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Effects of intracellular signals on Na^+/K^+-ATPase pump activity in the frog skin epithelium

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The effects of intracellular signals (pH_i, Na_i^+, Ca_i^{++}, and the electrical membrane potential), on Na^+ transport mediated by the Na^+/K^+ pump were investigated in the isolated *Rana esculenta* frog skin. In particular we focussed on pH_i sensitivity since protons act as an intrinsic regulator of transepithelial Na^+ transport (J_{Na}) by a simultaneous control of the apical membrane Na^+ conductance (g_{Na}) and the basolateral membrane K^+ conductance (g_K). pH_i changes which modify J_{Na}, g_{Na} and g_K, do not affect the Na^+ transport mediated by the pump as shown by kinetic and electrophysiological studies. In addition, no changes were observed in the number of ^{3}H-ouabain binding sites in acid-loaded epithelia. Our attempts to modify cellular Ca^{2+} (by using Ca^{2+}-free/EGTA Ringer solution or A23187 addition) also failed to produce any significant effects in the Na^+ pump turnover rate or the number of ^{3}H-ouabain binding sites. The Na^+ pump current was found to be sensitive to the basolateral membrane potential, saturating for very positive (cell) potentials and a reversal potential of -160 mV was calculated from *I-V* relationships of the pump. Changes in Na_i^+ considerably affected the Na^+ pump rate. A saturating relationship was found between pump rate and Na_i^+ with maximal activation at $Na_i^+ > 40$ mmol/l; a high dependence of the pump rate and of the number of ^{3}H-ouabain binding sites was observed in the physiological range of Na_i^+. We conclude that protons (in the physiological pH range) which act directly and simultaneously on the passive transport pathways (g_{Na} and g_K), have no direct effect on the Na^+/K^+ pump rate. After an acid load, the inhibition of J_{Na} is primarily due to the reduction of g_{Na}. This results in a reduction of Na_i and the pump turnover rate then becomes dependent on other pathways of Na^+ entry such as the basolateral membrane Na^+/H^+ exchanger.

Introduction

Intracellular pH (pH_i) has been reported to act as an intrinsic regulator of transepithelial Na^+ transport (J_{Na}) by controlling simultaneously and in parallel the apical membrane Na^+ conductance (g_{Na}) and the basolateral membrane K^+ conductance (g_K) [1]. Since the transepithelial Na^+ transport rate was found to follow the changes in g_{Na} and g_K, a simultaneous effect of pH_i on the Na^+/K^+-ATPase, responsible for the active step of transport, was suggested; this assumption was in accordance with the pH sensitivity of the pump activity in another tight epithelium, the rabbit urinary bladder [2,3]. However, there has been no direct experimental support for an effect of pH_i on the Na^+/K^+-ATPase pump in the frog skin epithelium. In the present study we investigated the effect of pH_i and of other intracellular signals such as Ca^{2+} and Na^+ on basolateral Na^+ transport mediated by the Na^+/K^+-ATPase, in order to elucidate the role of these signals in the coordination of passive and active transport processes involved in the net transepithelial Na^+ transport.

Different and complementary techniques for measurement of Na^+ transport via the Na^+/K^+ pump at the basolateral membrane including kinetic and electrophysiological approaches were developed in the isolated whole frog skin and on isolated epithelium. We also measured the number of Na^+-pump sites by specific ^{3}H-ouabain binding to the basolateral membranes.

Of the different intracellular signals tested, intracellular Na^+ alone produced an effect on the ouabain sensitive Na^+ transport and on the number of ^{3}H-ouabain binding sites, confirming the key role of this ion is modulating Na^+/K^+-ATPase activity. A model combining $[H^+]_i$ and $[Na]_i$ effects on the passive and active pathways is proposed to expiain the regulation

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of the transepithelial Na^+ transport through frog skin epithelium.

Materials and Methods

Frogs (*Rana esculenta*) were kept at 20°C under running tap water. In most experiments, the ventral part of the skin was separated into two symmetrical portions, (one to serve as a control) which were mounted as flat sheets (surface area 7 cm^2) in modified Ussing-type chambers with serosal and mucosal compartments of 7 ml. The isolation of the epithelium was obtained by treating the serosal side of the skin with 1.0 mg/ml collagenase (Worthington Biochemical Corp., St Louis, MO) at 30°C under a 10 cm hydrostatic pressure. Pieces of isolated epithelium using a combination of collagenase and hydrostatic pressure to detach the epithelium from the corium, as described previously [1], were then sandwiched between two nylon meshes (Millipore, USA) to maximise the exposed surface by avoiding curling and mounted in Ussing type chambers (surface area of 3.14 cm^2) or treated in beaker experiments (surface area of 1.12 cm^2).

Solutions

The control Ringer solution had the following composition (in mmol/l): NaCl 85; Na_2SO_4 12; KCl 2.5; $CaCl_2$ 2; $MgSO_4$ 2, Na_2HPO_4 2.5; KH_2PO_3 1.2 and glucose 11, pH 7.4; this solution was aerated. The 'acid Ringer' solution had the same composition as control Ringer but was gassed with a mixture of 5%CO_2/95%O_2 at pH 6.20. For the HCO_3^-/CO_2 Ringer solution, 24 mmol/l $NaHCO_3$ replaced the 12 mmol/l of Na_2SO_4 of the control Ringer and the solution was gased with a mixture of 5%CO_2/95%O_2, pH 7.40.

In nystatin experiments, the apical side of the skin was bathed in a saline solution designed to imitate the intracellular ion content (i.e. high K^+ concentration, very low Ca^{2+} concentration and low concentration of permeant anion) in order to maintain cell viability, since the cells equilibrate with the apical Ringer solution [4]. The nystatin bathing solution had the following composition: K_2SO_4 40 mM; $MgSO_4$ 3.2 mM; KH_2PO_4 1.2 mM; K_2HPO_4 2.9 mM; glucose 11 mM; sucrose 40 mM; Bes 10 mM. The final pH of this solution was adjusted to 7.40 with H_2SO_4 and the final amount of sodium controlled by addition of Na_2SO_4. The serosal side of the skin was bathed with control Ringer solution.

