J. Membrane Biol. 157, 117–126 (1997)

The Journal of
Membrane
Biology

The H^+ Pump in Frog Skin (*Rana esculenta*): Identification and Localization of a V-ATPase

U. Klein,[1] **M. Timme,**[1] **W. Zeiske,**[2] **J. Ehrenfeld**[3]

[1]Zoologisches Institut der Universität, München, Germany
[2]Laboratorium voor Fysiologie, Katholicke Universiteit Leuven, Belgium
[3]Laboratoire Jean Maetz, Villefranche sur-Mer, France

Received: 21 May 1996/Revised: 24 December 1996

Abstract. We here report on studies on the frog skin epithelium to identify the nature of its excretory H^+ pump by comparing transport studies, using inhibitors highly specific for V-ATPases, with results from immunocytochemistry using V-ATPase-directed antibodies. Bafilomycin A_1 (10 μM) blocked H^+ excretion (69 ± 8% inhibition) and therefore Na^+ absorption (61 ± 17% inhibition after 60 min application, $n = 6$) in open-circuited skins bathed on their apical side with a 1 mM Na_2SO_4 solution, ''low-Na^+ conditions'' under which H^+ and Na^+ fluxes are coupled 1:1. The electrogenic outward H^+ current measured in absence of Na^+ transport (in the presence of 50 μM amiloride) was also blocked by 10 μM bafilomycin A_1 or 5 μM concanamycin A. In contrast, no effects were found on the large and dominant Na^+ transport (short-circuit current), which develops with apical solutions containing 115 mM Na^+ (''high-Na^+ conditions''), demonstrating a specific action on H^+ transport. In immunocytochemistry, V-ATPase-like immunoreactivity to the monoclonal antibody E11 directed to the 31-kDa subunit E of the bovine renal V-ATPase was localized only in mitochondria-rich cells (i) in their apical region which corresponds to apical plasma membrane infoldings, and (ii) intracellularly in their neck region and apically around the nucleus. In membrane extracts of the isolated frog skin epithelium, the selectivity of the antibody binding was tested with immunoblots. The antibody labeled exclusively a band of about 31 kDa, very likely the corresponding subunit E of the frog V-ATPase. Our investigations now deliver conclusive evidence that H^+ excretion is mediated by a V-ATPase being the electrogenic H^+ pump in frog skin.

Key words: V-ATPase — H^+ transport — Na^+ transport — Epithelium — Bafilomycin A_1 — Concanamycin A — Immunocytochemistry

Correspondence to: U. Klein

Introduction

Like most freshwater animals including fish and crayfish, frogs actively reabsorb Na^+ from very dilute solutions (Krogh, 1938; Garcia-Romeu, Salibian & Pezzani-Hernandez, 1969; Garcia-Romeu & Ehrenfeld, 1972). It was demonstrated for the frog skin epithelium that this active Na^+ transport depended on the functioning of an electrogenic H^+ pump playing a key role in generating a favorable electrochemical gradient through the apical membranes of the outermost living cells (mitochondria-rich cells, MR cells, and granular cells, GR cells) and permitting passive electrodiffusion of Na^+ to occur (Ehrenfeld & Garcia-Romeu, 1977; Ehrenfeld, Garcia-Romeu & Harvey, 1985). Therefore, transepithelial Na^+ transport at low Na^+ concentrations in the external medium was found to result from the functioning of two pumps in series, a H^+ pump located apically on the MR cells and a Na^+, K^+ pump located basolaterally.

H^+ excretion is affected by several drugs (Ehrenfeld et al., 1985), known to inhibit one or more members of the three classes of ion motive ATPases (Pedersen & Carafoli, 1987): (i) oligomycin, a potent inhibitor of the mitochondrial F-ATPase; (ii) vanadate, a specific inhibitor of all P-ATPases, (iii) diethylstilbestrol (DES) and (iv) N,N′-dicyclohexylcarbodiimide (DCCD), both affecting H^+ pumps of all types and (v) N-ethylmaleimide (NEM), an inhibitor for both, V- and P-ATPases, but at different concentrations. Although the latter two inhibitors are not very specific for V-ATPases and may affect carboxyl- or sulfhydryl-groups, respectively, in other proteins as well, it was claimed (Harvey, 1992) without

further molecular or pharmacological evidences that the H^+ pump in the frog skin was a V-ATPase.

Therefore, we intended to identify the nature of the H^+ pump in the frog skin epithelium and tested successfully two highly specific V-ATPase inhibitors, bafilomycin A_1 (Bowman, Siebers & Altendorf, 1988) and concanamycin A (Dröse et al., 1993) on H^+ and Na^+ transport. In addition, we localized V-ATPase immunoreactivity in the mitochondria-rich cells (MR cells) with a monoclonal antibody directed against a single subunit of the bovine renal V-ATPase in cryosections of the skin and in whole-mount preparations of the isolated epithelium. Both approaches together deliver conclusive evidence that the H^+ pump in the frog skin epithelium is a V-ATPase and that it is localized apically in the MR cells.

Materials and Methods

Frogs (*Rana esculenta*) were kept at a temperature of ±15°C in running tap water at a day:night cycle of 12:12 hours. All investigations were carried out on ventral skins removed from double-pithed animals.

For transport experiments and physiological measurements, the skin was mounted between two lucite chambers of 7 ml capacity each, the exposed area being 7 cm^2. The skins were selected by their ability to maintain net Na^+ absorption from a dilute apical solution containing 1 mM $NaSO_4$.

For ion flux measurements, performed in open-circuit conditions (OCC), the skins were bathed apically in a continually aerated solution of 1 mM Na_2SO_4 buffered to pH 7.34 with 4 mM imidazole (pH adjustment with H_2SO_4), ''low-Na^+ conditions'': 1 mM Na^+, Cl^--free, HCO_3^--free. Normal Ringer solution was used on the serosal side and contained (in mM): NaCl 85; $NaHCO_3$ 24; KCl 2.5; $CaCl_2$ 2; $MgSO_4$ 2; Na_2HPO_4 3.2; KH_2PO_4 1.2; glucose 11; pH 7.34 after bubbling with 95% O_2 and 5% CO_2. Samples of the mucosal solutions were taken every 30 min. In these samples Na^+ concentrations were measured by flame photometry and H^+ concentrations by titration; details of both techniques have been described previously (Ehrenfeld & Garcia-Romeu, 1977). The analysis of the Na^+ and H^+ concentrations permitted the calculation of net Na^+ and H^+ fluxes (J_{nNa}+ and J_{nH}+). The fluxes are expressed in neq $\cdot h^{-1} \cdot cm^{-2} \pm$ SEM. Statistical analysis was carried out by the paired *t*-test.

Measurements of transepithelial currents were performed under short-circuit conditions (SCC) or at an imposed transepithelial potential difference (50 mV, serosal side positive). In these experiments, either ''low-Na^+ conditions'' were applied apically or ''high-Na^+ conditions,'' where normal Ringer solution was used in which 24 mM $NaHCO_3$ was replaced by 12 mM Na_2SO_4. The skin was clamped at a defined potential by using an automatic voltage clamp (Model VC 600, Physiological Instruments, Houston, TX). KNO_3-agar salt bridges were used to pass transepithelial current or to measure the spontaneous potential. The current was continuously recorded on a chart recorder (SEFRAM, Paris, France).

