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THE KEY ROLE OF THE H^+ V-ATPase IN ACID–BASE BALANCE AND Na^+ TRANSPORT PROCESSES IN FROG SKIN

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Summary

Frogs are faced with various osmoregulatory problems, such as compensation of salt and water loss or metabolic acidification. Being exposed both to air and to pond water of low salinity in their natural habitat, the epithelium of the frog skin serves as one of the major organs for body fluid homeostasis. For years, the frog skin has been the guiding model for ion transport processes in animal cells energized by a Na^+-motive force. Meanwhile, however, it was demonstrated that under natural conditions Na^+ uptake is electrically coupled to active H^+ secretion, mediated by an electrogenic H^+ pump. A proton-motive force generated at the apical membrane of the mitochondria-rich cells (MR cells) energizes Na^+ entry *via* apical Na^+ channels. The basolateral Na^+/K^+ P-ATPase then pumps Na^+ out of the cell into the body fluid. Thus, there are two pumps functioning in series, both involved in transepithelial Na^+ transport.

Our recent investigations provided conclusive evidence that the H^+ pump of the frog skin is an H^+ V-ATPase. In transport studies, Na^+ absorption and H^+ secretion were blocked by micromolar concentrations of bafilomycin A_1 or concanamycin A, two highly specific inhibitors of H^+ V-ATPases. Using immunofluorescence microscopy, H^+-V-ATPase-like immunoreactivity was found in MR cells in the region of their apical membrane foldings and intracellularly in the apical portion of the cell at so far unidentified locations. Besides the definition of its molecular nature, these results also confirmed the localization of the H^+ pump in the apical membrane of the MR cells. These cells were already candidates for H^+-V-ATPase localization mostly from correlations between their morphological features and their epithelial H^+ secretion capacity. So far, there is evidence for only one type of MR cell serving both H^+ and HCO_3^- secretion through an apical Cl^-/HCO_3^- antiporter.

H^+-V-ATPase-mediated H^+ secretion and thus Na^+ absorption can be modulated by complementary mechanisms. Changes in intracellular H^+ concentration linked to the animal's acid–base status will directly influence H^+ V-ATPase activity. Acute acidification increases H^+ current, probably as a result of the insertion of H^+-V-ATPase-bearing vesicles by exocytotic processes, while alkalization causes the reverse effects. Chronic metabolic acidosis induces an increase in MR cell number in response to hormonal signals.

Key words: H^+ pump, H^+ V-ATPase, epithelia, immunolabelling, Na^+ transport, frog skin.

Na^+ absorption by the Na^+-motive force

As amphibians, living frogs are exposed both to air and to low-salinity pond water. In the aquatic environment, they tend to loose ions, mainly Na^+ and Cl^-, from their body surfaces into the external milieu because of the large chemical gradients. In addition, they are faced with osmoregulatory problems caused by metabolic acidification. The frog skin evolved as a highly efficient transport epithelium for water and ions, and functions as one of the major organs involved in body fluid homeostasis. The stratified transport epithelium belongs to the tight epithelia (Whitear, 1975; Lindemann and Voute, 1979): the stratum germinativum is situated basally and generates the cells of the stratum spinosum by mitotic division. The spiny appearance is caused by numerous desmosomal contacts linked intracellularly to bundles of intermediate filaments which provide mechanical stability. From these two cell layers, the two types of polarized cells of the stratum granulosum develop, granular cells (GR cells) and flask-shaped mitochondria-rich cells (MR cells), connected to one another by tight junctions. Most of these cells are considered to form a functional syncytium; it has been difficult, however, to demonstrate the presence of gap junctions in these strata (Farqhar and Pallade, 1965). MR cells represent less than 6 % of the total epithelial cell population (Ehrenfeld *et al.* 1976) but are estimated to contribute as much as 60 % of the exposed apical transporting area because of numerous apical membrane foldings (Ehrenfeld *et al.* 1989). The transport properties of this layer determine the specific barrier functions of the epithelium. The epithelium is covered on the outside by dead cells, the stratum corneum, which are moulted periodically.

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The frog skin had been the guiding model for ion transport processes in animal cells (Koefoed-Johnsen and Ussing, 1958). The early history of ion transport studies started with Du Bois-Reymond (1848), who first reported that the frog skin was able to produce an electrical current. More than 50 years ago, pioneering work described the basic mechanisms of ion absorption and ion secretion using an external medium mimicking the physiological environment, showing that (i) salt-depleted frogs can absorb NaCl through their skin from solutions as low in NaCl concentration as 10 μmol l^{-1} (Krogh, 1937, 1938), (ii) *in vitro* frog skin can absorb Cl^- and water, depending on the supply of metabolic energy (Huf, 1935*a*,*b*) and (iii) the net influx of radioactive $^{24}Na^+$ was found to be larger than the outward flux (Katzin, 1939). These mechanisms were described in terms of net balances and couplings between ion transports since at this time no information concerning the type of transporters or the precise links between them was available. (For a more detailed review of the early discoveries, see Lindemann and Voute, 1979.)

The question of the origin of the ion-motive force was answered by the elegant experiments of Ussing and Zerahn (1951), who introduced the short-circuit current as a convenient measure of active ion transport. When skins were bathed on both sides with similar isotonic Ringer's solutions (with a high Na^+ concentration) to exclude chemical gradients and when the spontaneous transepithelial voltage was clamped at 0 mV to avoid electrical gradients, a clamping current (called the short-circuit current) could be measured and was equivalent to the net apical-to-basal flux of $^{24}Na^+$. Thus, they concluded that there must be a Na^+ pump which actively absorbs Na^+ into the blood. Koefoed-Johnsen and Ussing (1958) then explained the spontaneous transepithelial voltage, which largely depends upon apical Na^+ concentration, in terms of apical Na^+ and basolateral K^+ diffusion potentials arranged in series. In this two-barrier model, Na^+ enters the cell by diffusion through the apical membrane, driven by a basolateral Na^+/K^+ P-ATPase which actively pumps out Na^+ and thus keeps cellular Na^+ concentration low. In parallel, K^+ is pumped actively into the cell and cellular K^+ concentration is kept high; K^+ then exits the cell passively across the basolateral membrane. Thus, K^+ cycles and does not contribute to the short-circuit current. The passive apical Na^+ entry and active basal Na^+ extrusion together explain why all short-circuit current is carried by Na^+ under these symmetrical 'Ussing' conditions with apically high Na^+ concentration ('high-Na^+ conditions').

