**Bufo marinus** bladder H-K-ATPase carries out electroneutral ion transport

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**Burnay, Muriel, Gilles Crambert, Solange Kharoubi-Hess, Kathi Geering, and Jean-Daniel Horisberger.** *Bufo marinus* bladder H-K-ATPase carries out electroneutral ion transport. _Am J Physiol Renal Physiol_ 281: F869–F874, 2001.—_Bufo marinus_ bladder H-K-ATPase belongs to the Na-K-ATPase and H-K-ATPase subfamily of oligomeric P-type ATPases and is closely related to rat and human non-gastric H-K-ATPases. It has been demonstrated that this ATPase transports K⁺ into the cell in exchange for protons and sodium ions, but the stoichiometry of this cation exchange is not yet known. We studied the electrogenic properties of _B. marinus_ bladder H-K-ATPase expressed in _Xenopus laevis_ oocytes. In a HEPES-buffered solution, K⁺ transport activity of the nongastric _B. marinus_ H-K-ATPase, which was inhibited by 10-20 mM CO2 at a PCO₂ of 40 Torr, induced a slow-onset inward current that reached an amplitude of ~20 nA after 1–2 min. When measurements were performed in a solution containing 25 mM HCO₃⁻ at a PCO₂ of 40 Torr, the negative current activated by K⁺ was reduced. In noninjected oocytes, intracellular alkalization activated an inward current similar to that due to _B. marinus_ H-K-ATPase. We conclude that the transport activity of the nongastric _B. marinus_ H-K-ATPase is not intrinsically electrogenic but that the inward current observed in oocytes expressing this ion pump is secondary to intracellular alkalization induced by proton transport.

**Methods**

**cRNA synthesis and expression in _X. laevis_ oocytes.** The 3.7-kb full-length cDNA of the _B. marinus_ H-K-ATPase α-subunit (α₁b) (17) was subcloned into the pSD5 vector containing a 130-bp poly T tail. cRNA was obtained by in vitro transcription (21). Removal of 110 bp of the 5’-untranslated region containing a GC-rich region significantly improved expression of α₁b-subunits in _X. laevis_ oocytes. _X. laevis_ oocytes were obtained and prepared as described (11). Oocytes were injected with either 8 ng α₁b-cRNA or 8 ng of the _B. marinus_ Na-K-ATPase α₁-subunit (16) cRNA in combination with 1 ng _B. marinus_ bladder β-subunit (β₁) (18) cRNA. Other oocytes were injected with the cRNA of the α- and β-subunits of rabbit gastric H-K-ATPase, 12 and 1.5 ng respectively, or with 1.5 mg of cRNA of the β-subunit alone. The oocytes were then incubated at 19°C in modified Ham’s solution (11) until the electrophysiological or flux measurements.

Electrophysiological measurements. Three days after injection, the oocytes were loaded with Na⁺ by a 2-h exposure to a K⁺-free solution as described (19). The incubation solution contained 0.2 μM ouabain to inhibit endogenous _X. laevis_ oocyte Na-K-ATPase (29). The two-electrode, voltage-clamp technique, using a TEV-200 voltage clamp (Dagan, Minneapolis, MN), was applied to analyze putative H-K-ATPase pump currents. Current signals were filtered at 20 Hz and recorded on a Gould chart recorder (model 220, Gould, Cleveland, OH). The intracellular potential was held at ~50 mV. Current-voltage (I-V) curves were obtained by applying a balance of numbers of charges during each transport cycle.

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series of 500-ms voltage steps ranging from −150 to +30 mV. The H-K or Na-K pumps were activated by addition of 5 mM K+ to previously K+-free solution or inhibited by addition of 2 mM ouabain, in the presence of 5 mM K+.

In a first set of measurements, the bath solution contained (in mM) 92.4 Na+, 0.82 Mg2+, 5 BaCl2, 0.41 Ca2+, 10 tetraethylammonium, 22.46 Cl−, 2.4 HCO3−, 80 glucose, and 10 HEPES (pH 7.4).