Amiloride (Merck Sharp and Dohme Research Laboratories, Rayway, NJ) was used on the apical side of the skin at a final concentration of 50 μM. Bafilomycin A_1 or concanamycin A were dissolved in dimethylsulfoxide and used at final concentrations of 10 μM and 5 μM, respectively, added on the apical side of the frog skin epithelium. Controls in which equal-sized μl-volumes of dimethylsulfoxide were added, showed no reaction.

For biochemical analysis of the membrane proteins, epithelia were isolated from the skin by collagenase treatment as described in Aceves and Erlij (1971). Single epithelia of about 2 mg wet weight were carefully homogenized in a glass homogenizer with glass pistil at 4°C in 0.5 ml of 18 mM Tris-HCl, pH 7.4 containing 200 mM of sucrose, 5 mM of ethylenediaminetetraacetic acid, 0.2 mM of β-mercaptoethanol and 1 mM of phenylmethylsulfonyl fluoride (Giunta et al., 1984). The homogenate was centrifuged at 1000 × *g* for 10 min and the supernatant centrifuged at 50,000 × *g* for 20 min to obtain the membrane fraction. For protein extraction, the pellet was resuspended in 120 mM Tris-HCl, pH 6.8, containing 2% (w/v) of SDS (sodium dodecylsulfate), 2% (v/v) of β-mercaptoethanol and 20% (v/v) of glycerol, and treated for 5 min at 95°C. SDS polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as described in Schweikl et al. (1989) and Wieczorek et al. (1991). For protein labeling the mouse monoclonal antibody E11 was used directed to the 31-kDA subunit E of the bovine renal V-ATPase (Hemken et al., 1992). The cell culture supernatant was diluted 1:2 in blocking solution (1% bovine serum albumin in Tris-buffered saline; Wieczorek et al., 1991). Anti-mouse antibodies (whole molecules) conjugated with alkaline phosphatase (Sigma) were used as secondary antibodies, diluted in blocking solution as recommended by the manufacturer.

For immunocytochemical investigations, either cryosections of isolated skins or whole-mount preparations of isolated epithelia were used. For cryosections, small pieces of skin were frozen either unfixed or after fixation in 2 or 4% paraformaldehyde in 100 mM phosphate buffer, pH 7.2, for 1 hr on ice in cryoembedding medium (Microm) by quick immersion in melting isopentane after incubation in increasing concentrations 10, 20 and 30% (w/v) of sucrose for 20, 40, and 120 min, respectively. 7–15 μm cryosections were prepared using a cryostate (Microm HM 500 OM) at −25°C box temperature and at −30°C object temperature and collected on 0.01% polylysine coated coverslips. Immunolabeling was performed as described in Klein, Löffelmann & Wieczorek (1991). For primary antibody solution, the supernatant of the mouse monoclonal antibody E11 was used either undiluted or diluted 1:2 in blocking solution (Klein et al., 1991). Goat anti-mouse antibodies (whole molecules) conjugated with FITC (fluorescein isothiocyanate, Sigma) or with Cy3 (Sigma) were used as secondary antibodies diluted as recommended by the manufacturer.

For whole-mount preparations, epithelia were isolated as described above, cut into pieces of about 8 mm in diameter and immunolabeled after fixation in 2% or 4% phosphate-buffered formaldehyde, pH 7.4. The procedure followed in principle the same protocol as described for the cryosections, but all solutions were prepared with 5 mM HEPES (N-[2-hydroxymethyl]piperazine-N′-ethanesulfonic acid)-buffered normal Ringer solution, pH 7.45, without HCO_3^- (*see above*). From the blocking solution (3% gelatine in Ringer solution) on 0.1% saponin was added to antibody-containing or rinsing solutions in order to permeabilize the plasma membranes. The whole treatment was performed in 1.5 ml test tubes and the labeled epithelia were finally put on glass slides, apical side upwards. Immunofluorescence was evaluated with an Axioplan light microscope (Zeiss-Jena, Germany), either with differential interference contrast illumination or under epifluorescence illumination with the filters set for FITC fluorescence (BP 450-490, FT 510, LP 520). In some experiments the fluorescence pattern was analyzed with an Axiovert LSM 410 (Zeiss-Jena, Germany) with a laser excitation at 543 nm and the filters set for rhodamine fluorescence (FT 560, LP 570); optical sections were taken at 400 nm distance.

Results

Blocking of Na^+ and H^+ Transport by V-ATPase-specific Inhibitors

In isolated frog skins mounted at open circuit, bathed apically with a dilute Na^+-containing solution (1 mM

Na_2SO_4, ''low-Na^+ conditions'') and serosally with a HCO_3^-/CO_2-containing Ringer solution, Na^+ transport (absorption) is electrically counterbalanced by H^+ excretion (Ehrenfeld & Garcia-Romeu, 1977). When 10 μM bafilomycin A_1, a specific V-ATPase inhibitor, was added to the apical bath, a 61 ± 17% inhibition of Na^+ transport and a 69 ± 8% inhibition of H^+ transport was measured after two 30 min sample periods following application of the drug, as expressed relative to the two 30 min control periods (100%) measured before addition ($n = 6$; Fig. 1*a*). Na^+ absorption and H^+ excretion are highly correlated in these experimental conditions, either during the control periods or during the drug treated periods (Fig. 1*b*).

To investigate the specificity of this inhibition, Na^+ transport was blocked with 50 μM amiloride applied to the apical side in ''low-Na^+ conditions,'' clamping the transepithelial potential to zero (SCC). Under such conditions, the recorded clamping current mainly reflects H^+ excretion (Ehrenfeld et al., 1985). Addition of 10 μM bafilomycin A_1 to the apical side caused a marked inhibition of the clamping current (I_{SCC}) which decreased from 5.5 ± 1.8 μA/cm^2 in the control period to 0.4 ± 0.5 μA/cm^2 after 30 min of drug treatment ($n = 10$; Fig. 2*a*). Similar results (*data not given*) were found when the transepithelial potential was held at +50 mV (serosal side positive), a situation favoring H^+ excretion (Ehrenfeld et al., 1985). Concanamycin A was also tested on the current in a similar situation, the skin clamped to +50 mV. As with bafilomycin A_1, a clear inhibition of the current was observed after addition of 5 μM concanamycin A. This agent inhibited 75 ± 2% ($n = 7$) of the clamping current after 30 min application to the apical bathing solution (Fig. 2*b*).

The effect of both drugs was then tested on frog skins mounted in ''high-Na^+conditions,'' short-circuited skins bathed on both sides with Ringer solutions containing 115 mM Na^+ concentration. Under these experimental conditions, the large I_{SCC} matches with Na^+ transport, H^+ excretion being largely reduced by the lack of HCO_3^-/CO_2 in the Ringer solution and therefore negligible (Ehrenfeld & Garcia-Romeu, 1977). The I_{SCC} slightly decreased as a function of time in the control period and in the period of drug treatment, but it was not affected by addition of 10 μM bafilomycin A_1 (Fig. 3). In a similar way, no obvious effect was observed on the Na^+ current when 5 μ concanamycin A was added (*data not given*).

