1*). When α_{bl} and β_{bl} were co-injected (lane *4*), a more than 20-fold increase in rubidium uptake was observed with respect to oocytes injected with either subunit alone.

As shown in Fig. 4 *A*, rubidium uptake was activated in a dose-dependent manner by external K^+, with a $K_{1/2}$ of 370 μM. This value can be compared with those observed for the

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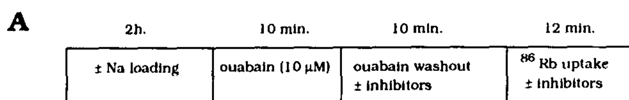

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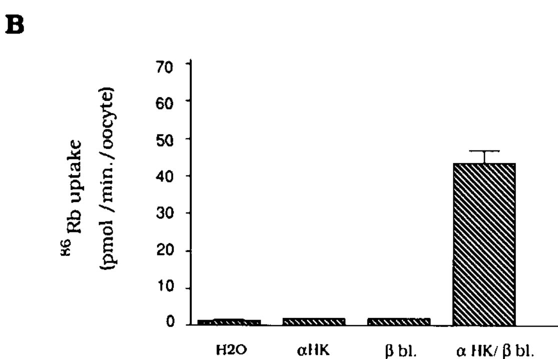

Figure 3. ^{86}Rb uptake in *Xenopus* oocytes mediated by the bladder H,K-ATPase. (*A*) Flow chart of the experimental protocol used to determine the ^{86}Rb uptake in *Xenopus* oocytes. (*B*) ^{86}Rb uptake in *Xenopus* oocytes injected with water (lane *1*), α_{bl} alone (lane *2*), β_{bl} alone (lane *3*) or α_{bl} and β_{bl} together (lane *4*). ^{86}Rb uptake was performed in the presence of 500 μM K^+ and after preliminary inhibition of the endogenous, ouabain-sensitive, Na,K-ATPase with 10 μM ouabain. Results are expressed as mean $\pm$ SE, n = 10–24 from three independent experiments.

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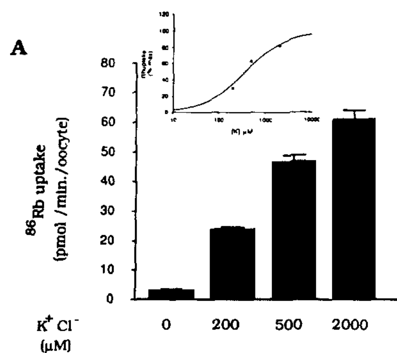

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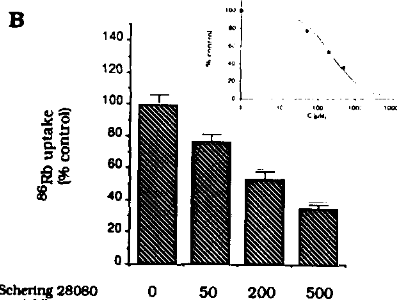

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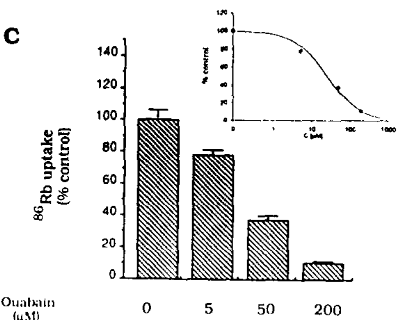

Figure 4. K^+-dependent activation of ^{86}Rb uptake (*A*), dose-dependent inhibition of ^{86}Rb uptake by SCH28080 (*B*) and by ouabain (*C*) in *Xenopus* oocytes injected with $\alpha_{bl}\beta_{bl}$ subunit cRNAs. SCH28080 and ouabain effects were determined in the presence of 200 μM KCl in the incubating solution. The final concentration of DMSO (diluent) was identical in all experimental conditions. Results are expressed as mean $\pm$ SE, n = 9–11. The activation ($K_{1/2}$) and inhibition K_i were obtained by fitting the ^{86}Rb uptake data to a single site model (*insets*).

amphibian Na,K- pump (~1 mM) (Jaisser et al., 1992) and those reported for the gastric H,K-ATPase (~750 μM) (Rabon et al., 1992). It is very close to the K_m value of 320 μM reported for the K-ATPase of amphibian distal nephron (Planelles et al., 1991). We thus propose that this rubidium transport is mediated by a K^+-activated ATPase.

