Na-K Pump Current in the *Amphiuma* Collecting Tubule

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ABSTRACT There is strong evidence supporting the hypothesis of an electrogenic Na-K pump in the basolateral membrane of several epithelia. Thermodynamic considerations and results in nonepithelial cells indicate that the current carried by the pump could be voltage dependent. In order to measure the pump current and to determine its voltage dependence in a tight epithelium, we have used the isolated perfused collecting tubule of *Amphiuma* and developed a technique for clamping the basolateral membrane potential ($V_{bl}$) through transepithelial current injection. The transcellular current was calculated by subtracting the paracellular current (calculated from the transepithelial conductance measured in the presence of luminal amiloride) from the total transepithelial current. Basolateral membrane current–voltage ($I-V$) curves were obtained in conditions where the ratio of the pump current to the total basolateral membrane current had been maximized by loading the cells with Na$^+$ (exposure to low-K$^+$ bath), and by blocking the basolateral K$^+$ conductance with barium. The pump current was defined as the difference of the current across the basolateral membrane measured before and 10–15 s after the addition of strophanthidin (20 $\mu$M) to the bath solution. With a bath solution containing 3 mM K$^+$, the pump current was nearly constant in the $V_m$ range of -20 to -80 mV (52 ± 5 $\mu$A/cm$^2$ at -60 mV) but showed a marked voltage dependence at higher negative $V_m$ (pump current decreased to 5 ± 9 $\mu$A/cm$^2$ at -180 mV). In a 1.0 mM K bath, the shape of the pump $I-V$ curve was similar but the amplitude of the current was decreased (24 ± 4 $\mu$A/cm$^2$ at -60 mV). In a 0.1 mM K bath, the pump current was not significantly different from 0.

Our results indicate that the basolateral Na-K pump generates a current which depends on the extracellular potassium concentration. With physiological peritubular concentration of K$^+$ and in the physiological range of potential, the pump activity, measured as the pump-generated current, was independent of the membrane potential.

INTRODUCTION

The electrogenic nature of the Na-K pump, a phenomenon demonstrated in several types of cells (subject reviewed in De Weer, 1985) means that one or several of the steps of the pump cycle must be carrying one or more charges across the mem-
brane. From thermodynamic considerations, such a charge-moving step may be dependent upon the potential difference across the membrane, and if this charge-moving step is rate limiting, then the pump activity will be voltage dependent (De Weer, 1984). Data supporting voltage dependence of the Na-K pump current have been obtained in the isolated cardiac cell (Gadsby et al., 1985), in the squid axon (De Weer et al., 1987; Rakowski et al., 1989) and frog oocyte (Lafaire and Schwarz, 1986; Rakowski and Paxson, 1988). Several investigators have reported data supporting the electrogenic nature of the Na-K pump of "leaky" or "tight" sodium-transporting epithelia of various origin (Sansom and O'Neil, 1986; Lewis et al., 1978; Kirk et al., 1980; Sackin and Boulpaep, 1983; Graf and Petersen, 1974; Nielsen, 1979a, b; Proverbio and Whittembury, 1975; Horisberger and Giebisch, 1988b). Na-K-ATPase, isolated from kidney medulla and reconstituted in artificial

**Figure 1.** Cellular model (upper panel) and equivalent electrical circuit (lower panel) of the transepithelial sodium transport by the principal cells of the *Amphiuma* collecting tubule. This model is based on the results of previously published studies (Hunter et al., 1987; Horisberger et al., 1987). The current across the cell ($I_c$) was calculated as the difference between the measured total transepithelial current ($I_{re}$) and the "shunt" current ($I_s$). The pump current was defined as the part of the transcellular current, measured under basolateral membrane voltage clamp conditions, that could be abolished by strophanthidin. In order to obtain optimal conditions to measure the pump current the following maneuvers were performed: (1) The pump activity was stimulated by a high intracellular Na⁺ activity obtained by previously inhibiting the pump for 4 min by a low-K⁺ bath. (2) The basolateral membrane conductance was reduced as a consequence of previous pump inhibition (Horisberger and Giebisch, 1988b) and by addition of 2 mM barium to the peritubular bath. (3) The paracellular conductance was decreased by potassium adaptation of the animals (Horisberger et al., 1987); (4) this maneuver also increased the apical membrane conductance and reduced the apical-basolateral membrane resistance ratio (Horisberger et al., 1987), a condition necessary to allow for voltage clamping of the basolateral membrane potential through transepithelial current injection.
membranes, has also been shown to be electrogenic (Borlinghaus et al., 1987; Fendler et al., 1985). Although depolarization of the basolateral membrane did not affect the basolateral ouabain-sensitive Na⁺ efflux (Cox and Helman, 1986c), no direct measurements of the voltage dependence of the pump current in epithelia have so far been reported. Considering the possible physiological relevance of the voltage dependence of the pump activity in sodium-transporting epithelia, we have developed a technique that allows measurements of the current across the basolateral membrane of the cells of an isolated perfused renal tubule, under voltage clamp conditions.

The voltage dependence of the pump current corresponds phenomenologically to a “pump conductance.” Under physiological conditions, this conductance amounts to only a small part of the total membrane conductance (De Weer, 1986). Therefore, for our study we have chosen conditions in which the pump conductance was maximized when compared with the total membrane conductance which was minimized. We had observed such conditions in previous experiments where membrane conductances and intracellular Na⁺ and K⁺ activities were measured (Horisberger and Giebisch, 1988b): after a 4-min exposure to a low-potassium bath, the principal cells of the collecting tubule of *Amphiuma* had a high Na⁺ and low K⁺ intracellular activity, and a markedly reduced basolateral membrane K⁺ conductance. We have further reduced the basolateral membrane conductance by adding barium to the peritubular solution: barium has been shown to be an inhibitor of this conductance (Hunter et al., 1987; Horisberger et al., 1987). As stated in previous papers from this laboratory (Horisberger et al., 1987; Horisberger and Giebisch, 1988b), exposing *Amphiuma* to a high potassium environment induces in the collecting tubule (a) an increase of the apical membrane conductance, largely a sodium-selective conductance and (b) a decrease of the paracellular conductance. Since these conditions allow for optimal measurements of the transcellular current, all the experiments reported here have been performed on potassium-adapted *Amphiuma* (see methods) (Horisberger et al., 1987). The strategy used to measure the pump current under optimal conditions is illustrated in Fig. 1. Under conditions described, we have measured the strophanthidin-sensitive basolateral membrane current in relation with the basolateral membrane potential. We found this pump current dependent on the peritubular bath K⁺ concentration and we describe its dependence on the membrane potential.