In summary, these physiological measurements give strong evidence for a V-ATPase as the H^+ pump in the frog skin epithelium, since bafilomycin A_1 and concanamycin A, both known to be highly specific inhibitors of V-ATPases, (i) were able to strongly inhibit H^+ excretion and electrogenic H^+ current and (ii) demonstrated an indirect inhibitory effect on Na^+ transport in open circuit and ''low-Na^+ conditions.''

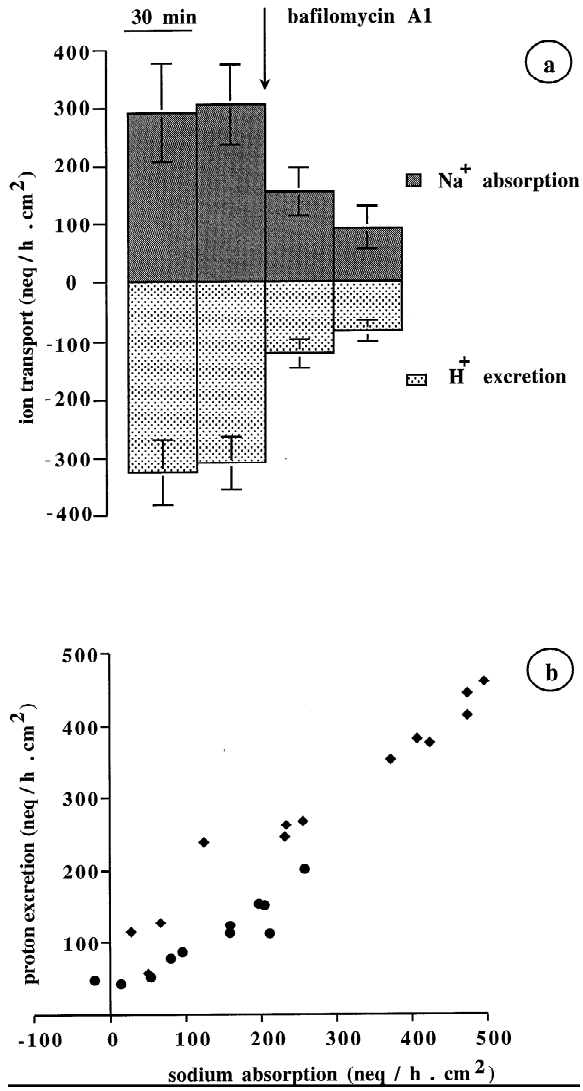

Fig. 1. H^+ excretion and Na^+ absorption are inhibited by bafilomycin A_1. Isolated frog skin, mounted under open-circuit ''low-Na^+ conditions'' in a chamber containing apically 1 mM Na_2SO_4 and serosally HCO_3^-/CO_2-containing normal Ringer solution with 115 mM Na^+ (*a*) Net transport of H^+ (measured by titration) and of Na^+ (measured by flame photometry) are given for 4 sampling periods. 10 μM bafilomycin A_1, added at the beginning of the third sampling period, reduced both transports significantly. (*b*) Values of individual skins for H^+ excretion are drawn against Na^+ absorption showing its 1:1 ($r = 0.95$) coupling in both periods before (♦) and after (●) addition of bafilomycin A_1.

Immunolabeling by V-ATPase-specific Antibodies

To localize V-ATPase immunoreactivity in the tissue, we investigated the frog skin by immunocytochemistry on cryosections of the isolated skin and in whole-mount preparations of the isolated epithelium. To probe the selectivity of the monoclonal antibody E11, we analyzed its binding pattern in membrane protein extracts of the isolated epithelium by immunoblots. SDS extracts con-

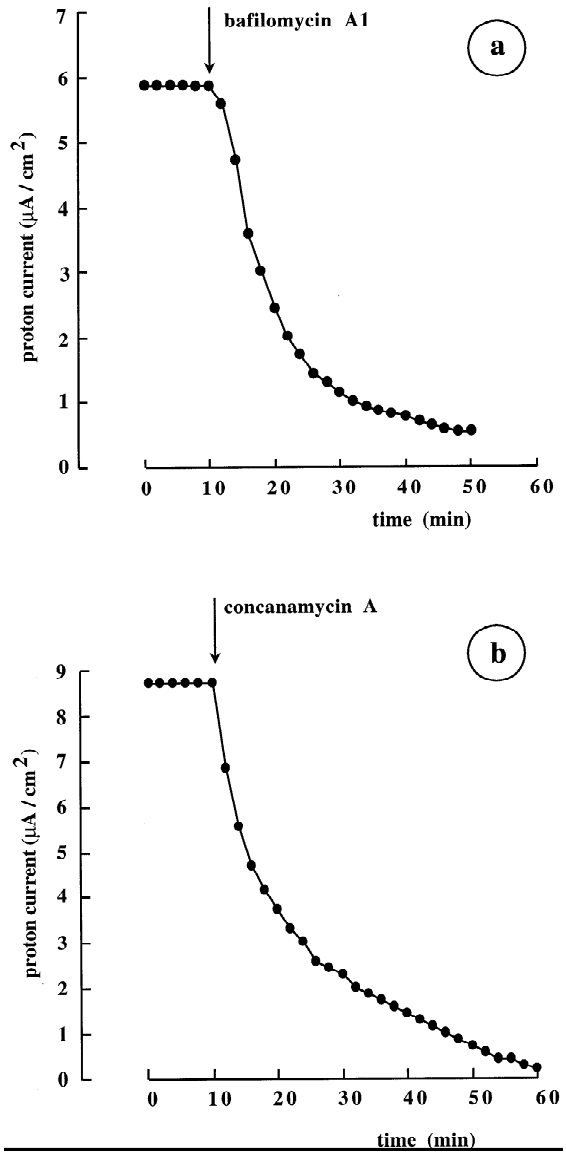

Fig. 2. Outward current in ''low-Na^+ conditions'' is inhibited by bafilomycin A_1 and concanamycin A. Isolated frog skin, mounted in a chamber under clamping conditions and with similar solutions as given in Fig. 1, but with 50 μM amiloride added to the apical side. The outward directed current is then carried mainly by H^+. (a) Clamping at 0 mV, I_{SCC} decreased when 10 μM bafilomycin A_1 had been added after a 10-min control period. (*b*) Clamping at 50 mV (serosal positive), clamping current also decreased when 5 μM concanamycin A had been added.