Ouabain-sensitive Na^+ transport

Transepithelial $^{22}Na^+$ fluxes were measured in nystatin-treated skins mounted in Ussing-type chambers. The skins were mounted in open circuit conditions or clamped at the desired electrical potential difference using an automatic voltage-current clamp (DVC 1000, WPI USA). Amiloride was added (10^{-4} mol/l) to the apical bathing solution prior to nystatin treatment (100 μg/ml) to block Na^+ transport through amiloride-sensitive Na^+ channels, so that the permeabilising effect of nystatin on the apical membrane could be assessed. $^{22}Na^+$ transepithelial fluxes were followed after addition of $^{22}Na^+$ (at a final concentration of 0.2 μCi/ml) to the apical Ringer solution. The cold side (serosal) was changed and collected after several 15-min sampling periods. The amount of $^{22}Na^+$ appearing on the cold side and that in aliquots (100 μl) of the apical Ringer solution were measured with a liquid scintillation counter (Packard Instrument Co., USA). Fluxes were calculated from the amount of radioactivity transferred per unit time and the specific radioactivity in the initial compartment. The difference between 'control' and 'ouabain' ($5 \cdot 10^{-4}$ mol/l) treated skins was used to calculate the ^{22}Na ouabain-sensitive fluxes expressed in nequiv. h^{-1} cm^{-2}.

A second procedure for measuring ouabain-sensitive $^{22}Na^+$ unidirectional fluxes was carried out on isolated epithelium which is equivalent to a short circuited preparation since the epithelium is isolated in beaker solution. In these experiments, 16 to 18 pieces (individual surface area of 1.13 cm^2) cut from the same isolated epithelium were loaded for 2 h in the presence of $^{22}Na^+$ (20 μCi/ml) in a K^+-free Ringer solution at 3°C in order to increase the Na^+ content of the epithelial cells. Epithelia were then washed at 3°C for 5 min in a K^+-free Ringer solution containing amiloride (10^{-3} mol/l) and furosemide (10^{-3} mol/l) in the presence or absence of ouabain ($5 \cdot 10^{-4}$ mol/l). The washout kinetics of $^{22}Na^+$ were then followed as a function of time (every 2 min for 20 min), in Na^+-Ringer solution containing amiloride and furosemide at 22°C, in the presence or absence of ouabain ($5 \cdot 10^{-4}$ M). Amiloride and furosemide served to block the apical Na^+ exit through Na^+ channels, Na^+ cotransporter and exchangers and to limit as much as possible the Na^+ efflux to the basolateral membranes (through the Na^+ pump). The analysis of the $^{22}Na^+$ efflux plotted as a function of time is that of a two compartment system corresponding to an extracellular space and a single cell Na^+ compartment. Considering the cell component, the unidirectional $^{22}Na^+$ efflux (J_{23}) was calculated as $J_{23} = 0.693\ A/t_{1/2}$ (where A represents the Na^+ pool and $t_{1/2}$ the half-time of the washout kinetics) and is expressed in nequiv. h^{-1} cm^{-2}. In the presence of ouabain J_{23} represents the Na^+ leak from the cell and in its absence J_{23} is the sum of this leak and of a ouabain-sensitive Na^+ efflux (J_{ouab}) assumed to be mediated by the Na^+/K^+-ATPase. The evaluation of the initial amount of ^{22}Na (Q_0) before washing the epithelium was calculated from linear regression analysis of the cell $^{22}Na^+$ washout component. The Na^+ pool (A) was estimated from the equivalent rela-

tionship between intra- and extracellular specific radioactivities and expressed as nequiv. cm^{-2}. The sodium pool(A) = $Q_0 \cdot A_t / Q_R \cdot S$ where Q_0 is the initial $^{22}Na^+$ radioactivity of the epithelium, A_R the amount of Na^+ of the Ringer solution, Q_R the ^{22}Na radioactivity of the corresponding Ringer solution and S the surface area of the epithelium. The cell sodium concentration was estimated from our measurements of the cell water content per cm^2 of tissue [5], and expressed in mmol/l.

^{3}H-Ouabain binding

The technique used for ^{3}H-ouabain binding to the split frog skin was derived from that of Cala et al. [6]. Briefly, pieces of epithelium of 1.13 cm^2 surface area (in beaker experiments) or of 1.54 cm^2 (when used in chambers) were mounted between two nylon meshes and bathed in the appropriate experimental solutions (specified in the text). The epithelia were then incubated for 30 min in a K^+-free Ringer solution containing 10^{-6} mol/l of cold ouabain and 10^{-7} mol/l ^{3}H-ouabain (3 μCi/ml). The choice of ouabain concentration and time of binding was a compromise between the need to saturate the Na^+/K^+-ATPase binding sites and the need to reduce to a minimum the non-specific ouabain binding sites. Although the non-specific binding at 10^{-6} mol/l ouabain is relatively small [6], we corrected for this binding by incubating the epithelia with $5 \cdot 10^{-4}$ mol/l ouabain and ^{3}H-ouabain (3 μCi/ml); furthermore, epithelia were washed in cold saccharose solution (240 mosmol/l) at 3°C for 15 min to eliminate extracellular fluid. The non-specific ^{3}H-ouabain binding measured in these conditions was less than 5% of the specific ^{3}H-ouabain binding.

The ^{3}H-ouabain binding was expressed in number of ouabain molecules bound to 1 cm^2 of epithelium surface area after correction for the non-specific binding. At the end of the washing period, the epithelia were placed in counting vials, extracted for 24 h by the addition of 1 ml NaOH (1 M) followed by neutralisation by 1 ml HCl (1 M) and counted after addition of 10 ml of scintillation liquid (Ready Safe, Beckman USA). Quench corrections were made for all samples using the external standard in the channels ratio mode and calculated from quench curves.

I-V relationship of the basolateral Na^+ pump

Microelectrode experiments were carried out under short-circuit conditions (SC) in which the spontaneous transepithelial electrical potential difference was clamped at zero volt with an automatic voltage clamp (Model VC 600, Physiologic Instrument, Houston, TX, USA). Impalements were performed from the basolateral side of the isolated epithelium with conventional KCl (1 mol/l) filled microelectrodes.

Current-voltage (I-V) relationships of the basolateral membranes were studied under SC with a computer program adapted from that described by Thomas et al. [7] and an Apple IIe computer for data storage and analysis. The *I-V* relationship of the basolateral membrane was obtained as follows: the total current across the tissue (I_t) at a given value of transepithelial potential (V_t) is the sum of transcellular current and shunt (paracellular) current I_s. When the tissue is short-circuited, i_t is transcellular since $I_s = 0$. At potentials $V_t \neq 0$, the cellular current component was calculated from amiloride-sensitive transcellular current (I_c). The *I-V* relationship of the Na^+ pump was obtained from the difference of the *I-V* curves recorded after inhibition of the Na^+ pump by perfusing the Ussing chamber with cold (4–6°C) and K^+-free medium and under control conditions perfusion with Ringer solution at room temperature. At any value of V_t, the basolateral membrane current I_b is given by $(I_b)_{V_t} = (I_c - I_c^{0k})_{V_t}$, where I_c is the amiloride-sensitive I_t measured 15 s after adding back K^+ to the bath (at 22°C), and I_c^{0k} is the amiloride-sensitive I_t measured in zero K^+ at 4°C. We make the assumption that the conductance of the pump (G_p) and its emf (E_p) are not affected over the time period (~ 1 s) it takes to generate the *I-V*, and are unchanged in the 15 s following readmission of K^+ to stimulate the pump. Thus at given V_t, the basolateral membrane pump current (I_p) is obtained from $(I_p)_{V_t} = (I_b)_{V_t} = (G_p \cdot V_b)_{V_t} - (G_p^{0k} \cdot V_b^{0k})_{V_t}$. In both conditions, Ba^{2+} (5 mmol/l) was present on the basolateral side to block the dominant K^+ channels.