Pharmacological Profile

Na,K- and H,K-ATPase bind important drugs such as the cardiotonic glycosides in the case of Na,K-ATPase (Forbush, 1983) and the SCH28080 compound for the gastric H,K-ATPase (Wallmark et al., 1987). The bladder K- pump is inhibited by SCH28080 in a dose-dependent manner with a K_i of 230 μM (Fig. 4 *B*). It is thus much less sensitive to this compound than the gastric H,K-ATPase (Wallmark et al., 1987), however, clearly more sensitive than the Na,K-

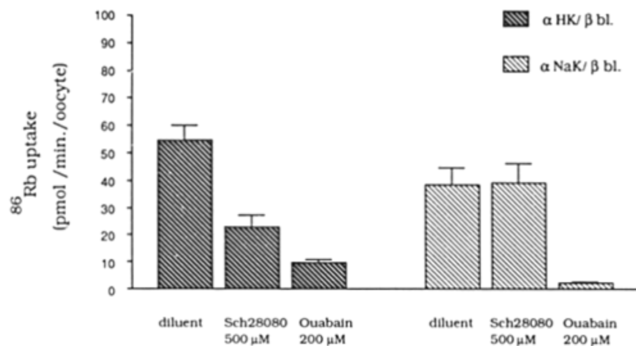

Figure 5. Differential effect of SCH28080 (500 μM) and ouabain (200 μM) on the ^{86}Rb uptake induced by the coinjection of $\alpha_{bl}\beta_{bl}$ subunit or $\alpha_{1NaK}\beta_{bl}$ subunit cRNAs in *X. laevis* oocytes. ^{86}Rb uptake was determined in the presence of 500 μM KCl in the incubating solution after Na^+ loading by a 2-h exposure to a K^+- and Ca^{2+}-free solution. Results are expressed as means $\pm$ SE, n = 9–11.

ATPase (Fig. 5). In addition, the bladder K- pump is also sensitive to ouabain with a K_i of 25 μM. This level of ouabain sensitivity is similar to that of the moderately ouabain-resistant toad Na,K- pump α_1 isoform, which has a K_i of 50 μM (Jaisser et al., 1992), when measured in the presence of 10 mM K^+, while ouabain-sensitive isoforms have a K_i of the order of 50 nM (Canessa et al., 1992). As both ouabain and SCH28080 are potassium competitive inhibitors (Wallmark et al., 1987; Forbush, 1983), we tested the effect of extracellular potassium on SCH28080 and ouabain-mediated inhibition of ^{86}Rb uptake. The inhibitory effect of 300 μM SCH28080 was antagonized by external K^+ (mean $\pm$ SE) (200 μM K^+: 56.3 $\pm$ 5.3%; 2.5 mM K^+:37.5 $\pm$ 3.5%, n = 11, $P < 0.05$), as well as the inhibitory effect of 1 mM ouabain (200 μM K^+: 95.1 $\pm$ 0.5%; 2.5 mM K^+: 85.5 $\pm$ 0.4%, n = 10, $P < 0.001$). We performed another set of experiments to compare the effects of SCH28080 and ouabain on both the toad Na,K-ATPase and bladder H,K-ATPase (Fig. 5). In the presence of 500 μM external potassium, the pharmacological profile of the two pumps is quite distinct. The Na,K- pump is not sensitive to 500 μM SCH28080 and is almost completely inhibited by 200 μM ouabain. In contrast, the bladder ATPase is partially inhibited by 500 μM SCH28080 and by 200 μM ouabain. Thus, the pharmacological profile of the bladder ATPase is quite unique and distinct from previously characterized Na,K- or H,K-ATPases.