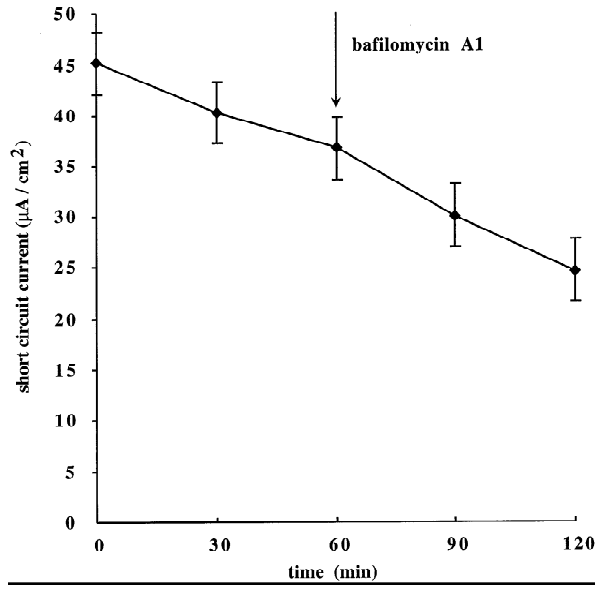

Fig. 3. Bafilomycin A_1 does not significantly affect Na^+ current under ''high-Na^+ conditions.'' Isolated frog skin, mounted under short-circuit conditions and bathed on both sides in HCO_3^-/CO_2-free normal Ringer solution (115 mM Na^+), conditions under which the I_{SCC} is carried mainly by Na^+ (H^+ current being negligible). After a control period of 60 min, 10 μM bafilomycin A_1 was added without obvious effect.

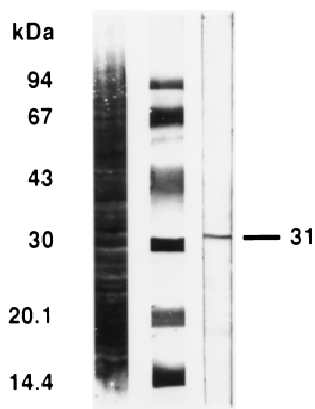

Fig. 4. Selectivity of anti 31-kDa antibody in membrane protein extracts of frog skin epithelium. Immunoblot, SDS-extract of membrane proteins from isolated frog skin epithelium. Left lane: protein extract, all proteins are stained by amidoblack; middle lane: pattern of standard proteins, amidoblack staining, the numbers represent molecular masses; right lane; protein extract, immunolabeling visualized by secondary antibodies coupled to alkaline phosphatase. Anti-31 kDa-antibody labeled only one band.

tained various protein bands as shown by general protein staining, but immunostaining with antibody E11 exhibited only one single band of 31 kDa (Fig. 4). Thus it may readily be concluded that the antibody specifically labeled a frog skin V-ATPase subunit corresponding to the 31-kDa subunit E of the bovine kidney V-ATPase, its proper immunogen.

In cryosections, showing the epithelial cells in a longitudinal view, only the flask-shaped MR cells, but no GR cells or other precursor cells in the epithelium were labeled (Fig. 5). A very intense immunofluorescence was detectable especially at the most apical border of the cells (Fig. 5*a–d*). When formaldehyde was used for fixation, the number of labeled MR cells increased and ad-

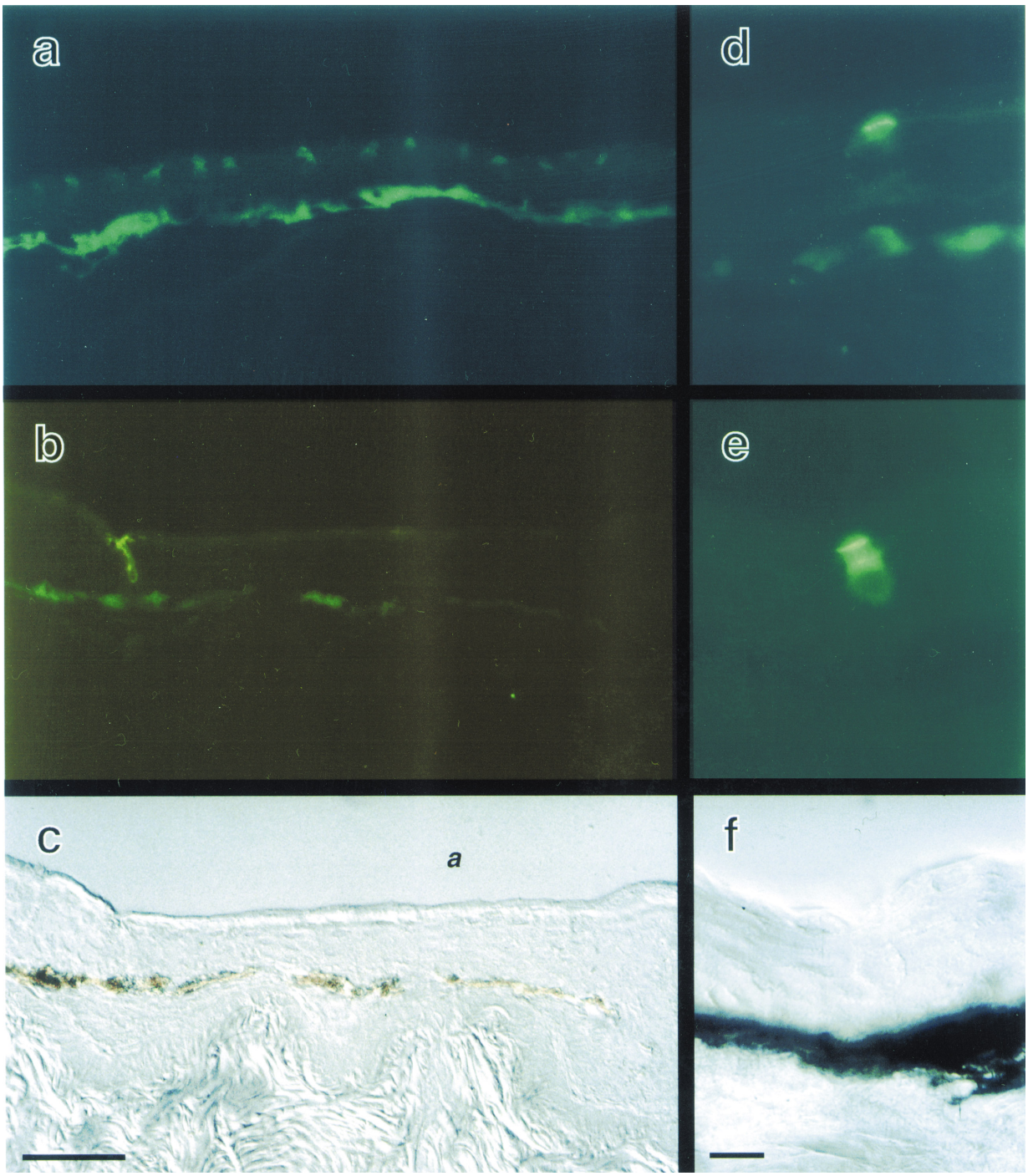

Fig. 5. V-ATPase immunoreactivity of MR cells in the frog skin epithelium. Cryosections of preparations fixed with 2% formaldehyde (*a*), (*b*), (*c*), and (*e*) or unfixed (*d*). (*a*), (*b*), (*d*) and (*e*) are viewed under epifluorescence illumination; (*c*) and (*f*) represent corresponding views to (*b*) and (*e*), respectively, focused at the same plane in differential interference contrast (DIC). (*a*), (*d*) and (*e*) show sections which are immunolabeled by anti 31-kDa antibody, visualized by FITC-conjugated secondary antibodies. (*a*) shows an overview of the epithelium demonstrating the density of the labeled MR cells. The fluorescence below the epithelial layer is due to the autofluorescence of pigment cells in varying appearance. (*b*) and (*c*) show sections after control incubation without primary antibody. In unfixed (*d*) and in fixed state (*e*) the strong labeling of the uppermost apical border of MR cells is obvious. In (a) and (e), the fixed preparations, nonapical labeling below the apical cell border but excluding the nucleus appears in addition. The flasklike overall shapes of the labeled MR cells can be extrapolated from this fluorescent pattern. *a*—apical. Scale bar in (*c*) means 50 μm for (*a*), (*b*) and (*c*); scale bar in (*f*) means 10 μm for (*d*), (*e*) and (*f*).