**METHODS**

*Amphiuma* of both sexes were obtained from C. Sullivan (Nashville, TE) and were K⁺ adapted by being kept for at least 4 d in aquaria containing a 10 mM NaCl + 50 mM KCl solution (Horisberger et al., 1987). The identification, preparation, and perfusion of single collecting tubules were carried out as described in previous papers from this laboratory (Hunter et al., 1987; Horisberger et al., 1987). The technique used to measure the transepithelial potential and to inject transepithelial current through the perfusion pipette has also been described in these papers. Short segments (range 182–270 μm) were dissected and cannulated at both ends for perfusion with control of the intratubular pressure (Horisberger and Giebisch, 1988b). In these short tubules with high transepithelial resistance, current injected at the proximal end of the tubule induced a nearly constant voltage deflection along the whole
length of the tubule (Horisberger and Giebisch, 1988a). Experiments were performed only when the voltage deflection measured at the distal end of the tubule was >80% of the voltage deflection measured at the proximal end. The effective transepithelial voltage (\(V_{t*}\)) was taken as the mean of the values measured at both ends of the tubule. Cellular impalements with conventional microelectrodes filled with 1 M KCl allowed for measurements of the basolateral membrane potential (\(V_{b0}\)) (Hunter et al., 1987). The mean distance of the impalement point of the intracellular electrode from the proximal end of the tubule was 35 ± 3% of the whole tubule length.

**Data Acquisition and Voltage Clamp**

Except for 25-s voltage-clamp experimental periods, the tubules were maintained under open circuit conditions. The transepithelial potentials (\(V_{t*}\)) measured at both ends of the tubule and the basolateral membrane potential (\(V_{b0}\)) were continuously recorded on two paper chart recorders (model 220, Gould, Inc., Cleveland, Ohio).

During \(V_{t*}\)-clamp periods, data were also recorded by means of a data acquisition system consisting of a microcomputer (IBM-XT), an analog-digital converter (RTI-815, Analog Devices, Inc., Norwood, MA) and a software package (UnkelScope, Unkel Software, Lexington, MA). Four channels sampled once every 50 ms: (a and b) \(V_{t*}\) recorded at both ends of the tubule, (c) \(V_{b0}\), and (d) the transepithelial current (\(I_{t*}\)). These data were stored digitally on the hard disk of the computer for later analysis. The controller included in the UnkelScope system also allowed us to adjust the current injected through the lumen with regard to the basolateral membrane potential every 50 ms. Thus, we were able to voltage clamp the basolateral membrane potential.

The paracellular conductance (\(G_{p}\)) was estimated as the transepithelial conductance measured with 10 \(\mu\)M amiloride present in the luminal fluid, using a 1-s positive luminal current injection. We have shown previously that this method yields valid estimates of the paracellular conductance (Hunter et al., 1987; Horisberger et al., 1987), and that this conductance is linear in the ±200 mV range (Horisberger and Giebisch, 1988a) and not influenced by the bath K+ concentration (Horisberger et al., 1987). \(G_{p}\) was measured immediately before and immediately after the maneuver described below and the mean of these two measurements was used to calculate the paracellular current (\(I_{p}\)) at each transepithelial potential value:

\[
I_{p} = V_{t*} \cdot G_{p}. \tag{1}
\]

The transcellular current (\(I_{c}\)) was then calculated as the difference between the total transepithelial current (\(I_{t*}\)) in the absence of amiloride and the paracellular current:

\[
I_{c} = I_{t*} - I_{p}. \tag{2}
\]

All currents and conductances are expressed per unit of luminal tubule surface area. The length and the luminal diameter of the tubule were measured optically by means of a calibrated eyepiece micrometer.

**Experimental Protocol**

After a stable cellular impalement had been obtained under open circuit condition, the tubule was exposed during a 4-min period to a 0.1 mM concentration of K+ in the peritubular bath solution (\([K^{+}]_{b}\)). Then 2 mM barium was added to the bath and simultaneously, \([K^{+}]_{b}\) was set to 3.0, 1.0, or 0.1 mM. As soon as the bath solution change had been completed (within 10 s), the control was switched to the basolateral membrane voltage clamp mode and \(V_{b0}\) was clamped at –60 mV for 2 s and then for four successive 0.5-s periods at –20, –100, –140, and –180 mV (15 experiments). Alternatively, \(V_{b0}\) was clamped at –50 mV for 2 s and...
then for four successive 0.5-s periods at -20, -80, -110, and -140 mV (11 experiments). The control potential was then set at -60 mV (or -50) for 18 s. During this time strophanthidin 20 μM was added to the bath solution. A second series of voltage steps at -20, -100, -140, -180, and -60 (or -20, -80, -110, -140, and -50) was carried out and then the control was switched back to the open circuit mode. Amiloride was added to the luminal solution to measure the shunt conductance and then the bath solution was changed for the control solution. A period of at least 10 min with luminal amiloride and another period of at least 5 min with control solutions were allowed to elapse. If the potential recording from the intracellular electrode was still stable after this recovery period, a second or a third experiment using a different bath K⁺ concentration was performed.

Basolateral membrane I-V curves (transcellular current vs. basolateral membrane potential) were established from the mean values of $V_w$, $V_b$, and $I_e$ sampled between 400 and 500 ms after the potential changes. The I-V curves were calculated only if $V_b$ (as measured from the intracellular microelectrode) had been effectively clamped within 3 mV of the command potential at all five set values of both series of voltage steps. Application of this criterion resulted in exclusion of 15 experiments out of 39 for the pump I-V curve results.