The Expression of α_{bl} and β_{bl} Induces H^+ Secretion

At this point, we do not know whether the bladder ATPase can exchange potassium ions against other cations. Evidence from SCH28080-sensitive rubidium uptake and K^+ transport in the collecting tubule cells indicates that this process is not electrogenic (Okusa et al., 1992; Wingo and Armitage, 1992). Thus K^+ ions are probably exchanged against an intracellular cation. One likely candidate for a counter ion is the proton. In this case the new ATPase would be a H,K-ATPase with transport functions similar to the gastric H,K-pump. Two sets of experiments indicate that this is the case. We measured intracellular pH in oocytes 2 or 3 d after injection with β_{bl} alone, α_{1NaK}/β_{bl} or α_{bl}/β_{bl} as described in Fig. 6

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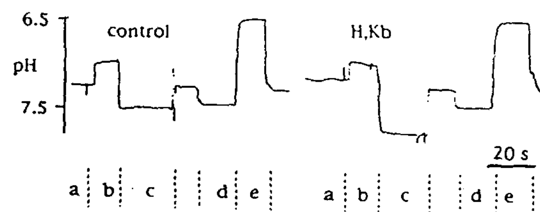

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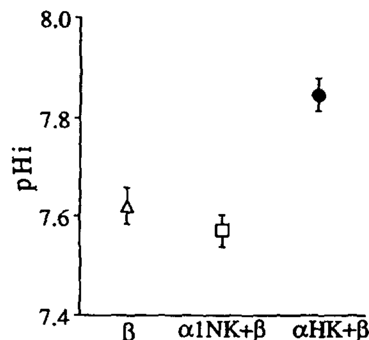

Figure 6. Expression of the *B. marinus* bladder H,K-ATPase in *Xenopus* oocytes: effect on intracellular pH (pH_i). (*A*) Original tracings of pH_i measurements in a control oocyte (injected with β_{bl}. Subunit cRNA alone) and an oocyte expressing the H,K-ATPase (injected with bladder α_{bl} and β_{bl} subunit cRNAs). The tracings show the following steps: (*a*) both electrodes in the bath solution; (*b*) potential electrode in the oocyte; (*c*) both electrodes in the oocyte, measurement of the pH_i; then both electrodes were withdrawn in the bath and the pH electrode was immediately calibrated by exposure to pH 7.5 (*d*) and pH 6.5 (*e*) buffers. (*B*) pH_i in oocytes injected with cRNAs from the β_{bl}, *Bufo* Na,K-ATPase $\alpha 1/\beta_{bl}$ or bladder H,K-ATPase α/β_{bl} subunits. was measured in oocytes injected with cRNA of the β_{bl} subunit alone (β, n = 26), the α_1 subunit of the *Bufo* Na,K-ATPase + β_{bl} subunit ($\alpha_{1NaK}+\beta$, n = 26), or the α subunit of the H,K-ATPase + the β_{bl} subunit ($\alpha_{bl}+\beta$, n = 27). Results are expressed as means $\pm$ SE (*vertical bars*).

A. A significant alkalization of the oocyte was observed only in cells coinjected with α_{bl} and β_{bl} (Fig. 6 *B*). The most likely explanation for this observation is that the bladder ATPase exchanges extracellular potassium ions against intracellular protons. We have tested directly H^+ secretion in oocytes two days after coinjection of α_{bl}/β_{bl} or $\alpha NAK/\beta_{bl}$ as shown in Fig. 7. Within 15 min of incubation, an important acidification of the external medium was clearly visualized by the color change of the pH indicator. In a second experiment, direct measurement of the pH in the external medium was carried out using liquid ion-exchanger pH-sensitive microelectrodes (Fig. 8). After a 15 to 20-min incubation, the pH of the external medium surrounding oocytes injected with the bladder ATPase cRNAs was significantly more acid than those of oocytes injected with the Na, K-ATPase cRNAs. The acidification of the external medium was completely blocked by ouabain (1 mM) and partially by