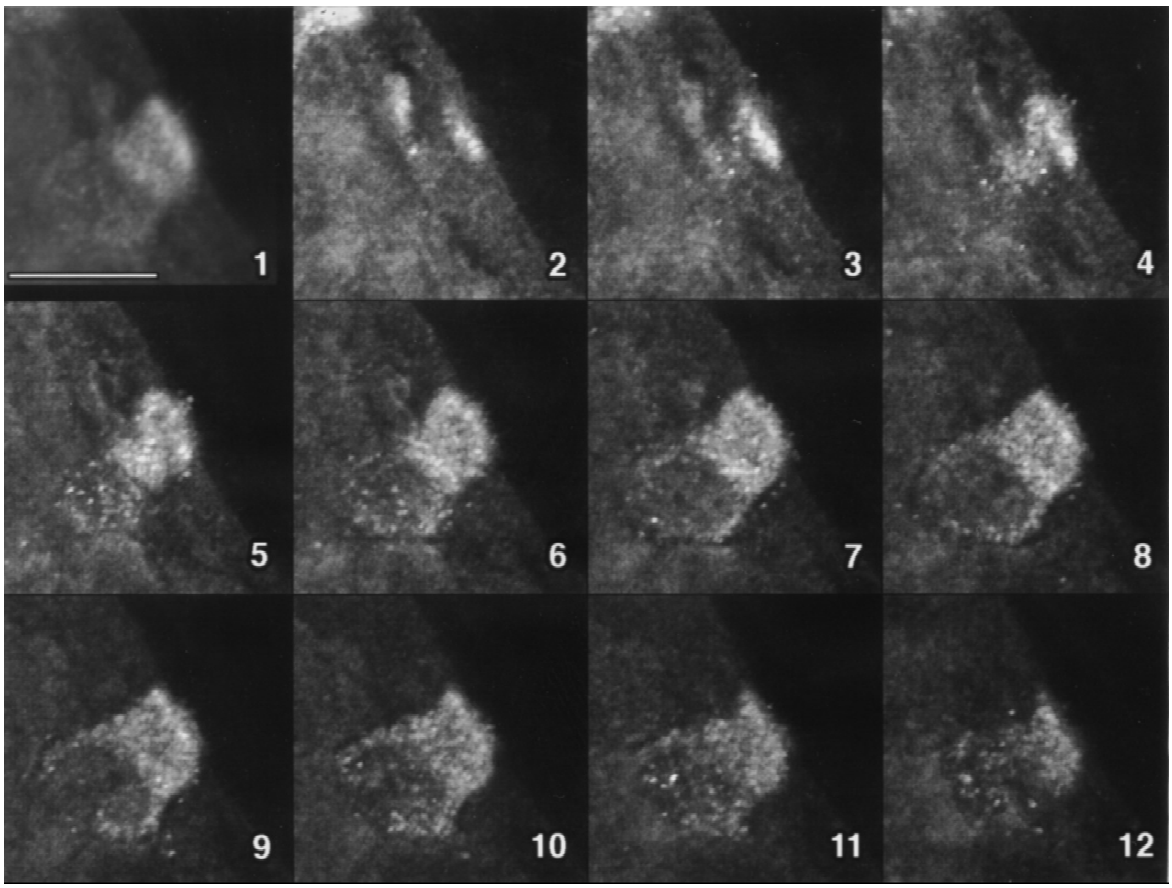

Fig. 6. V-ATPase immunoreactivity of MR cells in the frog skin epithelium. Preparation fixed with 2% formaldehyde, cryosection, Cy3-immunolabeling, confocal microscopy, epifluorescence. No 1: average image of the total stack of 24 images (distances in z-axis: 400 nm). No. 2 to 9 represent single confocal images numbered consecutively, distances in the z-axis: 800nm. The apical labeling can be seen in all sections at the right upper border of the flasklike cell; the nonapical labeling appears only intracellularly excluding the area of nucleus. Scale bar 10 μm.

ditional labeling in decreasing intensity from apical to basal appeared also in the upper region of the MR cell, the neck region and around the nucleus (Figs. 5*a*, *c;* 6). Controls with secondary goat antibody alone (Fig. 5*b*) or with other mouse monoclonal antibodies (*not shown*) failed to exhibit any fluorescence labeling in the epithelium.

However, it was not easy to distinguish if the nonapical labeling was membrane-bound or of intracellular origin (see e.g., Fig. 5*e*). For better spatial resolution of immunofluorescence, 15 μm-thick cryosections were also inspected in a confocal microscope. By analyzing the series of optical sections, it became obvious (i) that the apical membrane area was labeled at all levels and (ii) that the additional immunofluorescence was localized intracellularly and not at the basolateral plasma membrane (Fig. 6).

To screen for the density of labeled MR cells, whole-mount preparations of permeabilized isolated epithelia were probed with the antibody (Fig. 7). Here, the optical axis is perpendicular to the epithelial surface. Again, only MR cells were immunolabeled and such cells were found regularly in a dense spatial pattern, implying that all MR cells were labeled. Furthermore, all labeled cells exhibited a similar fluorescence distribution with a marked signal at the apical pole (Fig. 7).

Discussion

Pharmacology of H^+ Transport vs. H^+ ATPase

In the frog skin, active Na^+ absorption is known to occur in exchange for H^+ at low external concentrations of Na^+ resembling in vivo pond conditions. An electrogenic and active H^+ excretion mediated by an electrogenic H^+ pump plays a key role in the process of electrodiffusive Na^+ absorption at the apical side of the epithelium as a result of electrical coupling between both transports (Ehrenfeld & Garcia-Romeu, 1977; Ehrenfeld et al., 1985). The kinetic parameters of the frog skin H^+ pump