By blocking the pump, strophanthidin may induce changes of the K⁺ concentration in the unstirred layers along the basolateral membrane. As the basolateral membrane has been shown to be K⁺ selective, these changes in the unstirred layers could generate an inward K⁺ current which would be indistinguishable from the abolition of the outward pump current during simple transcellular current measurements. Thus, the "strophanthidin-sensitive" basolateral membrane current may not represent only a real pump current. However, as stated earlier, our experimental conditions were chosen to minimize the current flowing through the basolateral membrane K⁺ conductance. To estimate the magnitude of the residual K⁺ current flowing through the basolateral membrane under our experimental conditions, we have measured the transcellular current induced by changes of [K⁺]₀ under $V_e$-clamp conditions. Using a protocol analogous to that of the pump measurement, the pump was first blocked for 4 min by 20 μM strophanthidin, and then 2 mM barium was added to the peritubular bath solution. Then, [K⁺]₀ concentration was raised from 3.0 to 15 mM while $V_w$, $V_e$, and $I_e$ were recorded, during a 20-s experimental period under $V_w$ clamp conditions ($V_w$ set to -60 mV). The transcellular current was then calculated by the same method as described for the strophanthidin experiments.

Solutions and Drugs

The composition of the "control" solution was (in millimolar) Na⁺ 97.0, K⁺ 3.0, Ca⁺ 1.8, Mg²⁺ 1.0, Cl⁻ 89.6, HEPES/HEPES⁻ 14.4, and H₂PO₄⁻/HPO₄²⁻ 0.8. The "low-K⁺" solution was similar except for the 0.1 mM K⁺ and the 100.0 mM Na⁺ concentration. The same control solution was used for the bath and the perfusate except that 2.0 mM glucose and 0.25 mM glycine were added to the bath solutions. The pH of all solutions was adjusted to 7.6 with NaOH. The solutions were bubbled with O₂. Amiloride (gift of Merck, Sharp & Dohme, West Point, PA) was added to the luminal perfusion fluid at a concentration of 10 μM and strophanthidin (Sigma Chemical Co., St. Louis, MO) was added to the peritubular bath solution at a concentration of 20 μM.

Statistics

Results are expressed as mean ± SEM (n = number of observations). The statistical significance of the difference between mean values was determined by Student's t test. When appropriate, Student's t test for paired data was used, as indicated in the text. $P < 0.05$ was chosen as the level of significance.
RESULTS

Electrophysiological Characteristics

Under control conditions, the transepithelial \((V_{te})\) and basolateral membrane \((V_{bl})\) potential, \(-38 \pm 3\) and \(-61 \pm 2\) mV \((n = 39)\), respectively, and the apical \((G_a)\) and basolateral \((G_{ma})\) membrane conductances, \(2.2 \pm 0.3\) and \(2.9 \pm 0.4\) mS\(\cdot\)cm\(^{-2}\) \((n = 39)\), respectively, were similar to those measured previously in potassium-adapted \textit{Amphiuma} by the same method (Horisberger et al., 1987; Horisberger and Giebisch, 1988b). The response to exposure to the low-K\(^+\) bath was also similar to that described previously, consisting of a large reduction of \(G_a\) and \(G_{ma}\) to \(0.64 \pm 0.22\) and \(0.39 \pm 0.11\) mS\(\cdot\)cm\(^{-2}\) \((n = 39)\), respectively, so that we can assume that the intracellular Na\(^+\) and K\(^+\) activities were also similar to those measured under the same conditions (Horisberger and Giebisch, 1988b). The mean paracellular conductance—the transepithelial conductance measured with amiloride in the luminal fluid—was \(2.24 \pm 0.25\) mS\(\cdot\)cm\(^{-2}\) \((n = 39)\).

Effects of K\(^+\) Concentration and Strophanthidin on the Basolateral Membrane Potential

An original recording of the basolateral membrane potential \((V_{bl})\) during a typical experiment is presented in Fig. 2. We shall first consider the part of the experiment in which \(V_{bl}\) was recorded under open circuit conditions; the 25-s period during which potentials and current were recorded under basolateral membrane voltage clamp conditions (hatched bar) is described in more detail in Fig. 4 and will be discussed later.

Upon exposure to a peritubular bath solution in which the potassium concentration had been decreased to 0.1 mM, \(V_{bl}\) first rapidly hyperpolarized from \(-79\) to \(-112\) mV; this is due to the change of the K\(^+\) equilibrium potential across the K\(^+\)-selective basolateral membrane (Hunter et al., 1987; Horisberger and Giebisch, 1988b). There was then a slow depolarization of \(V_{bl}\) down to \(-57\) mV owing both to the decrease of the intracellular K\(^+\) activity and the decrease of the basolateral membrane K\(^+\)-selective conductance (Horisberger and Giebisch, 1988b) (Fig. 2, point A). At this point, adding 2 mM barium and raising \([K^+]_{b}\) to 3.0 mM produced a fast hyperpolarization to \(-106\) mV (Fig. 2, point B). The following 25-s period (hatched bar) was recorded under \(V_{bl}\)-clamp conditions; this part of the recording is shown magnified in Fig. 4 and will be discussed later (however, it can be seen in Fig. 2 that \(V_{bl}\) was effectively clamped at \(-60\) mV). During this voltage clamp period, 20 \(\mu\)M strophanthidin was added to the peritubular bath solution; it did not modify \(V_{bl}\) (which was clamped), but induced large changes of the transepithelial current and potential, as shown later in Fig. 4. When the recording mode was changed back to the open circuit conditions, \(V_{bl}\) had decreased to \(-22\) mV (Fig. 2, point C). Under control conditions, raising \([K^+]_{b}\) and addition of barium to the peritubular bath solution are two maneuvers which induce a depolarization of \(V_{bl}\) because, under control conditions, \(V_{bl}\) is mainly determined by the potassium-selective basolateral membrane conductance and the large negative equilibrium potential of potassium \((E_{K+})\) across this membrane (Hunter et al., 1987). The large hyperpolarization of \(V_{bl}\) produced by raising \([K^+]_{b}\) and adding barium after the cell has been loaded with
Na⁺ indicate that, in contrast under these circumstances, \( V_m \) is mainly dependent on another K⁺-dependent electrogenic process: the Na-K pump. This is confirmed by the very fast and large depolarization observed after the addition of strophanthidin.