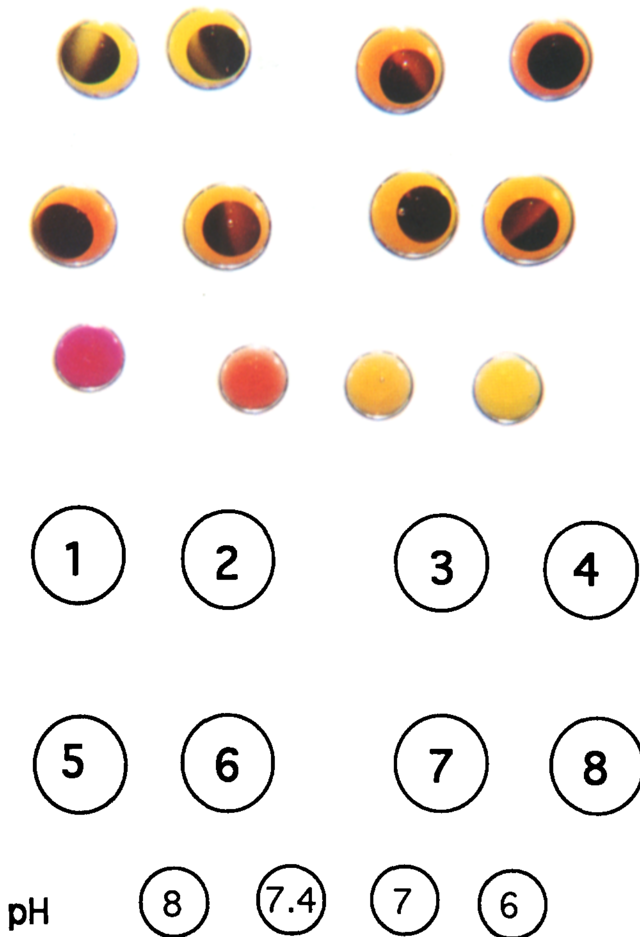

Figure 7. The expression of the bladder H,K-ATPase results in the acidification of the extracellular medium. Oocytes injected with α_{bl} and β_{bl} cRNAs (*1, 2, 5,* and *6*) or $\alpha_{Na,K}$ and β_{bl} cRNAs (*3, 4, 7,* and *8*) were placed under mineral oil in a 0.5–1.0-μl droplet of Red phenol–dyed solution, pH 7.8, without (*1–4*) or with 1 mM ouabain (*5–8*). The picture was taken after 15 min. The pH of the surrounding solution of oocytes 1 and 2 was around pH 6 whereas oocytes 3–8 were estimated to be between pH 7–7.5, when compared to standard pH.

SCH28080 (500 μM). The pH of the external medium incubating oocytes injected with $\alpha_{NaK}\beta_{bl}$ was not significantly changed by the presence of neither ouabain nor SCH28080. The data provide evidences for the expression of an H,K-pump in *Xenopus* oocytes.