The molecular nature of the ion transporters was unknown at that time. The Na^+/K^+ P-ATPase was first characterized by Skou (1957). Goldin (1977) later purified and reincorporated the pump into artificial lipid bilayer membranes and demonstrated the uphill transport of Na^+ and K^+ in opposite directions at the expense of ATP hydrolysis. The amiloride-sensitive pathway was conclusively demonstrated to be an ion channel by Lindemann and van Driessche (1977).

H^+ secretion by an electrogenic H^+ pump

For years, the 'frog skin model' served as a general model for energization of animal plasma membranes by a Na^+-motive force driving transepithelial transport of solutes and ions. Despite the success of this model and its wide applicability to animal cells and epithelia, it remained an open question as to how frogs can absorb Na^+ under natural conditions, when their skin is exposed to very dilute saline and transepithelial voltages range between 40 and 100 mV. Na^+ concentration in fresh water is as low as 0.1 mmol l^{-1} compared with approximately 105 mmol l^{-1} in blood. The answer was provided by the discovery of an apical H^+ pump energizing the apical plasma membrane and allowing Na^+ entry through this barrier (see below). In this article, we will concentrate on the nature of this H^+ pump, its properties, its functional mechanisms and its involvement in Na^+ absorption and acid–base balance.

In vivo experiments (Krogh, 1938; Garcia-Romeu *et al.* 1969; Garcia-Romeu and Ehrenfeld, 1972, 1975*a*; Kirschner *et al.* 1973) and experiments with isolated frog skin (Garcia-Romeu and Ehrenfeld, 1975*b*; Ehrenfeld and Garcia-Romeu, 1977) revealed that Na^+ and Cl^- uptake are independent transport processes, one being the exchange of Na^+ for secreted H^+ in a 1:1 stoichiometry (Fig. 1) and the other being the exchange of Cl^- for secreted HCO_3^- (or OH^-). H^+ secretion like that occurring through the *in vivo* frog skin still takes place *in vitro* if minimal conditions are met: i.e. (i) the basal bathing solution, mimicking plasma conditions, must contain HCO_3^-/CO_2; (ii) the apical bathing solution must be Cl^--free to avoid HCO_3^- secretion mediated by Cl^-/HCO_3^- exchange (HCO_3^- secretion would reduce the H^+ secretion rate since both ions could combine to form volatile CO_2); (iii) the apical bathing solution must be equilibrated with air to eliminate CO_2 (originating from cell metabolism or the blood), which would diffuse according to its concentration gradient. NH_3 (or NH_4^+) loss was found to be negligible and did not affect measurement of H^+ secretion (Garcia-Romeu *et al.* 1969). Under these experimental conditions, an H^+ secretion of 140–230 nequiv h^{-1} cm^{-2} was measured in the open-circuit mounted frog skin, which exhibited a mean transepithelial voltage of +30 mV (blood side positive; Ehrenfeld and Garcia-Romeu, 1977). The capacity for H^+ secretion in isolated skin *in vitro* is similar to that of a similar surface area of frog skin *in vivo*.

The main feature of H^+ secretion is its electrogenicity. The H^+ current contributes significantly to the short-circuit current measured in frog skin absorbing Na^+ from a dilute saline (Ehrenfeld and Garcia-Romeu, 1977). Blockers of H^+ secretion (see below) induce transepithelial hyperpolarizing effects (Ehrenfeld and Garcia-Romeu, 1977). In addition, when transepithelial Na^+ transport is inhibited by amiloride, the short-circuit current is carried mainly by H^+, since this current is correlated to H^+ secretion as measured by titration of the apical bathing solution (Ehrenfeld *et al.* 1985). In its electrogenicity, the frog skin H^+ pump resembles the H^+ pump of the turtle urinary bladder, another potent H^+-secreting epithelium taken as a model for renal epithelia (Steinmetz and Andersen, 1982).