Drugs

Amiloride was obtained from Merck, Sharp & Dohm, Research Laboratories, West Point, PA, USA, and ouabain and furosemide from Sigma Chemical Co. (St. Louis, MO, USA).

Results

Effects of intracellular acidification on the Na^+ pump transport rate and on the ^{3}H-ouabain binding

Two procedures of cell acidification were used: (1) substitution of a Ringer solution gassed in air (pH 7.40) by a Ringer solution equilibrated in a mixture of 5% CO_2, 95% O_2 (final pH 6.20) or (2) immersion of the epithelia in a Ringer solution containing 15 mmol/l of NH_4^+ for 15 min, followed by return into the control Ringer solution. These two procedures were found to decrease the cell pH by 0.4 pH unit and to partially block the transepithelial Na^+ transport [1]. An illustration of such a transepithelial Na^+ transport (J_{1s}) inhibition by NH_4^+ is given in Fig. 1; a 15 min period of serosal application of Ringer containing NH_4^+ (15 mmol/l) followed by reversal to normal Ringer solu-

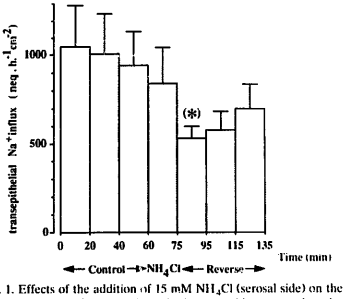

Fig. 1. Effects of the addition of 15 mM NH_4Cl (serosal side) on the transepithelial Na^+ influx through the frog skin mounted under open-circuit conditions. After 15 min of NH_4Cl treatment, the serosal Ringer solution is changed to the control Ringer solution, leading to an inhibition of the transepithelial Na^+ influx. The apical solution contained 115 mM of NaCl. Transepithelial fluxes were followed by the use of ^{22}Na (see Methods).

tion produced a prolonged partial inhibition of J_{13} (47 ± 17% inhibition after 20 min (* in Fig. 1) recovering from the ammonia load period, $P < 0.01$, $n = 9$ and 30% ± 11% inhibition after 40 to 60 min recovering, $P < 0.01$, $n = 9$). The transepithelial potential was 57 ± 9 mV during the control period, it decreased slightly during the NH_4^+ period (46 ± 8 mV), and returned to control values during the NH_4^+ washout period (58 ± 8 mV after 20 min) *.

The effects of cell acidification on the ouabain-sensitive Na^+ transport (J^{Na}_{ouab}) through the basolateral membranes of isolated epithelia were therefore investigated. Epithelia are spontaneously short-circuited since the apical and basolateral side are bathed in the same solution. Incubation of epithelia with a 5% CO_2 Ringer solution (pH 6.20) had no effect on J^{Na}_{ouab} or on the Na^+ leak (ouabain-insensitive Na^+ efflux) (Fig. 2A. Using the value for cell water content as 6.18 μl per cm^2 [5], the mean calculated Na^+ concentrations were not significantly different, 33.5 ± 8.5 mmol/l and 31.4 ± 9.7 mmol/l for control and acid-treated epithelia, respectively ($n = 5$).

The second acidification procedure of a NH_4^+ load followed by reversal to a normal Ringer solution gave a similar result: J^{Na}_{ouab} and the Na^+ leak were not affected by cell acidification (Fig. 2B). As in the preceding acidification technique, the mean cell Na^+ concentrations were identical in control (22.8 ± 4.8 mmol/l) and in NH_4^+ loaded epithelia (22.1 ± 4.1 mmol/l, n = 14).

With the $^{22}Na^+$ washout procedure, we pre-incubated the epithelia at 3°C with a K^+-free Ringer solution, in order to increase the cell Na^+ content by blocking the Na^+/K^+-ATPase; in previous experiments, the Na^+ content increase induced by such treatment was relatively variable from one group of epithelia to another. However, it is questionable whether an increase in cell Na^+ content would interfere with any effect of intracellular protons on the ouabain-dependent Na^+ efflux.

We therefore arbitrarily divided our results into two groups to analyse the data further; the first group had

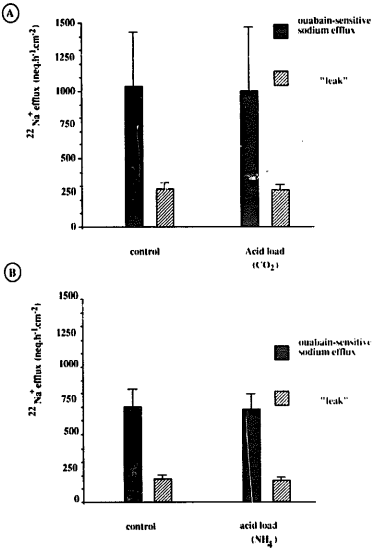

Fig. 2. Effects of two acid loading techniques (CO_2 and NH_4^+) on the ouabain-sensitive Na^+ effluxes and the Na^+ 'leak'. ^{22}Na efflux (expressed in nequiv. h^{-1} cm^{-2}) were measured by washout kinetics in the presence or absence of ouabain ($5 \cdot 10^{-4}$ mol/l); amiloride and furosemide were in both cases present in the Ringer solution (pH 7.4) to limit the ^{22}Na efflux to the Na^+ pump. Epithelia were acid loaded by incubation for 90 min in a 5% CO_2 Ringer solution pH 6.2 (A) or by addition of 15 mmol/l of NH_4Cl to the Ringer solution for 15 min prior to starting the ^{22}Na washout kinetics (B).

* Since the transepithelial voltage is little affected by acid load, increases in both cellular and junctional resistances must have occurred. The effects of intracellular acidification on apical and basolateral membrane resistances have been previously reported in detail [1]. Under open circuit condition junction resistance $R_j = V_t / J_{13}$ can be calculated to increase by 25% at most.