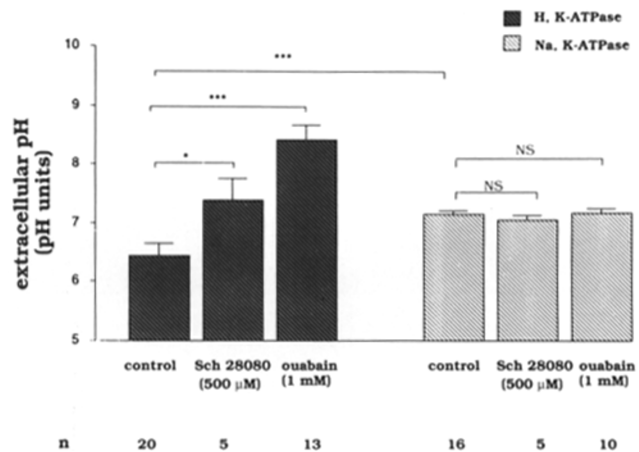

Figure 8. X. laevis oocytes were injected with α_{bl}/β_{bl}. or α_{NaK}/β_{bl}. cRNAs. Oocytes were incubated in a weakly buffered solution containing diluent (DMSO), SCH28080 (500 μM) or ouabain (1 mM). Extracellular pH were measured after 15–20 min. pH values are expressed as mean ± SE (*n*); * $P < 0.05$, *** $P < 0.005$, *t*-test.

Discussion

A New Member of the Na,K-/H,K-ATPase Gene Family

The primary structure of the new protein presented here clearly indicates that it is a new member of the large P-ATPase α subunit gene family. It is homologous with the gastric H,K-ATPase and any of the three known isoforms of the Na,K-ATPase, and slightly closer to another P-ATPase cloned from the rat colon, which function is not yet known. Both Na,K- and gastric H,K-ATPase are heterodimeric proteins, including a glycosylated β subunit that is essential to the function of the enzyme. The requirement of coexpression of a β subunit for expression of an active H,K- pump suggests that the novel ATPase is a new member of the Na,K-/H,K-ATPase subfamily.

The new H,K-ATPase is expressed specifically in urinary epithelia and could not be detected in colon. This suggests that the novel α subunit (which we term α_{bl}) is an isoform of the H,K-ATPase expressed specifically in the urinary tract, which was previously postulated to exist in several species (Doucet and Marsy, 1987; Garg and Narang, 1988; Cheval et al., 1991; Planelles et al., 1991; Okusa et al., 1992; Wingo and Armitage, 1992) and distinct from the putative H,K-ATPase identifed in the rat colon (Crowson and Shull, 1992; Jaisser et al., 1993*a*). It is not expressed in TBM cells (Fig. 2) which are sodium transporting cells derived from the toad bladder epithelium (Asher et al., 1988) that have the characteristics of the granular cells, the equivalent of the principal cells of the mammalian collecting tubule (CCT). The novel α subunit could therefore derive from any of the four other cell types composing the epithelium (Kraehenbuhl et al., 1979). Of special interest is the possibility that the α subunit is expressed in mitochondria rich cells. These cells are the amphibian counterpart of intercalated cells of CCT. Two types of intercallated cells have been described in mammals: α cells are believed to secrete protons while β cells are believed to be involved in bicarbonate secretion (Alpern et al., 1991; Schuster, 1993). Proton secretion in α cells could be mediated by a H,K-ATPase and/or by a vacuolar V-type ion motive ATPase (Alpern et al., 1991; Schuster, 1993). An H,K-ATPase has also been involved in the basolateral extrusion of the β intercalated cells (Schuster, 1993). Recently, SCH28080-sensitive proton transport has been described in intercalated cells of the CCT (Silver and Frindt, 1993).

Structure–Function Relationship

Na,K-ATPase and gastric H,K-ATPase are the receptors to important drugs such as the cardiotonic glycosides in the case of Na,K-ATPase (Forbush, 1983) and the SCH28080 compound for the H,K-ATPase (Wallmark et al., 1987). The