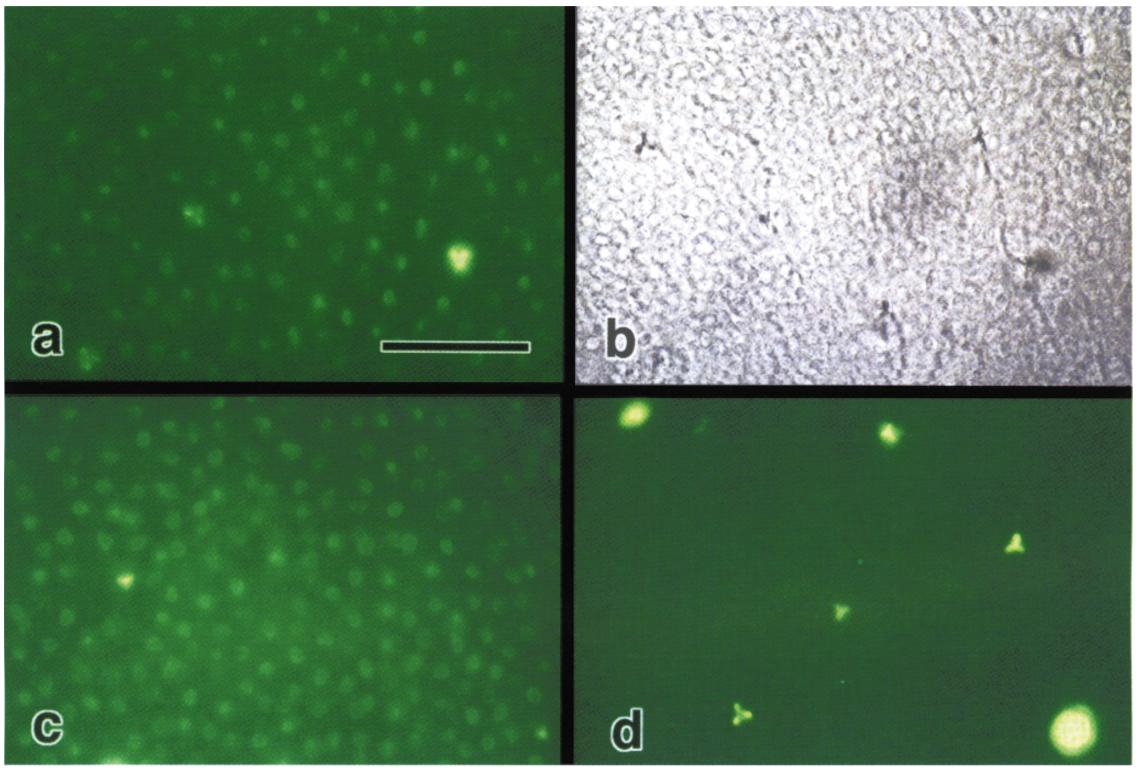

Fig. 7. V-ATPase immunoreactivity of MR cells in the frog skin epithelium. Isolated epithelium, fixed in 2% formaldehyde, treated with 0.1% saponin, immunolabeling visualized by FITC-conjugated secondary antibodies. (*a*), (*c*) and (*d*) represent views from the apical surface in epifluorescence illumination, (*b*) is the view correspondiung to (*a*) in DIC. Pictures (*a*) and (*b*) are focused on the optical section at the level of pits of MR cells, whereas (*c*) hits a lower level of nonapical immunofluorescence, surrounding the nonfluorescent nuclei; (*d*) control incubation without primary antibody. Yellow spots represent autofluorescence of apical gland openings. Scale bar 50 μm.

are relatively well defined and there are several evidences which localize the H^+ pump in the apical membrane domain of the MR cells in the outermost layer of living cells: H^+ flux is correlated with the number of MR cells and with the size of their apical surface area (Ehrenfeld et al., 1990), and whole-cell patch-clamp recordings of capacitance and current fluctuations from MR cells demonstrated insertion of DCCD-conductive membrane upon acidosis (Harvey, 1992). The H^+ pump current is strictly linked with aerobic metabolism (Ehrenfeld et al., 1985), greatly enhanced by HCO_3^-/CO_2 in the serosal bath (Ehrenfeld & Garcia-Romeu, 1977) and is blocked by carbonic anhydrase inhibitors (ethoxzolamide and acetazolamide; Garcia-Romeu & Ehrenfeld, 1972; Ehrenfeld & Garcia-Romeu, 1977). These properties may be readily explicable by the dependence of any H^+ ATPase on the supply of H^+ and of ATP. It is not easy, however, to deduce the molecular nature of the responsible H^+ ATPase relying on drug sensitivities influencing the transepithelial H^+ transport (H^+ excretion or H^+ current). Moreover, as already pointed out in the introduction, H^+ transport is affected by several drugs (Ehrenfeld, Lacoste & Harvey, 1989) which are known to affect more than one kind of ion motive ATPases.

Today, ion motive ATPases are classified into three groups, F-, P- and V-ATPases by their molecular structure and pharmacological properties (Pedersen & Carafoli, 1987). V-ATPases are specifically inhibited by bafilomycin A_1 and concanamycin A (Bowman, Siebers & Altendorf, 1988; Dröse et al., 1993) both interfering with the proton channel-bearing membrane sector V_0 (Zhang, Feng & Forgac, 1994). In this study we could demonstrate for the first time that these drugs block H^+ excretion in the frog skin epithelium when measured either by titration analysis of the apical bathing medium or by measuring the H^+-clamping current which develops in absence of Na^+ transport. In addition, at open circuit, Na^+ absorption was equally unpaired under ''low-Na^+ conditions'' apically. The effective concentrations of both drugs in μM range are higher than the one normally used in vitro, but such effects are well known for applications in vivo. In contrast, no significant inhibition of the Na^+ transport was measured at short-circuit in frog skins mounted in ''high-Na^+ conditions'' (both sides bathed with a 115 mM Na^+-containing Ringer solution). This demonstrates the specificity of these drugs with respect to a direct effect on H^+ excretion and strongly points to a V-ATPase nature of the respective H^+ pump.

It has to be mentioned that already in a previous paper (Ehrenfeld et al., 1985). The H^+ pump of the frog skin was tentatively grouped to ATPases, called ''F_0F_1-like'' at that time, which was found in other H^+ excreting epithelia of renal origin (Al-Awqati, 1978) and which later become famous as the first members of V-ATPases, located in the plasma membrane (Brown, Gluck & Hartwig, 1987). More recently, the frog skin H^+ pump was already claimed to belong to the class of V-ATPases (Harvey, 1992), since H^+ excretion was found to be sensitive to NEM and only by analogy with that of the plasma membrane of mammalian renal epithelia (Gluck, Cannon & Al-Awqati, 1982; Gluck & Caldwell, 1987; Brown, Gluck & Hartwig, 1987). Although NEM affects V-ATPases, it is a general sulfhydryl-reacting agent and may not act very specifically.

Nevertheless, the V-ATPase nature of the frog skin H^+ pump, which has now been clearly defined by our study, may be easily put in line with the drug sensitivities of H^+ transport, found so far (Ehrenfeld et al., 1985): NEM is known to inhibit P-ATPases in high micromolar concentration but V-ATPases in low micromolar concentration, in the latter by binding to cystein residues in the active ATP-binding site (Nelson, 1992); DCCD and DES interfere with H^+ conduction of H^+ ATPases of all three classes (Pedersen & Carafoli, 1987) and oligomycin, inhibiting mitochondrial F-ATPase, may act indirectly on the supply of ATP. Only the blocking action of vanadate stays questionable. Although it may act on the basolateral Na^+, K^+-ATPase, establishing the Na^+ gradient, or on another, so far not identified, apical H^+ pump of the P-type and thus may interfere indirectly with H^+ transport, it cannot be excluded that the V-ATPase of the frog skin itself is affected by vanadate, since this is already known from the V-ATPase of osteoclasts (Chatterjee et al., 1992).