Na^+ absorption by a proton-motive force

Information about the coupling mechanism linking Na^+ and

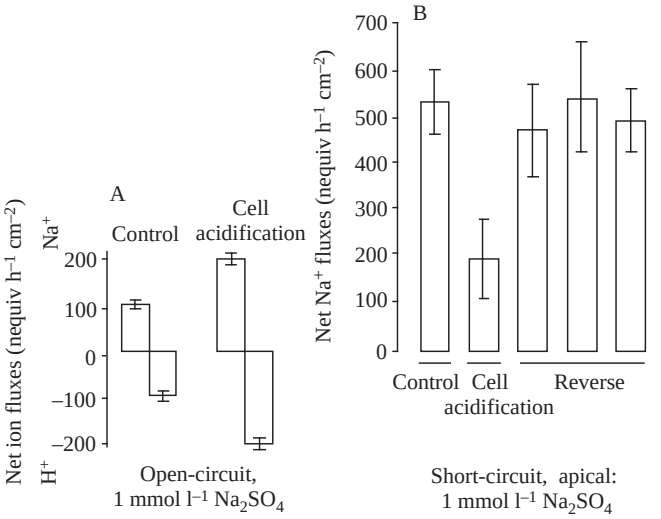

Fig. 1. Effect of cell acidification on Na^+ transport. Measurements were made on isolated frog skins mounted in a chamber under 'low-Na^+ conditions'. Apical solution, 1 mmol l^{-1} Na_2SO_4; basal solution, 115 mmol l^{-1} Na^+ and a HCO_3^-/CO_2-containing Ringer, pH 7.34. Na^+ absorption was measured by flame photometry, fluxes are drawn upwards; H^+ secretion was measured by titration, fluxes are drawn downwards. Cell acidification was achieved by changing the basal bathing solution from the normal Ringer to a HCO_3^--free Ringer, gassed with 5% CO_2 (pH 6.2). In open-circuit and 'low-Na^+ conditions', net Na^+ absorption is electrically linked to net H^+ secretion. Cell acidification stimulated H^+ secretion and thus the H^+ pump which, in turn, caused an increase in Na^+ absorption (A); data from Ehrenfeld *et al.* (1989). In short-circuit conditions (B), Na^+ transport is uncoupled from H^+ secretion and the Na^+ transport is driven by the Na^+-motive force. Cell acidification blocked Na^+ absorption in a reversible manner by a direct inhibiting effect on the apical membrane Na^+ permeability. In B, the columns represent the mean of the net fluxes of consecutive 30 min periods. Values are means ± S.E.M. N=6 for both A and B.

H^+ transport came from the observation that acetazolamide, a potent carbonic anhydrase inhibitor, blocked Na^+ absorption by frog skins mounted in open-circuit conditions but not in short-circuit conditions, while H^+ secretion was blocked in both cases (Ehrenfeld and Garcia-Romeu, 1977). Furthermore, the rates of transepithelial Na^+ and H^+ transport varied in opposite directions when the tissue was clamped over a large range of transepithelial voltages (Ehrenfeld *et al.* 1985). These findings imply that the two pathways are independent and that their coupling is indirect. They clearly exclude a direct coupling by a unique transporter such as a Na^+/H^+ antiporter. The functioning of such an antiporter in the apical membrane was in any case very unlikely for thermodynamic reasons. It was therefore proposed that the H^+ pump facilitated Na^+ entry at the apical membrane (Ehrenfeld and Garcia-Romeu, 1977; Ehrenfeld *et al.* 1985). Quantitative information about the intracellular ionic activities of Na^+ and H^+ and measurements of potential differences across single membranes permitted the calculation of the electrochemical gradients for both ions (Ehrenfeld *et al.* 1985). The contribution of the H^+ pump to the membrane potential across the apical membrane (20–30 mV, cell negative) was found to be essential for Na^+ entry from dilute saline. A revised frog skin model was therefore proposed to account for transepithelial Na^+ absorption under these conditions (Fig. 2). In this model, two ion pumps function in series; an electrogenic H^+ V-ATPase in the apical membrane of the MR cells establishes a favourable electrochemical gradient for Na^+ entry, while the electrogenic Na^+/K^+ P-ATPase in the basolateral membrane keeps cellular Na^+ concentration low and drives Na^+ from the cell to the body fluid. Thus, it is the activity of the H^+ pump that determines the rate of Na^+ absorption and not the apical Na^+ permeability, even when the latter is increased by exposure to neurohypophyseal hormones (see Fig. 6; Ehrenfeld *et al.* 1989). Na^+ entry is thought to take place through amiloride-sensitive Na^+ channels in MR and GR cells (Larsen *et al.* 1987; Harvey, 1992), although the participation of each of the two cell types is still a matter of debate. H^+ secretion is performed by an active electrogenic H^+ pump, which is thought to be localized only in the apical membrane of the MR cells (see below).

The molecular nature of the H^+ pump

H^+ secretion by the frog skin depends upon ATP and is totally blocked by incubation in an oxygen-free solution, indicating that the energy source for active H^+ transport is oxidative metabolism (Fig. 3; Ehrenfeld *et al.* 1985). In contrast, the turtle urinary bladder is capable of using either aerobic or anaerobic metabolism for fuelling active H^+ transport (Schwartz and Steinmetz, 1977). Drug sensitivities influencing transepithelial H^+ transport activity (H^+ secretion or H^+ current) are not good indicators of the molecular nature of the H^+ ATPase, since they may affect it indirectly by depletion of ATP. Moreover, the specificity of the effective drugs may be ambiguous, since some of them are known to act on more than one kind of ion-motive ATPase.

Ion-motive ATPases are classified into three groups according to their molecular structure and pharmacological properties (Pedersen and Carafoli, 1987): H^+ F-ATPases (ATP synthases) are specifically inhibited by sodium azide, P-ATPases by vanadate, acting as a phosphate analogue in the E_1E_2 reaction cycle (Pedersen and Carafoli, 1987), and H^+ V-ATPases by bafilomycin A_1 and concanamycin A (Bowman *et al.* 1988; Dröse *et al.* 1993). The latter two drugs are known to interfere with the membrane V_o sector of H^+ V-ATPases (Zhang *et al.* 1994). H^+ V-ATPases are ubiquitous in endomembranes of acidic organelles, 'vacuoles' such as lysosomes, but they are also found in the plasma membranes of certain ion-transporting epithelia such as renal proximal tubule, turtle urinary bladder and caterpillar midgut (Harvey and Wieczorek, 1997; Merzendorfer *et al.* 1997).

H^+ secretion by frog skin is inhibited by dicyclohexylcarbodiimide (DCCD), diethylstilboestrol (DES) and oligomycin (Fig. 3; Ehrenfeld *et al.* 1985, 1990). The latter is known to block ATP synthesis and thus ATP supply, while