For the experiments with CO2 and HCO3−, the solution contained less glucose (48 mM) but 25 mM HCO3− in addition; this solution was bubbled with 5% CO2 and had a pH of 7.4. All experiments were performed at room temperature.

Intracellular pH measurements. Double-barreled pH-sensitive microelectrodes, using the ion exchanger H-ionophore II cocktail A (Fluka), were prepared as described (14). These electrodes had a resistance of 2–10 GΩ. They were calibrated in HEPES-buffered solutions (pH 6.5 and 7.5) immediately before and after each intracellular pH (pHi) measurement. The pH electrodes were used only if they showed a pH response >52 mV/pH unit. pHi was calculated from the voltage read with the pH barrel minus the membrane voltage read from the reference barrel filled with 3 M KCl.

86Rb uptake measurements. 86Rb uptake was performed as previously described (4). In brief, oocytes were injected with either 8 ng αH1 cRNA or 8 ng B. marinus Na-K-ATPase α1-subunit cRNA in combination with 1 ng βH1 cRNA. After loading of oocytes with Na+ (see above) and their recovery in a solution containing (in mM) 90 NaCl, 2 CaCl2, 5 BaCl2, and 10 MOPS, pH 7.4, they were transferred to a solution containing (in mM) 5 KCl, 90 NaCl, 1 CaCl2, 5 BaCl2, 1 MgCl2, and 10 HEPES, pH 7.4, as well as 0.2 µM ouabain to inhibit the endogenous oocyte Na-K-ATPase. For measurements of 86Rb uptake by αH1-βH1 pumps, all solutions contained 10 µM bumetanide. After addition of 5 µCi/ml 86Rb (Amersham), oocytes were incubated for 12 min at room temperature before being washed in a solution containing (in mM) 90 NaCl, 1.0 CaCl2, 1.0 MgCl2, and 10 HEPES, pH 7.4. Individual oocytes were dissolved in 0.5% SDS and counted.

Drugs. DIDS and ouabain were obtained from Sigma.

Statistical analysis. Data are presented as means ± SE. Statistical analysis of the data was performed by a paired Student’s t-test when pairs of measurements obtained in the same oocyte were compared or by an unpaired Student’s t-test when different groups of oocytes were compared.

RESULTS

Electrogenic activity of the B. marinus Na-K- and H-K-ATPase. The type of β-subunit that is associated in situ with αH1 is not known. We coexpressed αH1 in X. laevis oocytes together with βH1, which had been cloned from the same organ and was used in earlier studies to analyze the function of this ATPase (17). Sequence comparison indicates that this β-subunit is the amphibian equivalent of the mammalian β2-isoform (13). The B. marinus Na-K-ATPase α-subunit was also coexpressed with βH1 to be able to attribute the observed functional differences between H-K- and Na-K-ATPases specifically to the α-subunit.

We first evaluated the functional expression of our ATPases using the 86Rb uptake assay. Figure 1 shows that B. marinus bladder H-K-ATPase was functionally expressed at a similar level to that for Na-K-ATPase, with 86Rb uptake values of ~40–50 pmol/min · oocyte−1 above the background uptake observed in control oocytes injected with the β-subunit alone and expressing only the endogenous X. laevis Na-K-ATPase α-subunit.

To determine the stoichiometry of B. marinus Na-K-ATPase, we studied the electrogenic activity of this ion pump compared with that of B. marinus Na-K-ATPase after expression in X. laevis oocytes. As expected, when Na-K-ATPase was stimulated by addition of 5 mM K+, an outward (positive) current was observed (Fig. 2A). In oocytes expressing the B. laevis H-K-ATPase, K+ induced the slow appearance of an inward (negative) current of small amplitude, which was sensitive to ouabain. The I-V curves of the K+-induced current and the ouabain-sensitive current are shown in Fig. 2B. B. marinus bladder H-K-ATPase (17), like B. marinus Na-K-ATPase (16), is moderately sensitive to ouabain. The ouabain-sensitive Na-K pump current was similar to the K+-activated Na-K pump current. On the other hand, in oocytes expressing H-K-ATPases, the amplitude of the ouabain-sensitive current was significantly larger than the K+-induced current. At present, the reason for this difference is not known. It is possible that low levels of extracellular K+ persist in the unstimulated layers close to the membrane due to a small K+ leak from the oocyte that maintains some activity of the H-K pump. No current resulting from endogenous oocyte Na-K-ATPase was observed, because this pump had been inhibited by exposure to 0.2 µM ouabain. In another set of experiments, the effect of 5 mM K+ in the absence and in the presence of 2 mM ouabain was compared. In oocytes expressing H-K-ATPase and at −50 mV, 5 mM K+ induced an inward current of 14.5 ± 3.2 nA in the control condition, which was reduced to 5.5 ± 1.6 nA in the presence of 2 mM ouabain (n = 6, P < 0.005, paired Student’s t-test).