Immunolabeling Localizing Subunit E of the Frog Skin V-ATPase

Immunocytochemistry labeled exclusively the MR cells in frog skin epithelium. The monoclonal mouse antibody used for this study was prepared against the last ten residues of the predicted protein sequence of the 31-kDA subunit E, part of the peripheral catalytical V_1 complex, of the bovine renal V-ATPase (Hirsch et al., 1988; Hemken et al., 1992). This antibody was already successfully used for immunolabeling in several mammalian tissues and exhibited a wide species reactivity, although so far, it did not show immunoreactivity in amphibian tissues (Hemken et al., 1992). Subunit E is an essential subunit of all V-ATPases and, although there seems to be only one gene, exhibited pronounced heterogeneity in different renal membrane compartments; therefore the resulting microheterogeneity of the V-ATPase is thought to provide a mechanisms for specificity of sorting and regulation (Hemken et al., 1992).

In the immunoblots of the membrane extracts from crude homogenate of isolated frog skin epithelia, antibody E11 labeled one single band. Therefore, this antibody seems to be very selective and showed no cross reactivity with other proteins in the denatured SDS extract. Furthermore, the size of the labeled protein perfectly matches with the expected size of the corresponding subunit E of a supposed frog skin V-ATPase. A similar labeling pattern in immunoblots was obtained, when this antibody was used to probe microsomal fractions of several mammalian species (Hemken et al., 1992). This may allow interpreting of the antibody-binding sites found in cryosections as V-ATPase-like, corresponding to subunit E of the frog skin V-ATPase. However, it is not possible to distinguish whether this subunit originates from endomembrane or plasma membrane V-ATPases, especially since antibody E11 recognizes both variations (Hemken et al., 1992).

In cytochemistry, strong immunofluorescence may be clearly attributed to the most apical region of the MR cells, an area, in which the apical membrane infoldings were located (Whitear, 1975). This corresponds to the membrane domain, in which the H^+ pump is expected to be situated with regard to the physiological investigations (*see above*). Labeling improved in intensity and in number of cells and with increasing fixative concentration, probably since the antigen is better preserved or trapped in the tissue. In addition in fixed tissue, diffuse immunofluorescence appeared also farther down in the cell. This labeling could be clearly localized intracellularly by confocal microscopy and was not bound to the basolateral membrane. The granular appearance of the labeling in light microscopy is very likely artificial and does not point to certain cellular structures. Therefore, there are several possibilities for interpretation of this immunolabeling: (i) It may be attributed to acidic endosomes in general and not involved in plasma membrane V-ATPase activity. Since GR cells are not labeled, such endosomes would be specific for MR cells or especially numerous there. (ii) It may visualize endocytotic vesicles, loaded with plasma membrane V-ATPase. Consecutive to changes in intracellular pH the H^+ pump activity appears to be regulated by endo/exocytotic recruitment of intracellularly stored molecules (Lacoste, Brochiero & Ehrenfeld, 1993). (iii) Finally, immunolabeling can point to soluble subunit-E peptides or to complete V_1 complexes in the cytosol. The soluble nature of the intracellular antigen is supported by its increased stability dependent on tissue fixation. Cytosolic V_1 complexes in a soluble form are meanwhile found in several cases, in yeast (Doherty & Kane, 1993), in a kidney epithelial cell line (Myers & Forgac, 1993) and in the insect larval midgut (Gräf, Har-

vey & Wieczorek, 1996). Furthermore, in the insect midgut (Sumner et al., 1994) and in yeast (Kane, 1995) it was demonstrated that dissociation of the peripheral V_1 complex is used in vivo for downregulation of V-ATPase activity. Since complete V_0V_1-holoenzyme reappear in the membrane without the need of protein biosynthesis (Kane, 1995; Jäger & Klein, 1996) it is assumed that free V_1 particles can also reassociate for restoration of activity in vivo. Altogether, the intracellular subunit E-immunoreactivity offers several possible mechanisms for regulation of the H^+ pump also in frog skin epithelium which should be confirmed by further analysis combining physiological and electron-immunocytochemical investigations.

No Evidence for Different Types of MR Cells in Frog Skin Epithelium

The pattern of V-ATPase immunolabeling does not suggest the existence of two or more different types of MR cells. In renal cortical collecting tubules, different subtypes of intercalated cells, also rich in mitochondria, have been found (Schwartz, Barasch & Al-Awqati, 1985) distinguished by the reverse location of the plasma membrane V-ATPase and a Cl^-/HCO_3^- exchanger, although always found on opposite membrane domains (Schwartz, Barasch & Al-Awqati, 1985; Brown, Sabolic & Gluck, 1992). For MR cells in the frog skin, the functional localization of a Cl^-/HCO_3^- exchanger is well established in the basolateral membrane (Duranti, Ehrenfeld & Harvey, 1986). Recently, a protein with erythrocyte band-3 immunoreactivity was demonstrated, which was found in the apical membrane of all MR cells in skins of toad and frog (Devuyst et al., 1993). The presence of an apical anionic exchanger would be consistent with the acetazolamide and HCO_3^-/CO_2 dependence of the HCO_3^- excretion in frog skin which is strictly linked to Cl^- absorption from low-salt solutions (Garcia-Romeu & Ehrenfeld, 1975a,b; Ehrenfeld & Garcia-Romeu, 1978). Therefore, both studies of immunolocalization of V-ATPase and Cl^-/HCO_3^- exchanger suggest only one type of MR cell in frog skin epithelium. It has to be mentioned that on the basis of differential sensitivities of Na^+ transport under ''high-Na^+ (Ussing) conditions'' to amiloride or ouabain, Rick (1992) suggested at least three different types of MR cells found in electron microprobe analysis.

The Proton Motive Force of a V-ATPase as Energy Source for Animal Epithelia

With our study we could show that the frog skin indeed has to be added to the increasing number of examples of ion transporting epithelia in which a V-ATPase is located in the plasma membrane (e.g., in crab gills; Onken & Putzenlechner, 1995; or in caterpillar midgut; Wieczorek et al., 1991). Besides its function for regulation of cell and systemic pH by actively secreting protons as known from the urinary epithelia (Gluck, 1992), the proton-motive force of plasma membrane V-ATPases is also used to energize transport of other ions by secondary processes.

We are very grateful to several kind gifts, enabling this study: Dr. S. Gluck, St. Louis, MO, USA, for the monoclonal antibodies, Dr. K. Altendorf, Osnabrück, Germany, for the bafilomycin A_1, and Dr. A. Zeeck, Göttingen, Germany, for the concanamycin A. Further thanks are due to Sandra Schmieder, Villefranche, France, for her help in part of the immunocytochemical work and to Dr. R. Albrecht of the department of Dr. G. Gerisch at the Max Planck Institute of Biochemistry, Munich, Germany, for providing the opportunity using the confocal microscope. This work was supported by the PROCOPE (Programme de Coopération Scientifique, France) and the DAAD (Deutscher Akademischer Austauschdienst, Germany) and by the German Research Foundation DFG (Kl 507-1, and Graduiertenkolleg ''Cellular and molecular aspects of development'')

References

Al-Awqati, Q. 1978. H^+ transport in urinary epithelia. *Am. J. Physiol.* **235:**F77–F88

Aceves, J., Erlij, D. 1971. Sodium transport across the isolated epithelium of the frog skin. *J. Physiol.* **212:**195–210