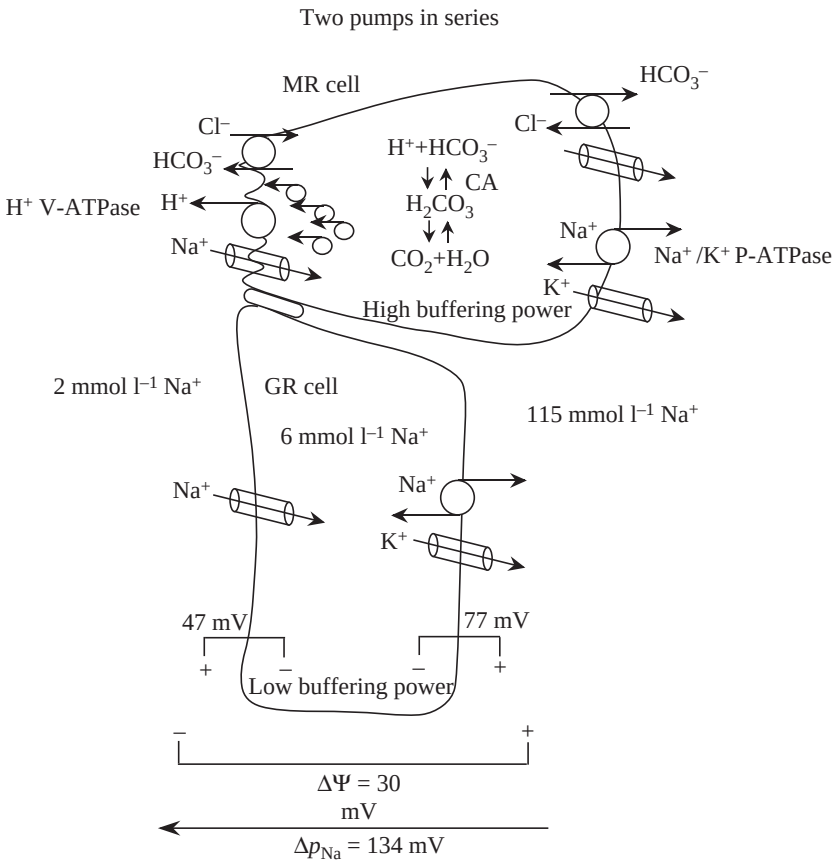

Fig. 2. Functional model of Na^+ uptake in frog skin epithelium under 'low-Na^+ conditions': two ion pumps working in series. In this schematic view, the outermost living cell layer of the frog skin epithelium is depicted, showing the two main cell types: mitochondria-rich cells (MR cell) and granular cells (GR cell) and the positions of the relevant membrane transporters. Transepithelial H^+ excretion is performed by an apical electrogenic H^+ V-ATPase in the MR cells in series with a basolateral Cl^-/HCO_3^- exchanger. The electrogenic H^+ V-ATPase hyperpolarizes the apical membrane, thus facilitating passive electrodiffusion of Na^+ into the cells by Na^+ channels present in both cell types; Na^+ is finally pumped out of the cells by the Na^+/K^+ P-ATPase located in the basolateral membranes. Different physiological conditions of acidosis or alkalosis modulate the number of H^+ pumps, probably by endo/exocytosis of endosomes storing H^+ V-ATPase, leading to an increase or decrease in the rate of H^+ secretion. Acute cell acidification induces inhibition of Na^+ channels in GR cells, but not in MR cells, in which the high buffering capacity of the carbonic anhydrase/HCO_3^- system 'protects' the Na^+ channels. With a high NaCl concentration in the apical bathing solution ('Ussing condition') or in short-circuit conditions, the H^+ pump does not facilitate Na^+ transport, which then is driven by the favourable electrochemical gradient and which passes mainly through GR cells. Active Cl^- absorption from diluted solutions is performed *via* a second Cl^-/HCO_3^- exchanger in the apical membrane of the MR cells. Transepithelial Cl^- fluxes which accompany Na^+ transport may occur through MR cells *via* concentration- and voltage-gated Cl^- channels (Larsen *et al.* 1987) or *via* paracellular shunt pathways. CA, carbonic anhydrase; $\Delta\Psi$, transepithelial potential and Δp_{Na}, electrochemical driving force.

the first two are known to block H^+ transport by H^+-ATPases in general, H^+ F-ATPases, H^+ P-ATPases and H^+ V-ATPases (Pedersen and Carafoli, 1987). *N*-ethylmaleimide (NEM), an alkylating agent that binds to sulphydryl groups, was also found to block H^+ secretion completely in frog skin at a concentration of 1 mmol l^{-1} (Fig. 3; Ehrenfeld *et al.* 1990). NEM does not affect H^+ F-ATPases, but is known to inhibit H^+ V-ATPases at low micromolar concentrations and also P-type ATPases, although only at high micromolar to low millimolar concentrations. However, the range of concentrations used in the study mentioned above was not sufficient to permit a precise estimate of inhibitor sensitivity.

The H^+ pump of the frog skin had already been tentatively assigned to a class of ATPases, called 'F_1F_o-like' in a previous paper (Ehrenfeld *et al.* 1985), which was also found in other H^+-secreting epithelia of renal origin (Al-Awqati, 1978), later known as plasma membrane H^+ V-ATPases. More recently, the frog skin H^+ pump was claimed to belong to the family of H^+ V-ATPases (Harvey, 1992), mainly by analogy with the H^+ pump of the plasma membrane in mammalian renal epithelia (Gluck *et al.* 1982; Gluck and Caldwell, 1987; Brown *et al.* 1987, 1992). Recently, we have been able to prove directly the

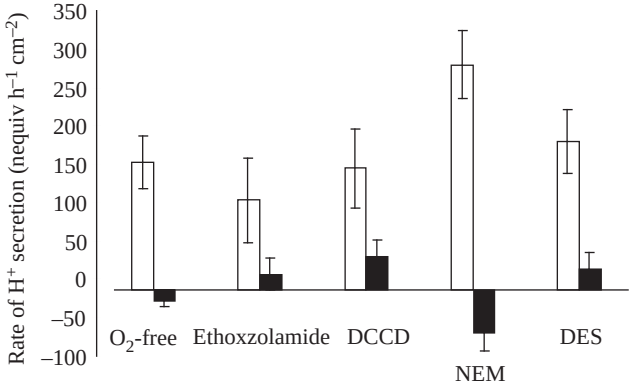

Fig. 3. Pharmacology of the proton pump in frog skin. Measurements of H^+ secretion under 'low-Na^+ conditions', see Fig. 1; experiments were performed in the presence of 50 µmol l^{-1} amiloride to inhibit Na^+ transport; frog skin was voltage-clamped to 50 mV, basal positive. Open columns show control transport values, filled columns represent H^+ secretion under the influence of (from left to right): lack of O_2 (*N*=9); 10^{-4} mol l^{-1} ethoxzolamide (*N*=6), 5×10^{-5} mol l^{-1} dicyclohexylcarbodiimide (DCCD) (*N*=5), 10^{-3} mol l^{-1} *N*-ethylmaleimide (NEM) (*N*=5) or 10^{-4} mol l^{-1} diethylstilboestrol (DES) (*N*=5) to the apical bathing solution. For details of the specificity of these agents, see text. Values are means ± S.E.M. Redrawn from Ehrenfeld *et al.* (1990).