As a control experiment for a nonelectrogenic ion pump, we also measured the 86Rb uptake and the K+-induced current in oocytes injected with cRNA of the α- and β-subunits of the rabbit gastric H-K-ATPase and in oocytes injected with gastric H-K-ATPase β-subunit cRNA alone. As described for the other groups, endogenous oocyte Na-K-ATPase was inhibited by ex-
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posure to ouabain, but these oocytes were not loaded with Na⁺. When compared with the baseline uptake observed in oocytes expressing the β-subunit alone (2.5 ± 0.2 pmol·min⁻¹·oocyte⁻¹, n = 11) or in noninjected oocytes (2.0 ± 0.2 pmol·min⁻¹·oocyte⁻¹, n = 12), coexpression of the gastric H-K-ATPase α- and β-subunits led to a significant increase in ⁸⁶Rb uptake (17.8 ± 1.1 pmol·min⁻¹·oocyte⁻¹, n = 18, P < 0.001 for both comparisons). In this latter group, addition of 20 μM SCH-28080 to 5 mM K⁺ reduced the ⁸⁶Rb uptake in oocytes expressing the Na-K-ATPase or the nongastric H-K-ATPase to 12.2 ± 0.8 pmol·min⁻¹·oocyte⁻¹ (n = 21, P < 0.001). In the same experiments, the ⁸⁶Rb uptake in oocytes expressing the Na-K-ATPase or the nongastric H-K-ATPase amounted to 78 ± 3 (n = 11) and 73 ± 5 pmol·min⁻¹·oocyte⁻¹ (n = 10), values similar to those observed in the experiments depicted in Fig. 1. In the same batches of oocytes, addition of 5 mM K⁺ produced a similar small inward current in the gastric H-K-ATPase α,β-subunit group (4.8 ± 1.0 nA, n = 10) and the β-subunit alone group (4.1 ± 1.0 nA, n = 7). Addition of 20 μM SCH-28080 to the 5 mM K⁺ solution did not induce any detectable current change. In the Na-K-ATPase group, K⁺ induced an outward current of 143 ± 22 nA (n = 7), whereas a small inward current of 11 ± 5 nA (n = 7) was detected in the B. marinus bladder H-K-ATPase group. Thus expression of gastric H-K-ATPase led to a small but significant rubidium transport that was not accompanied by any evidence for any evident electrogenic activity.