Fig. 3 shows the mean values of \( V_m \) at the end of the low-K⁺ exposure (open bars, corresponding to point A of Fig. 2), just after the addition of barium and K⁺ to the peritubular bath (black bars, corresponding to point B of Fig. 2) and 15 s after the addition of strophanthidin (hatched bars, corresponding to point C of Fig. 2). The three groups of bars summarize the results of experiments in which \([K⁺]_b\) was set to 3.0, 1.0, and 0.1 mM at the time when barium was added.

It can be seen, first, that the potential at the end of the low-K⁺ bath period (point A of Fig. 2, open bars of Fig. 3) was similar in the three groups. Secondly, addition of \([K⁺]_b\) of 3.0 mM produced a large depolarization (AV \( V_m \) 24 ± 6 mV \([n = 11]\) \( P < 0.005\), paired t test). In contrast, addition of barium and simultaneously raising \([K⁺]_b\) to 3.0 mM resulted in a large hyperpolarization (AV \( V_m \) - 24 ± 3 mV \([n = 18]\) \( P < 0.001\), paired t test). When \([K⁺]_b\) was set to 1.0 mM simultaneously with addition of barium, only a slight depolarization (AV \( V_m \) 8 ± 3 mV \([n = 10]\)) was observed. Thirdly, addition of strophanthidin to the peritubular bath solution resulted in a depolarization highly dependent on \([K⁺]_b\); the strophanthidin-induced depolarization (difference between black and hatched bars) amounted to 52 ± 5 mV \([n = 18]\), 28 ± 5 mV \([n = 10]\), and 3 ± 1 mV \([n = 11]\), with \([K⁺]_b\) of 3.0, 1.0, and 0.1, respectively.

Hence, before the addition of strophanthidin, \( V_m \) had a much larger negative value when \([K⁺]_b\) was at 3.0 mM than when it was at 0.1 mM (-68 ± 7 mV \([n = 18]\).
vs. $-13 \pm 3$ mV ($n = 11$, $P < 0.001$). This effect of $[K^+]_b$ was opposite to the effect predicted for a conductive, $K^+$-selective pathway. Rather than depolarizing the peritubular cell membrane, potassium activated an electrogenic hyperpolarizing process in the basolateral membrane. The fact that the $K^+$-induced hyperpolarization could be inhibited by strophanthidin strongly suggest that this process is a $Na^+/K^+$ countertransport with a higher number of $Na^+$ ions than $K^+$ ions being transported.

**Effect of Strophanthidin on the Basolateral Membrane I–V Curve**

We next consider the part of the experiments done under $V_{tr}$-clamp mode. Original recordings of the transepithelial potential, basolateral membrane potential and transepithelial current during the 25-s period of voltage clamp mode (corresponding to the hatched bar in Fig. 2) are presented in Fig. 4 (left panel). The middle trace shows that $V_m$ was clamped at five successive values ($-60, -20, -100, -140, -180$ mV) for 0.5 s at each step; then $V_m$ was maintained at $-60$ mV for 18 s and the voltage steps were repeated. The effect of 20 $\mu$M strophanthidin, added after the first series of voltage steps on the transepithelial current ($I_{tr}$) can be observed in the lower trace. Before strophanthidin, a positive transepithelial current of $-100$ nA had to be injected to maintain $V_m$ at $-60$ mV. After the addition of strophanthidin, a negative transepithelial current of about $-15$ nA was necessary to maintain $V_m$ at
the same value of \(-60\) mV. The upper trace \((V_{te})\) shows transepithelial potential. The shunt conductance \((G_s)\) was taken as the mean of the values obtained immediately before and after the experiment; these values were very similar \((G_s\) before \(2.21 \pm 0.25\) vs. after \(2.26 \pm 0.26\) mS \(\cdot\) cm\(^{-2}\), \(n = 39\), NS). Using the \(V_{te}\) and the \(I_{te}\) values corresponding to the five set potentials of each voltage step, and \(G_s\) the transcellular current \((I_{cel})\) was calculated for each \(V_{bl}\) value before and after strophanthidin. These values of \(I_{cel}\) are plotted against \(V_{bl}\) in Fig. 4 (right panel). After the addition of strophanthidin, the current across the basolateral membrane decreased by about 60 \(\mu\)A \(\cdot\) cm\(^{-2}\) in the low negative membrane potential range. The effect produced by

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\text{FIGURE 4. (Left panel) Original recordings of the transepithelial potential (}\ V_{te}\text{), the basolateral membrane potential (}\ V_{bl}\text{), and the transepithelial current (}\ I_{te}\text{) during the period when } V_{bl}\text{ was clamped. Two identical series of voltage steps of 0.5 s were performed, one at the beginning and one at the end of this period. Strophanthidin (20 \(\mu\)M) was added to the bath solution in the time between the series of voltage steps. (Right panel) Corresponding curves of the transcellular current (}\ I_{cel}\text{) vs. the basolateral membrane potential (}\ V_{bl}\text{). } I_{cel}\text{ was calculated for the voltage steps done before (open circles) and after (black circles) the addition of 20 \(\mu\)M strophanthidin to the peritubular bath solution.}
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Inasmuch as the transcellular current before and after strophanthidin were measured at the same measured basolateral membrane potentials (basolateral membrane voltage clamp conditions) and in the presence of the same concentration of barium, our measurements of the strophanthidin-sensitive current did not depend on the precise nature of block of the \(K^+\) conductance by barium. However, as shown in another paper (Horisberger and Giebisch, 1988a), at positive membrane
potential, the basolateral membrane conductance becomes very large even in the presence of barium. Under these conditions, the pump conductance becomes only a very small part of the total basolateral membrane conductance, and the measurement of the pump current becomes inaccurate. For this reason, pump current measurements were carried out only at negative membrane potentials.