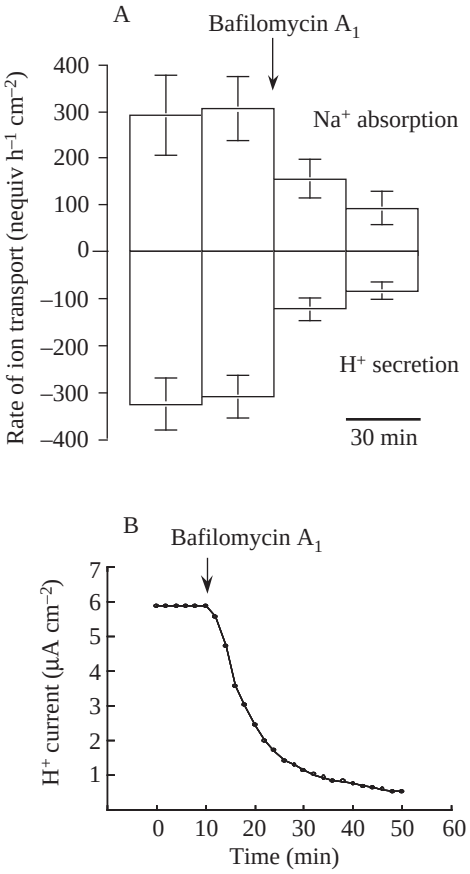

Fig. 4. Blocking of H^+ secretion by the V-ATPase-specific inhibitor bafilomycin A_1. Measurements of H^+ secretion under 'low-Na^+ conditions', see Fig. 1. (A) Open-circuit conditions: H^+ secretion and Na^+ absorption are electrically coupled. Addition of 5 μmol l^{-1} bafilomycin A_1 (arrow) to the apical bathing solution after two control periods of 30 min each dramatically decreased transport rates of both Na^+ and H^+ in the following two 30 min periods. Values are means ± S.E.M., *N*=6. (B) Short-circuit conditions with the apical solution containing 50 μmol l^{-1} amiloride to inhibit Na^+ transport. The current corresponds to H^+ secretion and is almost completely blocked by addition of 5 μmol l^{-1} bafilomycin A_1 (a representative experiment is shown).

H^+ V-ATPase nature of the H^+ pump in frog skin by pharmacological and immunological methods (U. Klein, W. Zeiske and J. Ehrenfeld, in preparation). 5 μmol l^{-1} bafilomycin A_1 (Fig. 4A) or 5 μmol l^{-1} concanamycin A inhibited H^+ secretion under open-circuit conditions measured by titration of the apical bathing solution. This inhibition was also found when transepithelial H^+ current was measured under short-circuit conditions in the absence of any Na^+ transport (inhibited by 50 μmol l^{-1} amiloride in the apical solution, Fig. 4B). The necessary concentrations of both drugs were higher than the ones used in biochemical studies, and they may not represent the effective concentrations at the membrane because of hampered access, e.g. hindered by the stratum corneum or trapped by surface mucous. With skins bathed on both sides by 'high-Na^+ conditions', the short-circuit current mainly reflects the large transepithelial Na^+ transport and the contribution of H^+ transport is barely detectable (see above). Under such conditions, bafilomycin A_1 and concanamycin A did not exert any inhibitory effect on short-circuit current. Taken together, these experiments show that both drugs act specifically on H^+ secretion and not indirectly on Na^+ transport, and that they do not act either directly on the Na^+/K^+ P-ATPase driving the clamping current under 'high-Na^+ conditions' or indirectly on the ATP supply and thus on the H^+ F-ATPase. From these observations, it may be readily concluded that H^+ secretion in frog skin is mediated by an H^+ V-ATPase.

The H^+ V-ATPase nature of the frog skin H^+ pump is in line with the drug sensitivities of H^+ transport already mentioned. Only the inhibitory effect of vanadate (Ehrenfeld *et al.* 1985) is ambiguous; it may act on the basolateral Na^+/K^+ P-ATPase or on another, so far unidentified, apical H^+ pump of the P-type and thus may interfere indirectly with H^+ transport. However, the contribution of an H^+ pump of the family of H^+/K^+ P-ATPases to H^+ secretion is unlikely since omeprazole and Sch-28080, two specific inhibitors of the H^+/K^+ P-ATPase (Lindberg *et al.* 1990; Wallmark *et al.* 1987), do not affect H^+ secretion in frog skin (J. Ehrenfeld, unpublished results). Furthermore, it cannot be excluded that the H^+ V-ATPase of frog skin itself is affected, since the H^+ V-ATPase of osteoclasts is known to be sensitive to vanadate (Chatterjee *et al.* 1992).