H-K-ATPase activity in the presence of CO₂-HCO₃⁻.
The current generated by the H-K-ATPase could be due to its electrogenic activity resulting from the transport of a larger inward than outward number of cations. Alternatively, the inward current could be due to another transporter activated by changes in the cellular ionic composition, i.e., by a modification of the intracellular pH resulting from the H-K-ATPase activity, for example. To distinguish between these two possibilities, we used the following reasoning. If the K⁺-induced inward current resulted from intracellular alkalization, preventing this alkalization should result in a smaller K⁺-induced inward current. In contrast, if the inward current were due to an electrogenic H⁺ (and Na⁺) exchange for K⁺, intracellular acidification may increase this current by providing more substrate (protons) to this ion pump. To test this hypothesis, we compared the effects of K⁺ activation of the H-K pump in a HEPES-buffered solution and in a solution containing 25 mM HCO₃⁻ bubbled with 5% CO₂ (pH 7.4). CO₂ is expected to produce intracellular acidification after diffusion across the plasma membrane and transformation into carbonic acid by carbonic anhydrase. The intracellular buffering power of the oocyte is also expected to be increased by the presence of CO₂ and HCO₃⁻. We observed that the inward current resulting from K⁺ activation of H-K-ATPase was reduced in the presence of the CO₂-HCO₃⁻ solution (see example in Fig. 3A). Figure 3B illustrates the reduction of the K⁺-induced inward current over the entire potential range. The mean values of the K⁺-induced inward currents at −50 mV were 11.7 ± 1.6 nA in the absence and 3.7 ± 1.3 nA (n = 9; P < 0.001) in the presence of CO₂-HCO₃⁻. As shown in Fig. 3C, the effect of CO₂-HCO₃⁻ was fully reversible, and the K⁺-induced inward current did not diminish with the time of the experiment. Moreover, the switch from the HEPES-buffered solution to the CO₂-HCO₃⁻ solution induced a large decrease in the baseline inward current, with a mean change of 60 ± 8 nA (n = 9). In contrast to the inward current observed in oocytes expressing H-K-ATPase, the electrogenic activity of Na-K-ATPase expressed in oocytes was barely modified by exposure of the oocytes to the CO₂-HCO₃⁻ solution: 123.5 ± 15.9 nA in the absence and 115.5 ± 15.8 nA in the presence of CO₂-HCO₃⁻ (n = 9; P > 0.7). These results support the hypothesis that the inward current is secondary to a change in pH. This hypothesis requires two premises: 1) the activation of H-K-ATPase induces an intracellu-

Fig. 2. K⁺-activated and ouabain-sensitive currents. A: current traces recorded from an oocyte expressing the B. marinus Na-K-ATPase (top) or bladder H-K-ATPase (bottom). After a stable current reading (at −50 mV) was reached, 5 mM K⁺ and 30–60 s later, 2 mM ouabain was added to the bathing solution. Activation of Na-K-ATPase produced a large outward current whereas activation of H-K-ATPase was associated with the slow appearance of a small inward current. B: current-voltage (I-V) relationship of the K⁺-induced (filled symbols) and ouabain-sensitive (open symbols) currents in oocytes expressing Na-K-ATPase (triangles) or H-K-ATPase (circles). Values are means ± SE of 9 measurements. Vm, membrane voltage.

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lar alkalization and 2) intracellular alkalization induces an inward current. The first assumption has been well established (17), and the second assumption was verified by the following experiments that were performed in native, noninjected oocytes to demonstrate the presence of an endogenous alkalinization-induced inward current.

**Effect of intracellular alkalization on the membrane current.** Noninjected oocytes were first incubated in a CO$_2$-HCO$_3^-$ solution for 2 h and placed into the same solution in the measurement chamber. In a first set of experiments, we monitored the effect on pHi of changing the bath to a CO$_2$-HCO$_3^-$-free solution. In a second set of experiments, we analyzed the effect of the same maneuver on inward membrane currents.

As shown in the example in Fig. 4A, removal of CO$_2$-HCO$_3^-$ produced a sizeable alkalization of the oocyte that was reversible on reexposure to the CO$_2$-HCO$_3^-$ solution. The mean pH$_i$ values in the CO$_2$-HCO$_3^-$ solution and 3 min after removal of CO$_2$ and HCO$_3^-$ were 7.02 ± 0.03 and 7.39 ± 0.05, respectively ($n = 8; P < 0.001$).

Figure 4B shows that pH$_i$ changes were paralleled by the appearance of an inward current on CO$_2$-HCO$_3^-$ removal. At a membrane potential of −50 mV, the amplitude of this current amounted to −113.6 ± 6.4 nA ($n = 11$). The shape of the I-V relationship of this current was similar to that of the K-induced current observed in oocytes expressing H-K-ATPase (compare Figs. 3B and 4C).
Effect of DIDS on the alkalinization-induced current. Many ion transport systems could be affected by intracellular alkalinization to produce the inward current observed after CO₂-HCO₃⁻ removal or after activation of H-K-ATPase in X. laevis oocytes. Even though endogenous activity of Na⁺-HCO₃⁻ cotransport is apparently low in X. laevis oocytes (28), we considered the implication of this electrogenic transport system by testing the effect of DIDS, a known inhibitor of the Na⁺-HCO₃⁻ cotransporter. DIDS, at a concentration of 500 μM, did not inhibit the K-induced inward current in oocytes expressing H-K-ATPase. The mean K-induced currents were $-28.1 \pm 7.60$ nA before and $-28.5 \pm 6.18$ nA after DIDS treatment ($n = 10$).