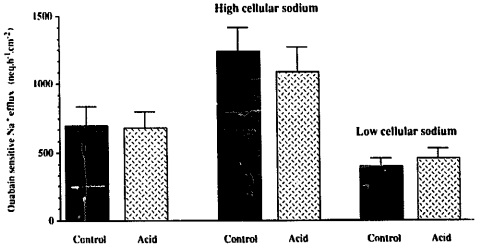

Fig. 3. The lack of effect of an acid load on the ouabain-sensitive Na^+ efflux is not dependent on the cell Na^+ content. Data from the two acidification techniques were pooled (first two columns, $n = 19$); data were then classified as epithelia with low Na^+ contents (< 12 mmol/l) or epithelia with high Na^+ contents (> 12 mmol/l).

a low Na^+ content ([Na] < 12 mmol/l, mean [Na], 10.5 ± 0.6 mmol/l, $n = 9$) and the second a high Na^+ content ([Na] > 12 mmol/l, mean [Na], 33.5 ± 8.5 mmol/l, $n = 10$). These epithelia were subjected at random to the first or second acidification procedure. From Fig. 3, it is apparent that the lack of effect of cell acidification on J^{Na}_{ouab} is not dependent on the cell Na^+ concentration.

Another experimental approach concerned intact frog skins treated with nystatin and mounted under open-circuit conditions. Nystatin permeabilizes the apical membranes of tight epithelia to monovalent cations [8,9] and with a 'cell-like' apical Ringer solution (high K^+, low Cl^- and Ca^{2+}) a 'one membrane' preparation, reflecting the basolateral membranes of the epithelium, can be obtained. The evolution of the transepithelial Na^+ influx (J_{13}) after nystatin addition to the apical solution (100 μg/ml) is given in Fig. 4. After blocking Na^+ transport by amiloride (10^{-4} mol/l), nystatin produced a rapid increase in the transepithelial Na^+ influx which remained stable over a long period of time (90 min). Subsequent addition of ouabain ($5 \cdot 10^{-4}$ mol/l) to the serosal solution reduced J_{13}, and a value for the ouabain-sensitive Na^+ influx was thus obtained. The transepithelial potential of nystatin-treated skins, dropped from 46 ± 5 mV to 30 ± 3 mV ($n = 6$) after ouabain addition, evidence of the electrogenicity of the pump.

We took advantage of the high CO_2 and low HCO_3^- permeabilities of the apical cell membranes to acidify the epithelial cells without changing the pH of the

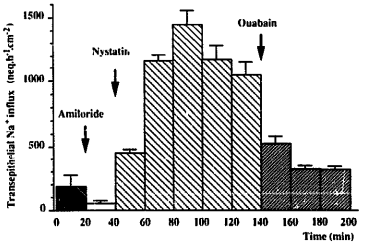

Fig. 4. Effect of nystatin and of ouabain on the transepithelial Na^+ influx, in frog skin mounted under open-circuit conditions. Nystatin (100 μg/ml) and ouabain (10^{-3} mmol/l) were added to the apical and serosal solution, respectively. The ouabain-sensitive Na^+ influx is taken to be the difference between the fluxes of nystatin-treated skins in the presence and absence of ouabain (at maximum inhibition).

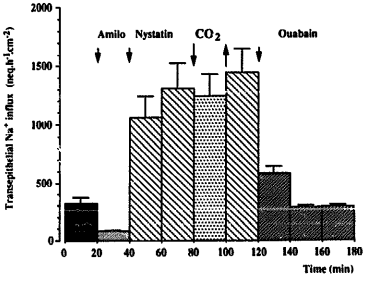

Fig. 5. Response of transepithelial Na^+ influx to substitution of control Ringer (HCO_3^--free Ringer solution equilibrated in air, pH 7.4) with Ringer gassed in 5% CO_2 containing 24 mM HCO_3^- (pH 7.4) on the apical side of nystatin-treated skins. Substitution, return to control, drug addition indicated by arrows. Frog skins under open-circuit conditions.

apical Ringer solution [1]. Thus, Na_2SO_4 (12 mmol/l) in the nystatin Ringer solution was replaced by $NaHCO_3$ (24 mol/l) and the Ringer solution gassed with 5% CO_2, keeping the pH constant at 7.40 (Fig. 5). Passing from the apical control solution to a HCO_3^-/CO_2-containing Ringer solution did not affect J_{13} (the difference between the CO_2/HCO_3^- period and the control period was 68 ± 71 nequiv. h^{-1} cm^{-2}, $n = 13$, n.s.); however, J_{13} increased slightly in the following reverse period (difference 218 ± 58 nequiv. h^{-1} cm^{-2}; $P < 0.0005$, $n = 13$).

Another approach was used to change the cell pH of nystatin-treated epithelia by varying the pH of the apical Ringer solution, since it has been reported that nystatin forms pores so permeable to univalent ions that the mobility of ions through the pore is almost the same as in water [10]. Fig. 6 illustrates the effect of apical pH changes on J_{13} measured on paired frog

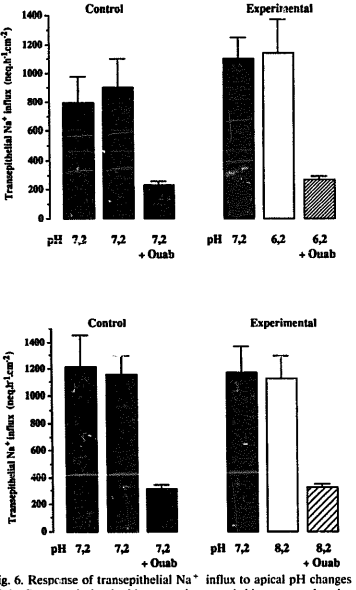

Fig. 6. Response of transepithelial Na^+ influx to apical pH changes of the Ringer solution bathing nystatin-treated skins mounted under open-circuit conditions. Upper part: changing from pH 7.2 to 6.2. Lower part: changing from pH 7.2 to 8.2. Each column is the mean of two 15-min periods.

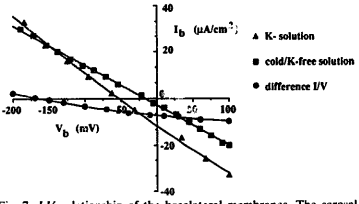

Fig. 7. *I-V* relationship of the basolateral membranes. The serosal side was perfused with a Ringer solution containing Ba^{2+} (10^{-4} M) to block the dominant basolateral membrane potassium conductance (g_K). A first *I-V* relationship of the basolateral membrane perfused with a cold (4°C), K^+-free Ringer solution was recorded. The serosal side was then perfused with a K^+-containing Ringer solution (3.5 mmol/l of K^+) and a second *I-V* relationship immediately recorded (15 s after perfusion with the K^+-containing Ringer solution). Amiloride (10^{-5} M) was finally added to the apical Ringer solution to block the apical Na^+ conductances and a third *I-V* relationship registered; the amiloride sensitive *I-V* relationships of the basolateral membranes perfusing with a cold, K^+-free Ringer solution (■) or with a K^+-containing Ringer solution (▲) were deduced. The basolateral *I-V* relations were determined at iso-transepithelial potentials. The pump current I_p (○) was taken as $(I_p)_{V_1} = (I_c - I_c^{0k})_{V_1} = (I_c)_{V_1} - (I_c^{0k})_{V_1^{0k}}$, $V_1 \# V_1^{0k}$. Abbreviations are given in Methods.

skins. Each column is the mean of two 15 min periods. Changing the pH of the apical Ringer solution from 7.20 to 6.20 or from 7.20 to 8.20 had no effect on the ouabain-sensitive Na^+ influx.