Localization of the H^+ pump

The H^+ pump has been tentatively localized in the apical membrane of the MR cells from both direct and indirect evidence. (i) H^+ secretion is inhibited by carbonic anhydrase inhibitors (Fig. 3; Garcia-Romeu and Ehrenfeld, 1972; Ehrenfeld and Garcia-Romeu, 1977) and it is the MR cells that have a high content of carbonic anhydrase, as shown by histochemical staining (Rosen and Friedley, 1973). (ii) The number of MR cells changes in parallel with the capacity of the skin to secrete H^+ after a metabolic acid load induced by NH_4^+ injection (Page and Frazier, 1987) or by pre-adaptation of living frogs to a 50 mmol l^{-1} KCl solution (Ehrenfeld *et al.* 1989). (iii) pH gradients measured by monitoring spatial pH profiles at the epithelial surface are found to be built up exclusively above MR cells in amphibian skins (Larsen *et al.* 1992; Harvey, 1992). (iv) Whole-cell patch-clamp recordings of membrane capacitance and current fluctuations from MR cells demonstrate that a respiratory acid load produces an additional stimulation of the DCCD-sensitive current (Harvey, 1992).

Unambiguous evidence for the localization of the H^+ pump has now been provided by our recent immunocytochemical investigation (U. Klein, W. Zeiske and J. Ehrenfeld, in preparation). Cryosections of frog skin or whole-mount preparations of isolated epithelia were probed with a monoclonal antibody (Hemken *et al.* 1992) directed against the 31 kDa subunit E of kidney microsomal H^+ V-ATPase. When

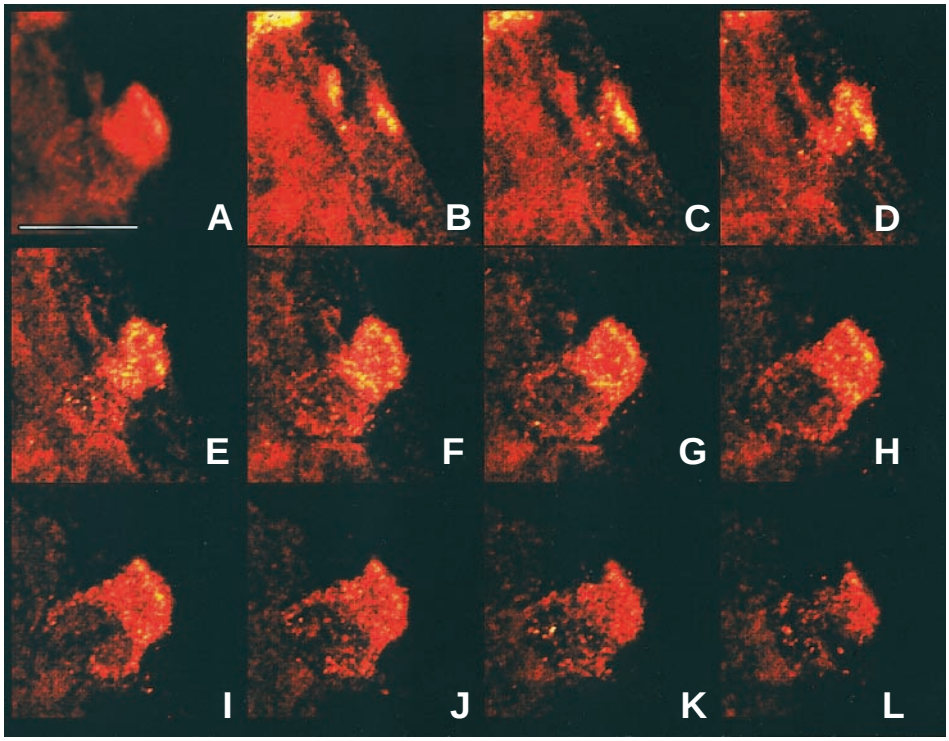

Fig. 5. Immunocytochemical labelling of mitochondria-rich (MR) cells by antibodies specific for H^+ V-ATPase. Cryosections of isolated frog skin epithelium, fixed with 2% formaldehyde, were probed with a monoclonal antibody to the 31 kDa subunit of the bovine kidney H^+ V-ATPase (Hemken *et al.* 1992) and visualized using secondary Cy3-conjugated antibodies; false colour representations of confocal micrographs, epifluorescence. (A) Average image of the total stack of 24 images (distance on *z*-axis, 400 nm). The flask-like overall shape of a labelled MR cells can be extrapolated from the fluorescence pattern, its large nucleus is visible as a less fluorescent rounded shape in the lower half of the cell. Immunolabelling appeared in the upper half of the MR cell. (B–L) A series of single confocal images numbered consecutively (distance on *z*-axis, 800 nm). Strong apical labelling can be seen in all sections at the right upper border of the flask-like MR cell just underneath the stratum corneum, demonstrating that the apical membrane of the MR cell exhibits H^+-V-ATPase-like immunoreactivity; non-apical labelling appeared only intracellularly, excluding the area of nucleus, and may be due to endosomes bearing H^+ V-ATPase or to soluble V_1 complexes of the H^+ V-ATPase in the cytosol. Scale bar, 10 μm.

applied in immunoblots of frog skin membrane protein extracts, this antibody was highly specific for a 31 kDa band, very likely the corresponding subunit of the frog skin H^+ V-ATPase. MR cells (and also several cells of the body of the gland underlying the epithelial layer, which also bear a high content of mitochondria; U. Klein and J. Ehrenfeld, unpublished observation), but not GR cells or their precursors, were specifically labelled by this H^+-V-ATPase-specific antibody. The immunofluorescence of the MR cells appeared in a characteristic pattern, which was confirmed by confocal microscopy (Fig. 5). Very intense labelling was found in the region of the apical membrane foldings. Additional labelling was found intracellularly in the apical portion of the cell, but could not be localized more precisely using light microscopy. Its localization either in endosomes or as soluble V_1 complexes in the cytosol may shed light on possible regulatory mechanisms of H^+ secretion, as will be discussed in detail below. However, the spatially defined immunoreactivity of the MR cells to an H^+-V-ATPase-specific antibody supports the conclusion that H^+ secretion in the frog skin is mediated by an H^+ V-ATPase localized in the apical membrane of the MR cells exclusively.