DISCUSSION

The activity of B. marinus bladder H-K-ATPase is associated with a small inward current, as demonstrated by the effect of K⁺ activation and ouabain inhibition of this cation pump. By themselves, these results indicate that the stoichiometry of the nongastric H-K pumps is different from that of the Na-K pump, for which a large outward current is observed.

The slow time course of the appearance of the inward current after K⁺ activation, or of its disappearance after ouabain inhibition, suggests that it is not intrinsically due to the activity of H-K-ATPase but rather results from changes in intracellular ionic conditions due to the ion pump function. We tested the hypothesis that the inward current is due to the activation of an electrogenic process by intracellular alkalinization produced by the activity of the H-K pump. We could demonstrate that intracellular alkalinization produced by CO₂-HCO₃⁻ removal is indeed accompanied by the appearance of an inward current in noninjected oocytes. This current and the K⁺-induced current in oocytes expressing H-K-ATPase have a similar I-V relationship. It should be noted that the pHₐ, measured by means of ion-sensitive microelectrodes, reflects the bulk pHₐ of the oocyte. Analogous to sizeable effects of submembraneous Na⁺ concentrations in X. laevis oocytes previously observed (1), it is certainly possible that the sudden activation of the highly expressed H-K-ATPases could result in larger local pH changes.

From the existence of the alkalinization-induced inward current and the intracellular alkalinization resulting from H-K-ATPase activity, we conclude that the inward current associated with the activity is essentially due to this mechanism.

We have not yet determined the nature of the electrogenic process activated by intracellular alkalinization. The I-V relationship of the current indicates a reversal potential close to 0 mV (see Figs. 2 and 4). This is not compatible with the activation of either a K⁺-selective or a Na⁺-selective channel under our experimental conditions. Our negative results with a Na⁺-HCO₃⁻ cotransport inhibitor also argue against the implication of such a transport system, although it cannot definitively be excluded that a DIDS-resistant Na⁺-HCO₃⁻ cotransporter exists in oocytes.

Similar to what has been described earlier (20), expression of gastric H-K-ATPase resulted in a lower expression of transport function (assayed as ⁸⁶⁸Rb uptake) than that obtained for either Na-K-ATPase or nongastric H-K-ATPase. However, this level of expression should have allowed us to detect a K⁺-induced or SCH-28080-sensitive activity if the activity of this ion pump was electrogenic in a way similar to that of Na-K-ATPase. However, the low level of expression may have prevented us from detecting an alkalinization-induced inward current similar to what we observed with nongastric H-K-ATPase. Thus our results confirm the hypothesis of an exchange of a symmetrical number of H⁺ and K⁺ ions by gastric H-K-ATPase (26).

Our results are thus compatible with the hypothesis that the activity of B. marinus bladder H-K-ATPase is not intrinsically electrogenic. In this regard, the bladder H-K pump is similar to the gastric H-K pump. What might be the actual ion stoichiometry of this transport system? The available evidence for all the highly related members of the H-K- and Na-K-ATPase family indicates that two K⁺ ions are transported into the cell for each hydrolyzed ATP, and we make the assumption that this is also the case for the nongastric H-K-ATPases. Because Na⁺ as well as protons seem to be transported by these pumps (9, 12), the simplest hypothesis is that one H⁺ and one Na⁺ ion are exchanged for two K⁺ ions, but this hypothesis has yet to be supported by experimental data. However, because both Na-K-ATPase and the gastric H-K-ATPase show some flexibility with regard to the stoichiometry of transported ions (5, 6, 24, 25), it is certainly possible that the ratio of Na⁺ ions to H⁺ ions is not fixed but varies, depending on the intracellular concentrations of both of these ions.

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