In Fig. 5, we have plotted the pump current ($I_{\text{pump}}$), calculated as the difference between the current across the basolateral membrane before and after strophanthidin, as a function of the basolateral membrane potential. These are the mean values of 14 measurements performed with $[K^+]_b$ at 3.0 mM, 6 measurements with $[K^+]_b$ of 1.0 mM, and 6 measurements with $[K^+]_b$ at 0.1 mM. With a $[K^+]_b$ of 3.0 mM, the pump current was nearly constant in the $-20$ to $-100$ mV $V_{bl}$ range. At larger negative potential, there was a sharp decrease of the strophanthidin-sensitive current, with a value not significantly different from 0 at $-180$ mV. As described by De Weer (1986), estimates of a reversal potential by extrapolation would be unreliable, because the $I-V$ curve may be S-shaped and cross the 0 current line at much higher potential. With a $[K^+]_b$ of 1.0 mM, the shape of the $I-V$ curve was roughly similar, with a strophanthidin-sensitive current of reduced amplitude when compared with the values obtained with $[K^+]_b$ at 3.0 mM. A reversal potential around $-160$ mV could be observed. The data obtained with a $[K^+]_b$ of 0.1 mM indicated that the strophanthidin current was abolished at low membrane potentials. With more negative membrane potentials, all the mean values of the strophanthidin-sensitive current were negative and these inward currents tended to be larger with higher negative potentials. The scatter of the data (no values significantly different from 0 under these conditions) did not allow to determine a reasonably accurate value for the reversal potential.
Potassium Current in the Presence of Barium

To estimate the K⁺ current flowing through the basolateral membrane conductance under our experimental conditions we also measured the transcellular current induced by changes of [K⁺]₀ in another group of tubules. After the pump had been blocked for 4 min and 2 mM barium had been added to the bath solution, and under voltage clamp conditions (Vₘ set to −60 mV), raising the bath K⁺ concentration from 3 to 15 mM induced an inward-directed current of 9 ± 3 μA·cm⁻² (n = 4), to be compared with an ouabain-sensitive current of >50 μA·cm⁻² measured with a K⁺ concentration of 3 mM. In this group of four tubules all the electrophysiological variables (Vₘ, −28 ± 6 mV; Vₘ, −55 ± 7 mV; Gₘ, 3.1 ± 0.4 mS·cm⁻²; Gₘ after 4-min pump block, 1.0 ± 0.3 mS·cm⁻²) were similar to those measured in the tubules used for the pump current measurements. Thus, a large change of [K⁺]₀ produced only minor changes of the current across the basolateral membrane after the K⁺ conductance had been blocked by barium. We show in the Appendix that this change of the peritubular bath concentration of potassium is much larger than the change of K⁺ concentration that can be expected in the unstirred layers from a sudden inhibition of the K⁺ flux carried by the pump.

Discussion

Our measurements of the current across the basolateral membrane under voltage-clamped conditions, and our recordings of the basolateral membrane potential after potassium readmission confirm the electrogenicity of the Na-K pump of the basolateral membrane of cells of a sodium-transporting “tight” epithelium. The electrogenicity of the K⁺-activated, strophanthidin-sensitive process in the basolateral membrane cannot be explained by the K⁺ conductance and K⁺ concentration gradient in the unstirred layers along the basolateral membrane because when [K⁺]₀ is increased the voltage change occurs in a direction opposite to that predicted by the K⁺ concentration changes in the bulk solution. Furthermore, when the basolateral membrane K⁺ conductance was blocked by barium, the hyperpolarizing effect of external K⁺ in the present experiment and the depolarizing effect of strophanthidin tended to be larger than what had been observed in similar studies performed in the absence of barium (Horisberger and Giebisch, 1988b). This fact strongly argues against a major role of the basolateral membrane potassium conductance in the potential changes observed when the pump was blocked or activated.

In order to consider the strophanthidin-sensitive current across the basolateral membrane as a reliable estimate of the current directly generated by the Na-K pump, we had to demonstrate that the strophanthidin-sensitive current was not significantly influenced by a potassium current flowing through the basolateral K⁺-selective conductance. To achieve this aim, our experimental conditions were chosen to minimize the effects of basolateral K⁺ conductance. We had previously shown that after a period of pump inhibition the basolateral K⁺ conductance was greatly reduced (Horisberger and Giebisch, 1988b). Furthermore, we added barium to the peritubular bath solution, a maneuver known to inhibit the basolateral K⁺ conductance (Hunter et al., 1987; Horisberger et al., 1987). To assess the magni-
tude of the K⁺ current flowing across the basolateral membrane in the presence of barium and strophanthidin, we tested the effect of a large change of the bath K⁺ concentration. For a peritubular bath concentration of potassium change from 3 to 15 mM, we observed an average current of 9 μA·cm⁻². Although the changes in K⁺ concentration were by far larger than those which might have been expected in the unstirred layers as a result of pump inhibition (see Appendix), the current change was only a modest fraction of the 50 μA·cm⁻² current change measured when the pump was inhibited by strophanthidin. Thus, the largest part of the strophanthidin-sensitive current is a pump-generated current.

The Pump I/V Curve

In accordance with thermodynamic considerations indicating a possible voltage dependence of an electrogenic process driven by a finite energy source (De Weer, 1984), the present results indicate that the Na-K pump current is related to the membrane potential. With a 3.0 mM concentration of K⁺ in the peritubular bath, the voltage dependence is small in the physiological membrane potential range (below -100 mV), but is marked between -100 and -180 mV. In this range, the voltage dependence of the pump current is roughly equivalent to a conductance of 0.5 mS·cm⁻² of tubular surface, a value of similar magnitude as the residual basolateral membrane conductance under these specific experimental conditions (basolateral conductance decreased by previously blocking the pump and by barium). It should be noticed that under normal conditions, the conductance of the pump amounts to a much smaller part of the total membrane conductance than under the conditions in which the present measurements were done, since under control conditions (a) the pump activity is probably lower because of the lower intracellular Na⁺ activity (Horisberger and Giebisch, 1988b), (b) the basolateral membrane conductance is about 10 times larger (Horisberger et al., 1987), and (c) the slope of the pump I-V curve is negligible in the physiological range of the basolateral membrane potential.