Regulation of H^+ transport

Electrogenic H^+ secretion in frog skin depends on electrical and chemical gradients, although clamping the transepithelial voltage at 0 mV under 'low-Na^+ conditions' induces only a slight reduction (15–20%) in H^+ flux, indicating that H^+ secretion is relatively independent of the transepithelial voltage over the range 0 to +50 mV (Ehrenfeld *et al.* 1985). Furthermore, H^+ excretion was also found to saturate at physiological transepithelial voltages (+50 mV), thus functioning as a source of constant current in this working range (Ehrenfeld *et al.* 1985). The H^+ pump current was greatly enhanced by HCO_3^-/CO_2 in the basal bathing solution (Ehrenfeld and Garcia-Romeu, 1977) and was blocked by the carbonic anhydrase inhibitors ethoxzolamide and acetazolamide (Garcia-Romeu and Ehrenfeld, 1972; Ehrenfeld and Garcia-Romeu, 1977). These properties are readily explicable by the dependence of any H^+-ATPase activity on the supply of H^+. Under 'low-Na^+ conditions', intracellular H^+ stimulates both H^+ V-ATPase activity and Na^+ transport, since the movements of these ions are electrically coupled (Ehrenfeld and Garcia Romeu, 1977; Ehrenfeld *et al.* 1990), whereas under 'high-Na^+ conditions' intracellular H^+ is known to block Na^+ channel activity and Na^+ transport (Funder *et al.* 1967; Palmer and Frindt, 1987; Harvey *et al.* 1988). Thus, in frogs facing chronic acidosis, the H^+ pump is directly involved in the regulation of cellular pH linked to concomitant Na^+ balance as a secondary benefit.

Besides influencing H^+ V-ATPase activity, MR cells seem to be able to control the number of active H^+ pumps in the apical membrane domain by endo/exocytotic shuttling of H^+-V-ATPase-bearing vesicles in response to the acid–base status of the cells (Lacoste *et al.* 1993). In skins bathed apically in a solution containing FITC–dextran as a fluid-phase marker, MR cells were preferentially labelled by the fluorochrome,

indicating a rapid apical membrane turnover. In addition, when acute acidosis was induced by CO_2 application in cells previously in an alkalotic state and exposed to FITC–dextran, the rate of FITC–dextran excretion was greatly stimulated in parallel with that of H^+ secretion. These findings were interpreted to indicate endo/exocytosis of endosomes, which store H^+ pumps (Lacoste *et al.* 1993). Increases in the apical membrane capacitance of isolated patch-clamped MR cells upon cell acidosis support this assumption (Harvey, 1992).

Vesicular trafficking in response to cellular acidification has been well documented in other H^+-secreting epithelia such as turtle urinary bladder (Cannon *et al.* 1985; Stetson and Steinmetz, 1985) and kidney collecting tubule (Schwartz and Al-Awqati, 1985). In these epithelia, the related mitochondria-rich cell populations, the intercalated cells in the kidney (Schwartz *et al.* 1985) or the carbonic-anhydrase-rich cells in the turtle (Stetson and Steinmetz, 1985), exist as different subtypes characterized by the different polarity of their membrane transporters (the H^+ pump or the Cl^-/HCO_3^- antiporter). In the kidney collecting duct, antibodies against the Cl^-/HCO_3^- antiporter (band 3) and against the H^+ V-ATPase differentiated the H^+-secreting cells into A-type cells, in which H^+ V-ATPase was labelled apically and band 3 basolaterally, and B-type cells, in which either basolateral or diffuse intracellular labelling for H^+ V-ATPase was observed but no labelling of band 3 (Alper *et al.* 1989; Schuster *et al.* 1991; Brown *et al.* 1992).

In the frog skin epithelium, there is no convincing morphological evidence for more than one type of MR cell (Whitear, 1975). However, Rick (1992) suggested on the basis of differential sensitivities of Na^+ transport under 'high-Na^+ conditions' to amiloride or ouabain, measured by electron microprobe analysis, that there are at least three functionally different types of MR cell. Immunolabelling of the H^+ V-ATPase does not support the assumption of different MR cell types, although immunolocalization cannot provide information about the functional status of the labelled pumps. In cryosections and in sheets of isolated epithelium, all detectable MR cells seemed to be labelled in the same pattern (U. Klein, W. Zeiske and J. Ehrenfeld, in preparation). Also, all MR cells bear an apical band-3-related protein (which may be a different isoform from the basolateral one), since Devuyst *et al.* (1993) have demonstrated an apical immunoreactivity of MR cells to antibodies directed to band 3 in skin of several amphibians (toad *Bufo marinus*, frog *Rana esculenta*, toad tadpole *Pelobatis syriacus*) and found that the labelling pattern was similar to that obtained after silver staining of all MR cells. The presence of an anion antiporter in the apical membranes of MR cells would be consistent with the strict link between HCO_3^- secretion and Cl^- absorption from a dilute saline and its dependence on CO_2/HCO_3^- and sensitivity to acetazolamide (Garcia-Romeu and Ehrenfeld, 1975*a*,*b*; Ehrenfeld and Garcia-Romeu, 1978). The functional localization of a Cl^-/HCO_3^- antiporter in the basolateral membrane is already well established (Duranti *et al.* 1986). It is therefore probable that the frog skin MR cells are different from A-type and B-type cells, since their apical membrane is labelled by both H^+ V-ATPase antibodies and band-3 antibodies, and that only a single type of functional MR cell is involved in H^+ and base secretion in frog skin. However, different states of differentiation of the MR cell may exist in the frog skin epithelium, as has been reported in toad skin (Budtz *et al.* 1995); these states could be modulated in response to the acid–base status of the animals or to environmental or hormonal stimuli.