Microelectrode impalements of the basolateral membranes were performed on the isolated epithelium in order to measure the *I-V* relationship of the pump current associated with the Na^+/K^+-ATPase activity under control conditions and during cell acidification. The epithelium was perfused on the apical side with a normal Ringer solution and on the basolateral side with a Ba^{2+}-containing Ringer solution in order to block the dominant K^+ conductance. After 15 min perfusion with a cold, K^+-free serosal Ringer solution (with Ba^{2+}), the *I-V* relationship of the basolateral membranes was recorded. The serosal side was then perfused with a K^+-containing Ringer solution (with Ba^{2+}) at room temperature (22°C) and a second *I-V* relationship of the basolateral side registered. The transcellular component (I_b) of the transepithelial current was obtained using amiloride sensitive *I-V* relations. A typical experiment is presented in Fig. 7. The difference between the two I_b-V_b relationships, i.e. in the presence or absence of K^+ in the serosal perfusion solution was taken to be the *I-V* relationship of the Na^+ pump. The presence of Ba^{2+} in both situations effectively reduces K^+ transference to zero, a sign that the K^+ current through channels has been eliminated. A mean reversal potential of 164 ± 4 mV (serosa posi-

TABLE I

Effects of $[H]_i$, $[Ca]_i$ and $[Na]_i$ changes on the number of ^{3}H-ouabain binding sites

Number of molecules bound expressed as number of sites per cm^2 ($\times 10^{-12}$) (assuming one site for each molecule of bound ouabain). Na^+ expressed in mmol/l. In A and B conditions, epithelia were treated in beakers (short-circuit conditions) and in the C condition, epithelia are mounted in Ussing chambers in open-circuit conditions.

A	Control	Acid load	Alkaline load		Non specific	
	29.8 ± 3.0	30.4 ± 3.4	29.0 ± 3.2		1.1 ± 0.25	$n = 10$
B	Control	A23187	EGTA Ca^{2+}-free		Non specific	
	29.0 ± 7.1	22.2 ± 5.3	21.0 ± 3.2		1.3 ± 0.3	$n = 4$
C	0 (Na^+)	5 (Na^+)	10 (Na^+)	20 (Na^+)	Non specific	
	17.1 ± 2.1	23.6 ± 3.7	30.4 ± 3.6	28.4 ± 5.9	2.1 ± 0.9	$n = 11$

tive) and a maximum pump current of 6.6 ± 0.4 $\mu A/cm^2$ was obtained in 12 experiments. In order to acidify the cells, the epithelium was perfused on its apical side with a CO_2/HCO_3^--containing Ringer solution [1]. A representative experiment is presented in Fig. 8. The mean pump currents prior to and during acid-loading of seven epithelia treated by a similar protocol were 10.3 ± 0.6 $\mu A/cm^2$ and 9.3 ± 0.8 $\mu A/cm^2$, respectively, and the mean reversal potentials 161 ± 6 mV and 162 ± 2 mV for control and acid-loaded epithelia, respectively; these values were not significantly different (difference 1 ± 1.4 $\mu A/cm^2$ and 1 ± 5 mV).

We can therefore conclude from the two different kinetic approaches and from the electrophysiological study, performed independently on isolated epithelia or on nystatin-treated skins, that small pH_i changes (which can affect transepithelial Na^+ transport) have no significant effects on the ouabain-sensitive Na^+ fluxes.

The number of ^{3}H-ouabain binding sites was also determined on isolated frog skin epithelia treated in different acid-base conditions. In control conditions, we found a mean number of ^{3}H-ouabain binding sites of (28.0 ± 3.1) · 10^{11} sites/cm^2; $n = 22$. This value is similar to that found in the *Rana catesbiana* epithelium ((2.1–2.5) · 10^{12} sites/cm^2) by Cala et al. [6]. As can be seen from Table IA there were no significant differences between control, acid-loaded (NH_4^+-treated) and alkaline-loaded epithelia (HCO_3^--Ringer gassed with air, pH 8.2).

Effect of calcium on the Na^+ pump transport rate and on ^{3}H-ouabain binding sites

The possible role of Ca^{2+} as a regulator of epithelial Na^+ transport has recently been reviewed [1,13–16]. We therefore investigated the possible role of intracellular Ca^{2+} on ouabain-dependent Na^+ transport by incubating epithelia bathed on both sides in a Ca^{2+}-free Ringer solution containing 0.5 mmol/l EGTA or by addition of the A23187 ionophore (10^{-5} mmol/l, both sides) to the Ringer solution, in order to decrease or increase the cell Ca^{2+} content. Use of fura-2 as a Ca^{2+} probe confirmed this last statement (unpublished results).

Incubation of epithelia in Ca^{2+}-free Ringer solution containing 0.5 mmol/l EGTA produced a significant increase by 221 ± 58 nequiv. h^{-1} cm^{-2} ($P < 0.025$, $n = 6$) of the ouabain-insensitive Na^+ efflux (control 196 ± 29 nequiv. h^{-1} cm^{-2} and Ca^{2+} free 416 ± 70 nequiv. h^{-1} cm^{-2}), indicating that Ca^{2+} plays a role in maintaining cell membrane integrity or controls non selective cation permeability on the apical membranes as reported by Van Driessche and Zeiske [11]. Of the six epithelia studied, four showed an increase of the ouabain-dependent Na^+ efflux but owing to high individual variability, the mean J_{ouab}^{Na} did not significantly differ in the two experimental conditions (difference = 353 ± 353 nequiv. h^{-1} cm^{-2}; Fig. 9). However, the calculated cell Na^+ content of the Ca^{2+}-free and EGTA-treated epithelia, increased significantly ($P < 0.005$, $n = 6$) from 29.9 ± 10 mmol/l (control) to 38.8 ± 11 mmol/l in the Ca^{2+}-free experiments. These last data make interpretation of the results difficult since

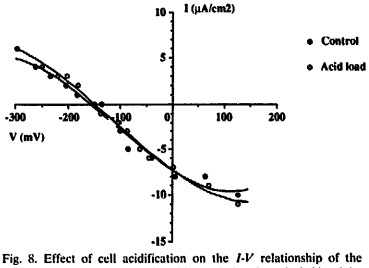

Fig. 8. Effect of cell acidification on the *I-V* relationship of the pump. Acidification was produced by perfusing the apical side of the skin with a CO_2/HCO_3^- Ringer solution. The same protocol as that reported in the preceding figure was used to determine the *I-V* relationship of the pump.