The dependence of the pump current on the membrane potential was roughly similar to that observed by Gadsby et al. (1985) in cardiac myocyte, De Weer et al. (1987) in squid giant axon (1987), and Rakowski and Paxson (1988) in Xenopus oocyte. Since we could not explore the positive membrane voltage range, we cannot compare our data with those of Lafaire and Schwartz (1986), who observed a biphasic I-V curve with a negative slope conductance in the positive membrane voltage range.

An apparent voltage dependence can be due to the effect of the potential on one or several charge translocating steps of the pump cycle (Chapman et al., 1983; De Weer, 1986), and thus on the pump turnover rate. However, the possibility of a change of the Na⁺/K⁺ coupling ratio towards unity (electroneutral Na⁺/K⁺ exchange) with high negative membrane potential cannot be excluded. The constancy of the coupling ratio of the Na-K pump is still a controversial matter: in nerve and cardiac cells, parallel changes of the pump-generated current and Na⁺ transport rate indicate a constant coupling ratio (Abercrombie and De Weer, 1978; Eisner and Lederer, 1980; Rakowski et al., 1989); similarly in rabbit proximal tubules (Avison et al., 1987), the 3 Na⁺ to 2 K⁺ ratio appears to be constant over a
wide range of transport rate. However, a coupling ratio changing with the transport rate has been reported in frog skin (Cox and Helman, 1986a, b), amphibian proximal tubule (Sackin and Boulpaep, 1983), and rabbit cortical collecting tubule (Sansom and O’Neil, 1986). In the collecting tubule of *Amphiuma*, estimates of the pump coupling ratio, by comparison of the pump current to the transcellular sodium current under steady-state conditions, are compatible with a 3 Na⁺ to 2 K⁺ ratio (Horisberger, 1989). However, the measurements of the present study were made under transient conditions, far from a steady state, and give no information about the pump coupling ratio.

Assuming a constant stoichiometry, we can tentatively apply our data to a very simplified pump model described by De Weer (1986). The small slope conductance of the pump at physiological membrane potentials indicates that the pump rate is limited by a voltage-insensitive step (pump of the “rheogenic type”). Goldshlegger et al. (1987) have provided evidence that this rate-limiting, voltage-insensitive step is the potassium translocation step (conformational change \( E_{f}(2K) \rightarrow E_{i}(2K) \)), in accordance with the data by Nakao and Gadsby (1986) indicating that the sodium translocating step is charged.

Voltage Dependence of the Pump: Physiological Relevance

From the observed \( I-V \) relation of the pump current, variations of the basolateral membrane potential of the same magnitude as those observed under hormonal stimulation (in the range of \(-50\) to \(-80\) mV [Horisberger et al., 1987]) would entail only minor changes of the pump activity. These findings are in accordance with the results of Na⁺ flux measurements by Cox and Helman (1986c). Thus, in the physiological range of the basolateral membrane potential, the assumption that the Na-K pump is electrically equivalent to a pure current source is appropriate (Boulpaep and Sackin, 1979). This property of the Na-K pump contrasts with that of the H⁺ pump in acid secreting cells which is markedly voltage dependent in the physiological range of membrane potential and H⁺ concentrations (Andersen et al., 1985; Ehrenfeld et al., 1985; Harvey and Ehrenfeld, 1986).

Dependence on Peritubular K⁺ Concentration

The dependence of the pump current on \([K^+]_b\) could theoretically be due either to a change of the stoichiometry of the pump or to a change of the activity of the pump with a constant stoichiometry. The current dependence on external potassium appears to be similar to the well-known K⁺ dependence of the enzymatic (ATPase) activity of the Na-K pump (Jorgensen, 1986). This parallel dependence of the pump current and enzymatic activity on external \([K^+]\) suggests a constant pump stoichiometry.

Reversal Potential and Reverse Pump Current

By choosing appropriate experimental conditions, De Weer and Rakowski (1984) have been able, in the squid giant axon, to record current generated by the backward-running electrogenic Na⁺ pump. With low peritubular K⁺ concentrations we have also recorded negative (inwardly directed) strophanthidin-sensitive currents,
which tended to be larger with higher negative potentials. In a peritubular bath containing 1.0 mM K⁺ the pump reversal potential was about −160 mV. Assuming a constant pump stoichiometry (3 Na⁺/2 K⁺/1 ATP) and using the values of intracellular Na⁺ (45 mM) and K⁺ (16 mM) activities measured previously under similar conditions—after inhibition of the Na-K pump for 4 min—(Horisberger and Giebisch, 1988b), an excess of free energy of ATP hydrolysis (ΔG_{ATP}) can be calculated according to the equations given by De Weer (1986). This yields a ΔG_{ATP} of 360 mV. This value is lower than those reported in amphibian nonepithelial tissues (muscle), 480 mV (Kushmerik, 1969) and 560 mV (Dawson et al., 1980). The lower ΔG_{ATP} observed in the present study and the finding that all the strophanthidin-sensitive currents measured at membrane potentials lower than −50 mV at 0.1 mM [K⁺]₀ were negative suggest the possibility of a systematic bias in pump current estimates. For instance, if the basolateral membrane conductance were to decrease between the I–V curves established before and after strophanthidin, this could lead to an overestimate of the pump conductance and to larger apparent negative pump currents at high negative membrane potentials. This possibility must be considered, but we believe that such conductance changes must be small in view of the short intervals (20 s) between the time of the two I–V curves taken before and after strophanthidin exposure. We cannot, at present, evaluate to what extent other factors, such as the uncertainty concerning the precise intracellular ionic activities, the intrinsic difference of the tissues and the experimental conditions (isolated perfused tubule, at room temperature) may have contributed to our finding lower ΔG_{ATP} values than those reported in nonepithelial tissues.

In summary, our data confirm that the cardiac steroid-sensitive Na-K pump of the basolateral membrane of "tight" epithelia is electrogenic and transports an asymmetrical number of charges. In agreement with thermodynamic considerations, the activity of this pump is voltage sensitive, with the largest voltage dependence in the range of −100 to −200 mV of the membrane potential. This voltage dependence is similar to that reported in nonepithelial cells. As expected from measurements of its enzymatic activity, the electrical activity of the pump is also dependent on the external K⁺ concentration.