A long-term regulatory mechanism of H^+ secretion, over the range of days and thus involving moulting, affects the pattern of cell type differentiation. Metabolic acidification induced either by NH_4^+ injections (Page and Frazier, 1987) or by pre-adaptation of living frogs to a 50 mmol l^{-1} KCl solution (Ehrenfeld *et al.* 1989; Lacoste *et al.* 1991) increased the number of MR cells compared with GR cells. This increase was paralleled by an increase in the rate of H^+ secretion and, consequently, in Na^+ absorption under 'low-Na^+ conditions' (Ehrenfeld *et al.* 1989). The correlation between changes in the number and morphology of MR cells and the H^+ secretion capacity induced by various environmental salinities or acid–base status in frog skin was similar to that for carbonic-anhydrase-rich cells in turtle urinary bladder (Husted *et al.* 1981; Schwartz and Al-Awqati, 1985; Steinmetz and Stetson, 1987) and intercalated cells in renal collecting duct (Madsen and Tisher, 1984).

The development and differentiation of more MR cells may also be mediated by aldosterone (which is also known to induce moulting), since plasma levels of this hormone increase after KCl adaptation (Oberleithner *et al.* 1983). Furthermore, aldosterone induces rapid morphological changes in the shape of MR cells in frog skin in 1–4 h (Voute *et al.* 1972; Voute and Meier, 1978). Short-term effects of aldosterone are also implicated since this hormone stimulated H^+ secretion (measured by titration) within 1 h (Ehrenfeld *et al.* 1989) and produced an additional stimulation of the DCCD-sensitive current which was paralleled by an increase in cell capacitance in whole-cell patch-clamp studies of MR cells (Harvey, 1992). These findings argue in favour of insertion of membranes containing H^+ pumps in response to aldosterone treatment. Neurohypophyseal hormones, such as arginine vasotocin and oxytocin, do not act upon the H^+ V-ATPase and thus do not stimulate Na^+ transport from a dilute NaCl medium, as they would otherwise do in 'high-Na^+ conditions' (Fig. 6; Ehrenfeld *et al.* 1989). Likewise, Brown *et al.* (1981) could find no consistent pattern between MR cell numbers and electrical parameters related to the hormonal effect of oxytocin. MR cell differentiation is also thought to be induced by prostaglandins, since ibuprofen injection for 3 days significantly increased MR cell numbers (Page *et al.* 1990).

Regarding possible regulatory mechanisms, the intracellular H^+-V-ATPase-like immunoreactivity in MR cells may be interpreted in several ways. (i) Most simply, it may indicate acidic endosomes, in general, which would not be involved in regulating plasma membrane H^+ V-ATPase activity. Since GR cells are not labelled, such endosomes would have to be specific for MR cells or especially numerous in these cells. (ii) It may visualize endocytotic vesicles loaded with plasma membrane H^+ V-ATPase, as discussed above. (iii) Most interestingly, it

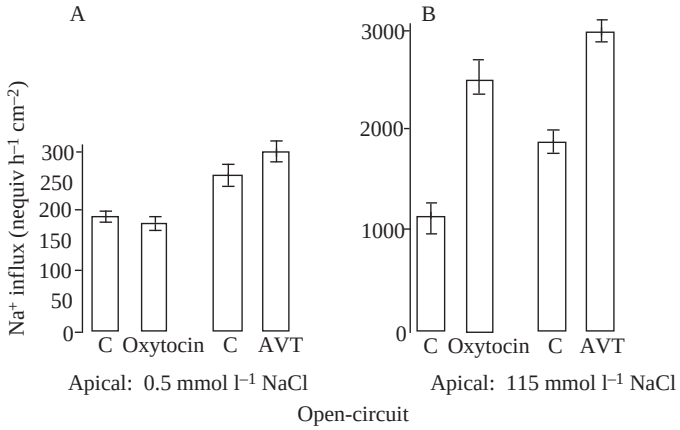

Fig. 6. Effect of neurohypophyseal hormones on Na^+ absorption in frog skin epithelium. (A) Under open-circuit and 'low-Na^+ conditions' (as given in Fig. 1, but with apical 0.5 mmol l^{-1} NaCl), oxytocin or arginine vasotocin (AVT) do not affect Na^+ transport, which is then electrically linked to H^+ secretion. Thus, H^+ secretion is independent of both hormones. (B) In contrast, the Na^+ permeability is influenced by both hormones. Under open-circuit and 'high-Na^+ conditions' (see Fig. 1), Na^+ absorption is increased when oxytocin or AVT is added. Under these conditions, the large Na^+ absorption is not dependent on H^+ secretion and Cl^- serves as the counter-ion for Na^+ transport. Values are means ± S.E.M., *N*=8. *C*, control. Data from Ehrenfeld *et al.* (1989).

may indicate soluble subunit-E peptides or complete V_1 complexes in the cytosol, since subunit E is part of the peripheral catalytic V_1 complex of the H^+ V-ATPase and not of the membrane-inserted V_o complex. Cytosolic V_1 complexes in a soluble form are found in several instances: in yeast (Doherty and Kane, 1993), in a kidney epithelial cell line (Myers and Forgac, 1993) and in the insect larval midgut (Gräf *et al.* 1996). Furthermore, in insect midgut (Sumner *et al.* 1995) and in yeast (Kane, 1995), it has been demonstrated that dissociation of the cytosolic V_1 complex inhibits H^+ V-ATPase activity. Since complete V_1V_o holoenzyme reappears in the membrane without the need for protein biosynthesis (Kane, 1995; Jäger and Klein, 1996), it is assumed that free V_1 particles can also reassociate to restore ATPase activity *in vivo*.

We have outlined the key roles of an H^+ V-ATPase present in the MR cell of the frog skin epithelium in H^+ secretion and as a driving force for Na^+ absorption from a dilute external medium. In this respect, the frog skin constitutes a model for freshwater animals including fishes and crustaceans, which have recently been found to possess similar mechanisms (for a review, see Lin and Randall, 1995). The frog skin is another example of the increasing number of ion-transporting epithelia in which an H^+ V-ATPase provides a proton-motive force energizing animal plasma membranes (for a review, see Wieczoreck and Harvey, 1995).

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