changes in Na^+ content affect the Na^+/K^+ pump activity, as will be discussed below. We therefore decided to increase the cell Ca^{2+} content by incubating the epithelia with the ionophore A23187. This treatment did not produce any significative change in the cell Na^+ content (control: 35.7 ± 7.2 mmol/l and A23187-treated epithelia: 41.22 ± 9.2 mmol/l, $n = 4$) and did not affect either J^{Na}_{ouab} (Fig. 9) or the Na^+ leak (control: 307 ± 64 nequiv. h^{-1} cm^{-2} and A23187-treated epithelia: 323 ± 66 nequiv. h^{-1} cm^{-2}, $n = 6$, difference 16 ± 24, n.s.). In addition, no differences were observed in the number of ^{3}H-ouabain binding sites in epithelia treated by the ionophore A23187 or with Ca^{2+}-free Ringer solution containing 0.5 mmol/l EGTA (Table IB) as compared with control epithelia.

Effect of intracellular [Na^+] content on the Na^+ pump transport rate and on ^{3}H-ouabain binding sites

It is apparent from the results presented in Fig. 3 and from a plot of the calculated Na^+ content as a function of ouabain-dependent Na^+ efflux (Fig. 10), that a relationship exists between cell Na^+ content and ouabain-dependent Na^+ transport. However, since in order to obtain accurate measurements of the $^{22}Na^+$ washout, Na_i^+ was increased by cold, K^+-free incubation of the epithelia, few values of the ouabain-dependent Na^+ efflux were available for epithelia of low cell Na^+ content. The 'nystatin technique' was therefore preferred to the $^{22}Na^+$-washout technique to analyse the dependence of the ouabain-sensitive Na^+ fluxes on cell Na_i^+. The transepithelial potential was clamped to +80 mV (serosal positive), a value expected to be close to the electrical potential of the basolateral membranes in short-circuited skins (since in nystatin treated epithelia, the apical membrane resistance is considerably reduced [8,9]). With this method, the Na^+ concentration of the apical Ringer solution is precisely controlled and can be expected to equilibrate with the cell Na^+ after nystatin-treatment of the apical membranes. A wide range of Na^+ concentrations was investigated with emphasis on the physiological Na^+ concentrations at the lower end of the range. Ouabain-insensitive Na^+ fluxes (Y) were found to have a linear relationship with Na_i. Similar relationships were found for 'nystatin-treated skins' ($Y = (6.4 \pm 0.4)[Na] + 16.1 \pm 11$, $r = 0.99$, $P < 0.001$) and for Na^+-washout experiments ($Y = (7.9 \pm 0.6)[Na] + 24.2 \pm 20$, $r = 0.85$, $P < 0.001$). On the other hand, the ouabain-sensitive Na^+ fluxes presented a saturating function (Fig. 11, upper part) with increasing Na^+ concentrations of the mucosal bathing solution (J^{Na}_{ouab} is maximal at Na^+ concentrations higher than 40 mmol/l. At the lowest Na^+ concentrations (inset of Fig. 11, upper part) which correspond to the normal cell Na^+ activity range (below 15 mmol/l [17,18]), the ouabain-sensitive transepithelial Na^+ fluxes displayed a sharp dependence on apical Na^+ concentration (expected to be identical to Na_i). It may be objected, however, that the reported values of Na_i (in particular for low Na_i) could be underestimated since Na^+ leak pathways at the basolateral membranes of the epithelium are present [5,12].

The number of ^{3}H-ouabain binding sites in the basolateral membranes of nystatin-treated epithelia (isolated epithelia mounted in Ussing chambers) was measured while changing the Na^+ concentration of the apical Ringer solution from 0 to 20 mmol/l Na^+; these Na^+ concentrations were chosen since the ouabain-sensitive transepithelial Na^+ fluxes were most sensitive to differences within this range. In the absence of Na^+ in the apical Ringer solution, the number of ^{3}H-ouabain sites was low, being little more than half the number

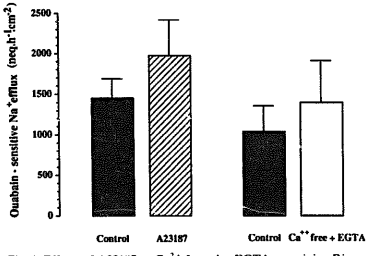

Fig. 9. Effects of A23187 or Ca^{2+}-free plus EGTA-containing Ringer solutions on the ouabain-sensitive Na^+ efflux. Prior to the $^{22}Na^+$ washout, epithelia were incubated for 90 min in a Ringer solution containing 10^{-5} mol/l A23187 (left part of the figure) or 90 min in a Ca^{2+}-free Ringer solution containing 0.5 mmol/l of EGTA (right of the figure). Epithelia in beakers (short-circuit conditions).

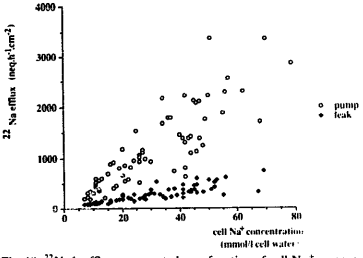

Fig. 10. $^{22}Na^+$ efflux represented as a function of cell Na^+ concentration. The ouabain-sensitive Na^+ efflux (pump, (○)) and the ouabain-insensitive efflux ('leak', (●)) were plotted as functions of the calculated Na^+ concentration (mmol/l cell water).

present at apical Na^+ concentrations of 10 or 20 mmol/l (Table 1C); an intermediate value of ^3H-ouabain binding sites was found at 5 mmol/l Na^+.

Effect of voltage on the ouabain-dependent Na^+ transport through nystatin-treated skins

Nystatin-treated skins were voltage-clamped from 0 mV to 120 mV, with a low (1 mmol/l) or high Na^+ concentration (40 mmol/l) in the apical Ringer solution (Fig. 12). The ouabain-sensitive Na^+ transport was largely independent of the clamping potential between 0 mV and 60 mV, but at higher clamping potentials, the $J_{13,ouab}$ was sensitive to the imposed potential (56% and 70% inhibition at 40 mmol/l and 1 mmol/ of Na^+, respectively, for a voltage clamp of 120 mV). It is of note that the approximate clamping potential extrapolated from the zero intercept of the abscissa (indicating a complete blockage of $J_{13,ouab}$) is close to the reversal potential of the pump measured with microelectrodes (164 ± 4 mV, $n = 13$).