**APPENDIX**

We try below to estimate the magnitude of the possible change of the concentration of K⁺ produced by sudden blocking of the Na-K pump in the unstirred layers along the basolateral membrane. The basolateral membrane of the principal cells of the collecting tubule of *Amphiuma* has a very complicated geometry; numerous infoldings of the basolateral membrane amplify its surface by a factor larger than 10 (in K⁺-adapted animals) (Stanton et al., 1984). Thus, to analyze the unstirred layers effect within the lateral spaces and the basolateral membrane invaginations, we use a very simplified, "worst case" model: all the active transport sites, pumps and K⁺ channels, are assumed to be located at the deep end of the membrane infoldings and therefore separated from the well stirred peritubular solution by the whole length of the basolateral membrane infoldings (Fig. 6). We use also the simplifying assumptions of a linear gradient of K⁺ along the membrane infoldings.

Starting from a steady-state situation, where the inward K⁺ flux carried by the pump (J_K) pump is equal to the outward "leak" flux through the conductance, sudden blocking of the
Na-K pump produces the appearance of a net outward K⁺ flux equal to \(-J_k\) pump. We now use the Fick equation (Davson, 1970):

\[
\frac{ds}{dt} = -D \cdot A \frac{dc}{dx},
\]

where \(ds/dt\) expresses the rate of transport, \(D\) the diffusion coefficient, and \(dc/dx\) the K⁺ concentration gradient. \(A\) is the mean cross-sectional area of the extracellular spaces between the cells and within the basolateral membrane infoldings. From inspection of histologic pic-
tures of the *Amphiuma* collecting tubule (Stanton et al., 1984), we can see that the width of the lateral spaces and of the basolateral membrane invaginations is not negligible and that these spaces contain a sizeable amount of extracellular fluid; we assume that this cross-sectional area of the extracellular spaces ($A$) amounts at least to 1/100 of the peritubular surface area. Let us take, for example, a $K^+$ concentration gradient of 1 mM between the well-stirred bath and the deep end of the infoldings and a depth of the infoldings of 20 $\mu$m. For 1 cm$^2$ of tubular surface, ($A$ equal to 0.01 cm$^2$), with a $f_K$ pump equivalent to twice the pump current (2.60 $\mu$A/F = 1.3 nmol s$^{-1}$) and for a $K^+$ concentration gradient of 1 mM/20 $\mu$M (0.5 mol liter$^{-1}$ cm$^{-1}$), the Fick equation yields a value of $D$ amounting to $2.6 \times 10^{-7}$ cm$^2$s$^{-1}$. This value is two orders of magnitude lower than the diffusion coefficient of $K^+$ in free solution ($1.99 \times 10^{-5}$ cm$^2$s$^{-1}$ [Robinson and Stokes, 1959]). It is also about 10 times lower than estimates of the diffusion coefficient of $K^+$ in kidney slices ($2.3 \times 10^{-6}$ cm$^2$s$^{-1}$ [Proverbio and Whitembury, 1975]). Thus, the assumed 1 mM $K^+$ concentration gradient in the unstirred layers when the pump is blocked was greatly overestimated. As we have shown that an increase of the concentration of $K^+$ in the bath of 12 mM (from 3 to 15 mM) produced an inward $K^+$ current amounting to only a modest fraction (<20%) of the measured strophanthidin sensitive current, we may safely conclude that, under our experimental conditions, the potassium current flowing through the $K^+$ conductance and produced by the effect of strophanthidin on the $K^+$ concentration in the unstirred layers is negligible.

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**References**

Abercrombie, R. F., and P. De Weer. 1978. Electrical current generated by squid giant axon sodium pump: external potassium and internal ADP effects. *American Journal of Physiology.* 235:C63–C68.

Andersen, O. S., J. E. N. Silveira, and P. R. Steinmetz. 1985. Intrinsic characteristics of the proton pump in the luminal membrane of a tight epithelium. *Journal of General Physiology.* 86:215–234.

Avison, M. J., S. R. Gullans, T. Ogino, G. Giebisch, and R. G. Shulman. 1987. Measurement of Na$^+$-K$^+$ coupling ratio of Na$^+$-K$^+$-ATPase in rabbit proximal tubule. *American Journal of Physiology.* 253:C126–C136.

Borlinghaus, R., H.-J. Apell, and P. Läuger. 1987. Fast charge translocations associated with partial reactions of the Na-K-pump. I. Current and voltage transients after photochemical release of ATP. *Journal of Membrane Biology.* 97:161–178.

Boulpaep, E. L., and H. Sackin. 1979. Equivalent electrical circuit analysis and rheogenic pump in epithelia. *Federation Proceedings.* 38:2030–2036.

Chapman, B. J., E. A. Johnson, and J. M. Kootsey. 1983. Electrical and biochemical properties of an enzyme model of the sodium pump. *Journal of Membrane Biology.* 74:139–153.

Cox, T. C., and S. I. Helman. 1986a. Na$^+$ and K$^+$ transport at basolateral membranes of epithelial cells. I. Stoichiometry of the Na,K-ATPase. *Journal of General Physiology.* 87:467–483.

Cox, T. C., and S. I. Helman. 1986b. Na$^+$ and K$^+$ transport at basolateral membranes of epithelial cells. II. K$^+$ efflux and stoichiometry of the Na,K-ATPase. *Journal of General Physiology.* 87:485–502.
Cox, T. C., and S. I. Helman. 1986c. Na\(^+\) and K\(^+\) transport at basolateral membranes of epithelial cells. III. Voltage independence of basolateral membrane Na\(^+\) efflux. *Journal of General Physiology.* 87:503–509.

Dawson, H. 1970. A Textbook of General Physiology. Williams & Wilkins Co., Baltimore. 395–507.

Dawson, M. J., D. G. Gadian, and D. R. Wilkie. 1980. Mechanical relaxation rate and metabolism studied in fatiguing muscle by phosphorus nuclear magnetic resonance. *Journal of Physiology.* 299:465–484.

De Weer, P. 1984. Electrogenic pumps: theoretical and practical considerations. In Electrogenic Transport: Fundamental Principles and Physiological Implications. M. P. Blaustein and M. Lieberman, editors. Raven Press, New York. 1–15.