pharmacological profile of the bladder H,K-ATPase is of special interest in view of what is already known for the Na,K-ATPase and the gastric H,K-ATPase. In Fig. 9, the amino acid sequence of the α subunit of the bladder H,K-ATPase is compared with those of the rat colon putative H,K-ATPase (lane *2*) (Crowson and Shull, 1992), the rat gastric H,K-ATPase (lane *3*) (Shull and Lingrel, 1987), and the α_1 subunit of the *Bufo* Na,K-ATPase (Jaisser et al., 1992). The ouabain-binding site of Na,K-ATPase is localized on the H_1 transmembrane segment (Canessa et al., 1992), the H1-H2 (Price et al., 1990) and the H3-H4 ectodomain (Mc Parland et al., 1991; Canessa et al., 1993). Site-specific mutagenesis studies have shown that a cysteine (Cys113; *X. laevis* sequence) (Canessa et al., 1992) and an adjacent phenylalanine (Shultheis and Lingrel, 1993) are important in conferring ouabain sensitivity. Charged amino acids at the border of H1-H2 ectodomain are also critical in conferring ouabain resistance in rat and toad (Price et al., 1990; Jaisser et al., 1992). On the other hand, site specific chemical modification of gastric H,K-ATPase α subunit by a photoactivable analogue of SCH28080 showed that the H1-H2 ectodomain was also involved in the binding of this compound and modelling suggested that a phenylalanine (Phe124, pig gastric sequence) in the H1 transmembrane segment (just at the interface with the ectoplasmic loop) and an aspartic acid (Asp136, Pig gastric sequence) in the ectoplasmic loop were participating in the docking of the drug (Munson et al., 1991). Inspection of the sequences shown in Fig. 9 reveals a striking diversity in this domain of the protein. In the bladder pump, a tyrosine residue (Tyr 133), similar to the Na,K-ATPase, is substituted by the phenylalanine (Phe124, pig gastric sequence) involved in SCH28080 binding. The extracytoplasmic loop itself is extremely divergent between the four sequences discussed here. Similar observations can be made for the H3-H4 ectocytoplasmic loop. A highly conserved tyrosine (Tyr313, *X. laevis* sequence) has been recently shown to be also involved in ouabain binding (Canessa et al., 1993). On the other hand, the tryptophane in the H3-H4 ectodomain which can be chemically labeled by an ouabain analogue (McParland et al., 1991) is substituted by different residues in the H,K- pumps discussed here. Thus, the α_{bl} shares sequences and residues with both Na,K- and H,K-ATPase. This mosaic at the primary structure level could well explain the pharmacological profile observed in this study. A more detailed analysis by chimeric-and site-directed mutagenesis approaches should allow to define the binding sites for ouabain and SCH28080 but also should help in designing specific drugs for the three types of pumps.

There are two other domains quite divergent between the sequences presented in Fig. 9 and of potential interest in the context of a discussion of structure–function relationships. First, the amino-terminal segment is strikingly divergent. This domain has been involved in controlling cation binding during the catalytic cycle of Na,K-ATPase by changing the activation of the pump by external potassium (Burgener-Kairuz et al., 1991), or by changing the potassium dependence of the current voltage relationship (Vasilets et al., 1991) or changing the nucleotide dependence of potassium deocclusion (Wierzbicki and Blostein, 1993). Second, the last hydrophobic segments (H7-H10) at the carboxy terminus of the molecule are also quite divergent. Omeprazole, another inhibitor of the gastric H,K-ATPase, is able to bind