Discussion

The effects of intracellular signals previously reported to modulate Na^+- and K^+-channel conduc-

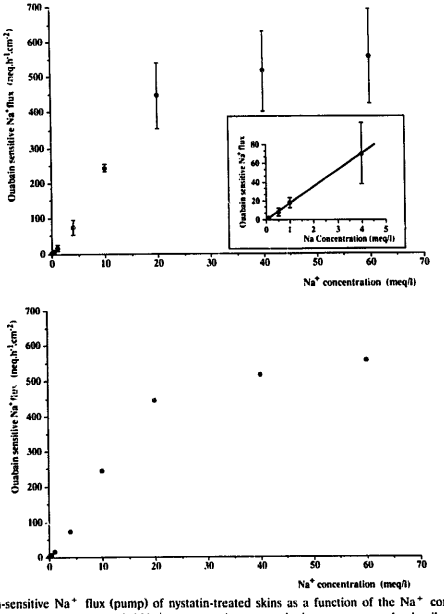

Fig. 11. Transepithelial ouabain-sensitive Na^+ flux (pump) of nystatin-treated skins as a function of the Na^+ concentration of the mucosal bathing solution. Upper part: data in the 0.1 to 60 mmol/l Na^+ concentration range; the inset represents the detail of the 0.1 to 4 mmol/l Na^+ range. Paired nystatin-treated skins were used in these experiments, one served as a control and the other was incubated with ouabain (10^{-3} mmol/l) on its serosal side. Each point is the mean of 4–10 different skins; four or five different Na^+ concentrations were tested in successive 15 min periods on each nystatin-treated skin. Lower part: Curve fitting of the data. The following equation was used for the Hill plot (dashed line): ouabain-sensitive Na^+ transport = $Min + (Max - Min)/(1 + ((Na/Na_{50})^{-n})$; the best fit was for $Na_{50} = 11.5 \pm 0.5$ mmol/l; $Max = 570 \pm 14$ nequiv. h^{-1} cm^{-2}; $Min = 8 \pm 7$ nequiv. h^{-1} cm^{-2} and $n = 2$ ($\chi^2 = 649$). The Langmuir equation ouabain-sensitive Na^+ transport = $V_{max}/((1 + Na_{50}/Na)^n)$ is shown as a solid line. It gave the best fit for $Na_{50} = 3.8 \pm 0.3$ mmol/l, $V_{max} = 685 \pm 26$ nequiv. h^{-1} cm^{-2} and, $n = 3$ ($\chi^2 = 2648$). 'P-fit' software (Biosoft, Cambridge, UK) was used to fit the experimental data to the reported equations.

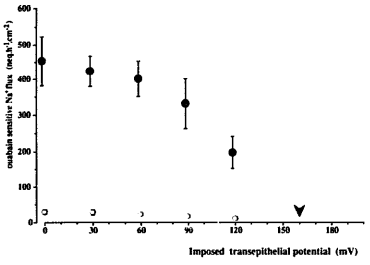

Fig. 12. Dependence of the ouabain-sensitive Na^+ fluxes on transepithelial voltage clamping potential. Nystatin-treated skins were clamped between 0 to 150 mV (serosal side positive) with low I mmol/l Na^+ (white circles) or 40 mmol/l Na^+ (black circles) in the apical bathing solution. The arrow represents the value calculated from the *I-V* relationship of the pump (164 ± 54 mV) determined with microelectrode impalements.

tances were tested on the ouabain-sensitive Na^+ transport mediated by Na^+/K^+-ATPase.

Our attempts to modify the intracellular Ca^{2+} content (utilisation of Ca^{2+}-free/EGTA-containing medium or A23187 addition) failed to detect any effect of Ca^{2+} on the rate of Na^+ transported by the pump or on the number of ^3H-ouabain binding sites.

The main aim of this study was to examine the effects of H^+ ions, since the passive Na^+ and K^+ conductances in the frog skin epithelium were found to be extremely sensitive of pH_i changes. In this epithelium, a sharp sigmoidal relationship was found between g_{Na} or g_K and pH_i with an apparent pK of 7.20 (1); a similar pH_i sensitivity of g_{Na} has also been found in other tight epithelia such as the toad urinary bladder and rat cortical collecting tubule [19,20], and of g_K in the diluting segment of the amphibian tubule and in turtle colon [21,22]. In the frog epithelium, we have determined the effects of intracellular H^+ on Na^+ pump activity over a range of pH_i (~ 0.4 pH_i units by CO_2 or NH_4^+ addition) which we have previously shown to block g_{Na} or g_K. The ouabain-sensitive Na^+ effluxes measured in isolated epithelia were unaffected by pH_i changes producing an inhibition of g_{Na}, g_K or transepithelial Na^+ transport (see Figs. 2 and 3). It could be argued that a Na^+/H^+ regulatory mechanism is stimulated during the ^{22}Na washout period, buffering any pH_i changes with consequently a similar pH_i in control and 'acidified' epithelia. This is most unlikely since: (1) amiloride (10^{-3} mol/l) was added to the Ringer solution during the ^{22}Na washout period to block the functioning of the Na^+/H^+ exchanger present on the basolateral membranes of the frog skin epithelium [5,12], (2) the NH_4^+-loading technique, induces a prolonged inhibition of the transepithelial Na^+ transport (see Fig. 1), and pH_i recovery is slow even when the Na^+/H^+ exchanger is not blocked by amiloride.

The effect of changes in the membrane potential has also to be taken into account. The reversal potential of the pump deduced from the *I-V* relationships or estimated from the extrapolation in 'nystatin experiments' was twice that reported in the toad bladder by Garty [9] who also used the nystatin approach. The value of − 160 mV which we found, is, however, much lower than that predicted (− 477 mV) from a pump with a reversal potential equal to $1/F \cdot G_{ATP}$ [23] with a G_{ATP} equal to − 11 kcal/mol (a reasonable value found in various cells). However, using physiological ionic activities (cell Na^+: 12 mequiv./l; cell K: 70 mequiv./l; serosal Na^+: 89 mequiv./l and serosal K^+: 2.9 mequiv./l) and a Na/K coupling ratio of 3:2, free energy − 11 kcal/mol ATP gives a reversal potential of − 160 mV at 23°C. This is close to the value found in our study. Recently, Wu et al. [24] found that the pump in *Rana* oocytes, depended directly on the membrane potential up to a plateau at 0 mV with interpolated zero current at − 165 mV.