De Weer, P. 1985. Cellular sodium-potassium transport. In The Kidney: Physiology and Pathophysiology. D. W. Seldin and G. Giebisch, editors. Raven Press, New York. 31–48.

De Weer, P. 1986. The electrogenic sodium pump: thermodynamics and kinetics. In Progress in Zoology, Volume 33. H. C. Luttgau, editor. Gustav Fischer Verlag, New York. 387–399.

De Weer, P., and R. F. Rakowski. 1984. Current generated by backward-running electrogenic Na pump in squid giant axon. *Nature.* 309:450–452.

De Weer, P., R. F. Rakowski, and D. C. Gadsby. 1987. Current-voltage relationships for the electrogenic sodium pump of squid giant axon. *Biophysical Journal.* 51:385a. (Abstr.)

Ehrenfeld, J., F. Garcia-Romeu, and B. J. Harvey. 1985. Electrogenic active proton pump in Rana esculenta skin and its role in sodium ion transport. *Journal of Physiology.* 359:331–355.

Eisner, D. A., and W. J. Lederer. 1980. Characterization of the electrogenic sodium pump in cardiac Purkinje fibers. *Journal of Physiology.* 303:441–474.

Fendler, K., E. Grell, and E. Bamberg. 1985. Pump currents generated by the purified Na\(^+\)/K\(^+\)-ATPase from kidney on black lipid membrane. *EMBO (European Molecular Biology Organization) Journal.* 4:3079–3085.

Gadsby, D. C., J. Kimura, and A. Noma. 1985. Voltage dependence of Na/K pump current in isolated heart cells. *Nature.* 315:63–65.

Goldshlegger, R., S. D. J. Karlish, A. Rephaelia, and W. D. Stein. 1987. The effect of membrane potential on the mammalian sodium-potassium pump reconstituted into phospholipid vesicles. *Journal of Physiology.* 387:331–355.

Graf, J., and O. H. Petersen. 1974. Electrogenic sodium pump in liver parenchymal cells. *Proceedings of the Royal Society of London, Series B.* 187:363–367.

Harvey, B. J., and J. Ehrenfeld. 1986. Regulation of intracellular sodium and pH by the electrogenic H\(^+\) pump in frog skin. *Pflügers Archiv.* 406:362–366.

Horisberger, J.-D. 1989. The sodium pump current in renal tubular cells. *Current Topics in Membranes and Transport.* 34:253–267.

Horisberger, J.-D., and G. Giebisch. 1988a. Voltage dependence of the basolateral membrane in the *Amphiuma* collecting tubule. *Journal of Membrane Biology.* 105:257–263.

Horisberger, J.-D., and G. Giebisch. 1988b. Intracellular Na\(^+\) and K\(^+\) activities and membrane conductances in the collecting tubule of *Amphiuma.* *Journal of General Physiology.* 92:643–665.

Horisberger, J.-D., M. Hunter, B. A. Stanton, and G. Giebisch. 1987. The collecting tubule of *Amphiuma.* II. Effects of potassium adaptation. *American Journal of Physiology.* 253:F1273–F1282.

Hunter, M., J.-D. Horisberger, B. A. Stanton, and G. Giebisch. 1987. The collecting tubule of *Amphiuma.* I. Electrophysiological characterization. *American Journal of Physiology.* 253:F1263–F1272.
Jorgensen, P. L. 1986. Structure, function and regulation of Na,K-ATPase in the kidney. *Kidney International*. 29:10-20.

Kirk, K. L., D. R. Halm, and D. C. Dawson. 1980. Active sodium transport by turtle colon via an electrogenic Na-K exchange pump. *Nature*. 287:237-239.

Kushmerik, M. J. 1969. Free energy and enthalpy of ATP hydrolysis in the sarcoplasm. *Proceedings of the Royal Society of London, Series B*. 174:348-353.

Lafaire, A. V., and W. Schwarz. 1986. The voltage dependence of the rheogenic Na⁺/K⁺ ATPase in the membrane of oocytes of Xenopus laevis. *Journal of Membrane Biology*. 91:43-51.

Lewis, S. A., N. K. Wills, and D. C. Eaton. 1978. Basolateral membrane potential of a tight epithelium: Ionic diffusion and electrogenic pumps. *Journal of Membrane Biology*. 41:117-148.

Nakao, M., and D. C. Gadsby. 1986. Voltage dependence of the Na translocation by the Na/K pump. *Nature*. 323:628-630.

Nielsen, R. 1979a. Coupled transepithelial sodium and potassium transport across isolated frog skin: Effect of ouabain, amiloride and the polyene antibiotic filipin. *Journal of Membrane Biology*. 51:161-184.

Nielsen, R. 1979b. A 3 to 2 coupling of the Na-K pump responsible for the transepithelial Na transport in frog skin disclosed by the effect of barium. *Acta Physiologica Scandinavica*. 107:189-191.

Proverbio, F., and G. Whittembury. 1975. Cell electrical potentials during enhanced sodium extrusion in guinea-pig kidney cortex slices. *Journal of Physiology*. 250:559-578.

Rakowski, R. F., D. C. Gadsby, and P. De Weer. 1989. Stochiometry and voltage dependence of the sodium pump in voltage-clamped, internally dialyzed squid giant axon. *Journal of General Physiology*. 93:903-941.

Rakowski, R. F., and C. L. Paxson. 1988. Voltage dependence of the Na/K pump current in Xenopus oocytes. *Journal of Membrane Biology*. 106:173-182.

Robinson, R. A., and R. H. Stokes. 1959. Electrolytes Solutions. Butterworths, London.

Sackin, H., and E. L. Boulpaep. 1983. Rheogenic transport in the renal proximal tubule. *Journal of General Physiology*. 82:819-851.

Sansom, S. C., and R. G. O’Neil. 1986. Effects of mineralocorticoids on transport properties of cortical collecting duct basolateral membrane. *American Journal of Physiology*. 251:F743-F757.

Stanton, B. A., D. Biemesderfer, D. L. Stetson, M. Kashgarian, and G. Giebisch. 1984. Cellular ultrastructure of Amphiuma distal nephron: effects of exposure to potassium. *American Journal of Physiology*. 247:C204-C216.