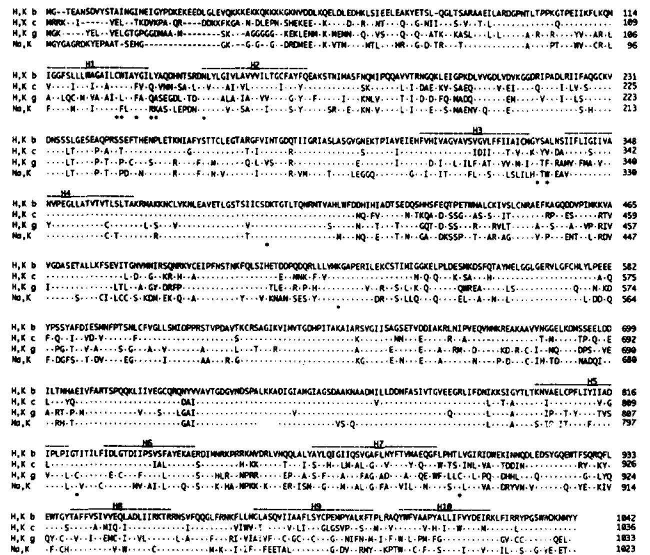

Figure 9. Primary sequence of the α_{bl} subunit of *B. marinus* (*top line*) and comparison with the rat distal colon putative H, K-ATPase (line *2*) (Crowson and Shull, 1992), the rat gastric H,K-ATPase (line *3*) (Shull and Lingrel, 1987), and the *Bufo* Na,K-ATPase (line *4*) (Jaisser et al., 1992) α subunits. Gaps (*dashes*) were introduced to maintain alignment. Dots indicate identity to the corresponding residues of H,K-ATPase α_{bl}. Putative transmembrane domains (*H1–H10*) are indicated. The amino acid numbers are shown on the right. Stars indicate amino acids discussed in the text.

covalently with SH groups of two cysteines (Cys813 [or Cys822] and Cys892) predicted to be in the extracytoplasmic loops connecting the hydrophobic segment H5/H6 and H7/H8 (Besancon et al., 1993). These cysteine residues are not found in the bladder H,K-ATPase, the Na,K-ATPase or the rat colon putative H,K-ATPase.

It has been recently proposed that negatively charged amino acids, present in the putative transmembrane segments of Na,K-ATPase are involved in cation occlusion (Karlish et al., 1992). The charge distribution within these last transmembrane domains is different for the four sequences compared in Fig. 9, suggesting that important functional differences in cation transport may be linked to these charges.

Relationship with the Renal H, K-ATPase

The relationship between the bladder H,K-ATPase described here and the H,K-ATPase studied in the mammalian cortical collecting tubule (Doucet and Marsy, 1987; Garg and Narang, 1988; Cheval et al., 1991; Wingo and Armitage, 1992) or the distal nephron of amphibia (Planelles et al., 1991) is not yet clear. In these studies, the K-ATPase activity was reported to be moderately sensitive to SCH28080 (K_i in the μM range) but insensitive to ouabain (up to 1 mM) (Cheval et al., 1991; Planelles et al., 1991) whereas the bladder H,K-ATPase reported here is moderately sensitive to both drugs. It should be noticed that experiments designed to probe the activity of the kidney K- or H,K-ATPase were performed in the presence of high concentrations of ouabain to eliminate any Na,K-ATPase activity. These protocols might have overlooked the type of isoform described here.

On the other hand, it is conceivable that some ouabain-sensitive ATPase activity described in the cortical and medullary collecting duct that had been attributed to the Na,K- pumps (Hayashi and Katz, 1987; Feraille et al., 1993) were in fact due to a moderately ouabain-resistant H,K-pump. An apical ouabain-sensitive K^+ pump in the distal part of the nephron postulated by Giebisch and co-workers more than 20 yr ago could represent the H,K-ATPase described here (Wiederholt et al., 1971; Strieder et al., 1974). Finally, the diversity of the Na,K-/H,K-ATPase gene subfamily may be larger than anticipated. For instance a human gene (ATPlAL1) (Modyanov et al., 1991) has been partially characterized. The gene is expressed in brain and kidney. The sequences of exon 5, 9, and 20 suggest that it could be another member of the Na,K-/H,K-ATPase subfamily described here. The precise relationship between the human gene and the amphibian H,K-ATPase will require knowledge of the sequence of a full-length cDNA and its functional expression.

The renal H,K-ATPase of the CCT has been physiologically located at the apical membrane of α cells (Alpern et al., 1991, Schuster, 1993). Likewise gastric H,K-ATPase is functionally expressed at the apical membrane of parietal cells. Thus, H, K-ATPases are in principle targeted to the apical membrane of epithelial cells whereas Na, K-ATPases are generally routed to the basolateral membrane (Gottardi and Caplan, 1993). The specific targeting to each of the two plasma membrane domains appears to involve information encoded on both the α and β subunits (Gottardi and Caplan, 1993). The sequence of α_{bl} and β_{bl} (Jaisser et al., 1993*b*) should help in designing experiments addressing this important question.

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