Cell acidification induces an approximate 20 mV to 30 mV depolarisation of the basolateral membranes [1]; in acid-loaded epithelia, such a depolarisation would to a certain degree favour the Na^+ exit through the pump in view of its potential dependence (Figs. 7 and 12) and could compensate for possible partial inhibition by protons of the Na^+ pump. In the two other techniques used (nystatin treatment and the electrophysiological approach in which pH_i effects were studied over a range of ~ 400 mV), this difficulty was not present and in these studies, we also failed to detect significant effects of pH_i on the Na^+ transport mediated by the pump. Thus, small pH_i changes which greatly affect g_{Na}, g_K and transepithelial Na^+ transport rate have no direct effect on the Na^+ transport mediated by the Na^+ pump nor on the number of ^3H-ouabain binding sites. Since transepithelial Na^+ transport is the result of passive and active processes located at opposite barriers, other intracellular signals in addition to protons may be involved in regulating the Na^+ pump rate after pH_i changes. The Na^+ pump was found to be extremely sensitive to Na^+ ions. We found a sigmoidal saturating relationship between the ouabain-sensitive Na^+ flux and the $[Na^+]$ of the mucosal solution in equilibrium with Na_i^+ in nystatin-treated skins. On the other hand, the ouabain-insensitive Na^+ flux displays a linear relationship as a function of Na^+. We analyzed the Na^+ pump data presented in Fig. 11 according to multiple site interaction formulae (Hill plot), and the Langmuir equation (see Ref. 4). The best fit (see Fig. 11, lower part) was

obtained with the Hill plot which gave a maximum Na^+ transport rate * of 570 nequiv. h^{-1} cm^{-2}, with a K_{Na} (Na_i leading to half-maximal flux) of 11.6 nequiv./l and a number of multiple site interactions of 2.02 ± 0.18 ($\chi^2 = 649$). The best fit obtained with the Langmuir equation gave a number of equivalent binding sites for Na^+ ions of 3, with a K_{Na} of 3.8 nequiv./l and a maximum Na^+ transport of 685 nequiv. h^{-1} cm^{-2}, with a number of multiple site interactions of 3 ($\chi^2 = 2649$). The maximum current found from the *I-V* relationship of the pump was 6.6 $\mu A/cm^2$ (246 nequiv. h^{-1} cm^{-2}). From these two maximum Na^+ transport rates found in our $^{22}Na^+$ flux studies (570 nequiv. h^{-1} cm^{-2} and 685 nequiv. h^{-1} cm^{-2}, mean 650 nequiv. h^{-1} cm^{-2}), we calculated a ratio of 3 Na^+ in exchange for 2 K^+. A ratio of 1.5 is generally found at physiological ion concentrations in frog skin [25], rabbit urinary bladder [26], turtle colon [27] and rabbit colon in symmetrical Na^+ and K^+ solutions [28]. The presence of multiple Na^+ sites on the ATPase at the inner surface of the membrane (generally three sites) has been reported in a number of epithelial and nonepithelial cells [4,29–31]. A similar saturating relationship was also reported in the rabbit urinary bladder [4,32], *Necturus* gallbladder [33] and the cortical collecting tubule [34].

Cellular Na^+ activity (a^i_{Na}) of the frog skin epithelium, measured with ion-selective microelectrodes, varied from 6 mmol/l to 12 mmol/l with low (2 mequiv./l) and high (115 mequiv./l) Na^+ concentrations in the apical bathing solution respectively [17,18,25] and was reduced to very low values (1 mmol/l) with amiloride or phenamil in this solution [5,17,18,36]. The 'physiological range' of a^i_{Na} is therefore certainly below 12 mmol/l, a value at which the Na^+ pump is far from saturation and extremely sensitive to Na^+ variations. A similar observation was made for the cortical collecting tubules of rabbits in which the pump was found to operate far below its maximal activity in physiological intracellular concentrations [34]. In addition, we found that the number of ^3H-ouabain-binding sites was a function of the cell Na^+ content and increased 2-fold when the apical Ringer solution bathing nystatin-treated epithelia passed from a Na^+ concentration of 0 mmol/l to one of 10 mmol/l. A low ^3H-ouabain-binding site at a low cellular Na^+ has already been reported by Cala et al. [6] in the bullfrog *Rana catesbiana*. Insertion of new Na^+ pumps into the basolateral membranes from an intracellular pool or a higher turnover rate of already existing pumps with increased a^i_{Na} could both explain our results. A direct effect of a^i_{Na} on the affinity of the ouabain-binding site of the Na^+ pump, reported in cultured chick cardiac myocytes [37], is also a possible explanation.

We previously found [12] that a^i_{Na} was reduced from 11 ± 2 mequiv./l to 5 ± 2 mmol/l when cells were acidified from a pH of 7.23 to 6.85 (0.4 pH unit changes). In view of the insensitivity of the Na^+ pump to protons (over the pH_i range studied here), the blocking of the transepithelial Na^+ transport can be explained as follows: in acid load conditions, the Na^+ conductance of the apical membranes is blocked, resulting in an immediate drop of a^i_{Na} and of the Na^+ pump turnover rate (in view of the Na^+ dependence of the pump). Thus if a proton represents a direct intracellular mediator for 'cross-talk' between opposite membranes (acting directly on Na^+ and K^+ channels), a^i_{Na} which is highly dependent on the apical g_{Na}, could modulate the functioning of the pump. With prolonged acidification, the 'switch on' of a Na^+/H^+ exchanger located on the basolateral membranes increases the Na^+ pool (which however remains smaller than in control acid-base conditions; [5]). Therefore, the Na^+ pump, in prolonged acid conditions, by increasing its turnover rate (which is far from saturation) regulates a^i_{Na} and maintains the Na^+ gradient necessary for the functioning of the Na^+/H^+ exchanger. A schematic model of the coordination of passive and active transport in the frog skin epithelium following acid-base disturbances is given in Fig. 13.

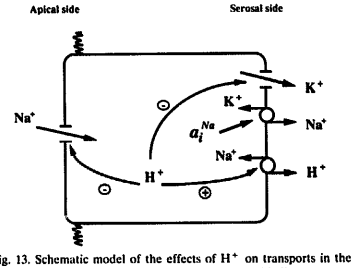

Fig. 13. Schematic model of the effects of H^+ on transports in the frog skin epithelium. After an acid load of the epithelium, protons inhibit the Na^+ and K^+ conductive pathways and stimulate the Na^+/H^+ exchanger located on the basolateral membrane. At first, the a^i_{Na} decreases as a result of the blockage of the Na^+ channels and Na/K activity is therefore reduced. With prolonged acidification the Na^+/H^+ exchanger is stimulated, leading to an increase of a^i_{Na}. Thus the Na^+/K^+-ATPase pump which is very sensitive to a^i_{Na} variations increases its turnover rate, regulating a^i_{Na} and maintaining the Na^+ gradient for the necessary functioning of the Na^+/H^+ exchanger.

* Maximum Na^+ transport rates are lower in nystatin-treated tissues compared to intact whole skins. This may be due to low permeability of the accompanying anion (SO_4^{2-}) in addition to possible unknown perturbations of the cytosol by the 'intracellular like' nystatin bathing solution.

Acknowledgments

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