Bowman, E.J., Siebers, A., Altendorf K. 1988. Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells and plant cells. *Proc. Natl. Acad. Sci. USA* **85:**7972–7976

Brown, D., Gluck, S., Hartwig, J. 1987. Structure of novel membrane coating material in proton secreting epithelial cells and identification as H^+-ATPase. *J. Cell Biol.* **105:**1637–1648

Brown, D., Hirsch, S., Gluck, S. 1988. Localization of a H^+ pumping ATPase in rat kidney. *J. Clin. Invest.* **82:**2114–2126

Brown, D., Sabolic, I., Gluck, S. 1992. Polarized targeting of V-ATPase in kidney epithelial cells. *J. Exp. Biol.* **172:**231–243

Chatterjee, D., Chakraborty, M., Leit, M., Neff, L., Jamsakellokumpu, S., Fuchs, R., Bartkiewicz, M., Hernando, N., Baron, R. 1992. The osteoclast proton pump differs in its pharmacology and catalytic subunits from other vacuolar H^+-ATPases. *J. Exp. Biol.* **172:**193–204

Devuyst, O., Rott, R., Denef, J.F., Crabbe, J., Katz, U. 1993. Localization of a Band 3-related protein in the mitochondria-rich cells of amphibian skin epithelium. *Biol. Cell.* **78:**217–221

Doherty, R.D., Kane, P. 1993. Partial assembly of the yeast vacuolar H^+ ATPase in mutants lacking one subunit of the enzyme. *J. Biol. Chem.* **268:**16845–16851

Dröse, S., Bindseil, K.U., Bowman, E.J., Siebers, A., Zeeck, A., Altendorf K. 1993. Inhibitory effect of modified bafilomycins and concanamycins on P- and V-adenosine triphosphatases. *Biochemistry* **32:**3902-3906

Duranti, E., Ehrenfeld, J., Harvey, B.J. 1986. Acid secretion through the *Rana esculenta* skin: Involvement of an anion exchanger mechanism is at the basolateral membrane. *J. Physiol.* **378:**195–211

Ehrenfeld, J., Garcia-Romeu, F. 1977. Active hydrogen excretion and sodium absorption through isolated frog skin. *Am. J. Physiol.* **233:**F46–F54

Ehrenfeld, J., Garcia-Romeu, F. 1978. Coupling between chloride absorption and base excretion in isolated skin of *Rana esculenta*. *Am. J. Physiol.* **4:**F33–F39

Ehrenfeld, J., Garcia-Romeu, F., Harvey, B.J. 1985. Electrogenic active H^+ pump in *Rana esculenta* skin and its role in Na^+ ion transport. *J. Physiol.* **359:**331–355

Ehrenfeld, J., Lacoste, I., Harvey, B.J. 1989. The key role of the mitochondria-rich cell in Na^+ and H^+ transport across the frog skin epithelium. *Pfluegers Arch.* **414:**59–67

Ehrenfeld, J., Lacoste, I., Garcia-Romeu, F., Harvey, B.J. 1990. Interdependence of Na^+ and H^+ transport in frog skin. *Comp. Physiol.* **6:**152–170

Garcia-Romeu, F., Ehrenfeld, J. 1972. The role of ionic exchangers and pumps in transepithelial sodium and chloride transport across frog skin. *In*: Role of membranes in secreting processes. L. Bolis, R.D. Keynes, and W. Wilbrandt, editors. pp. 264–268. Amsterdam, North Holland

Garcia-Romeu, F., Ehrenfeld, J. 1975*a*. In vivo Na^+ and Cl^-- independent transport across the skin of *Rana esculenta*. *Am. J. Physiol.* **228:**839–844

Garcia-Romeu, F., Ehrenfeld, J. 1975*b*. Chloride transport through the non short-circuited isolated skin of *Rana esculenta*. *Am. J. Physiol.* **228:**845–849

Garcia-Romeu, F., Salibian, A., Pezzani-Hernandez, S. 1969. The nature of the in vivo sodium and chloride uptake mechanisms through the epithelium of the chilean frog *Calyptocephalella gayi* (Dum et Bibr. 1841). *J. Gen. Physiol.* **53:**816–35

Giunta, C., De Bortoli, M., Ytacchini, A., Sanchinni, M. 1984. Na^+/K^+-ATPase from *Xenopus laevis* (Daudin) kidney and epidermis: high sensitivity towards regulatory compounds. *Comp. Biochem. Physiol.* **79:**71–74

Gluck, S. 1992. V-ATPases of the plasma membrane. *J. Exp. Biol.* **172:**29–37

Gluck, S., Caldwell, J. 1987. Immunoaffinity purification and characterization of vacuolar H^+-ATPase from bovine kidney. *J. Biol. Chem.* **262:**15780–15789

Gluck, S., Cannon, C., Al-Awqati, Q. 1982. Exocytosis regulates urinary acidification in turtle urinary bladder by rapid insertion of H^+ pumps into the luminal membrane. *Proc. Natl. Acad. Sci. USA* **79:**4327–4331

Gräf, R., Harvey, W.R., Wieczorek H. 1996. Purification and properties of a cytosolic V_1-ATPase. *J. Biol. Chem.* **271:**20908–20913

Harvey, B.J. 1992. Energization of sodium absorption by the H^+-ATPase pump in mitochondria-rich cells of frog skin. *J. Exp. Biol.* **172:**289–309

Hemken, P., Guo, X.-L., Wang, Z.-Q., Zhang, K., Gluck, S. 1992. Immunological evidence that vacuolar H^+-ATPase with heterogeneous forms of M_r = 31,000 subunit have different membrane distributions in mammalian kidney. *J. Biol. Chem.* **267:**9948–9957

Hirsch, S., Strauss, A., Masood, K., Lee, S., Sukhatme, V., Gluck, S. 1988. Isolation and sequence of a cDNA clone encoding the 31-kDa subunit of bovine kidney vacuolar H^+-ATPase. *Proc. Natl. Acad. Sci. USA* **85:**3004–3008

Jäger, D., Klein, U. 1996. Biogenesis and regulation of V-ATPase in the midgut of the tobacco hornworm. *Verh. Dtsch. Zool. Ges.* **89:**327

Kane, P.M. 1995. Disassembly and reassembly of the yeast vacuolar H^+ ATPase in vivo. *J. Biol. Chem.* **270:**17025–17032

Klein, U., Löffelmann, G., Wieczorek, H. 1991. The midgut as a model system for insect potassium transporting epithelia: immunocytochemical localization of a vacuolar-type H^+ pump. *J. Exp. Biol.* **161:**61–75

Krogh, A. 1938. The active absorption of ions in some fresh water animals. *Z. Vergl. Physiol.* **25:**335–350

Lacoste, I., Brochiero, E., Ehrenfeld, J. 1993. Control of Na^+ and H^+ transports by exocytosis/endocytosis phenomena in a tight epithelium. *J. Membrane Biol.* **134:**197–212

Myers, M., Forgac, M. 1993. Assembly of the peripheral domain of the bovine vacuolar H^+ ATPase. *J. Cell Physiol.* **156:**35–42

Nelson, N. 1992. The vacuolar H^+-ATPase—one of the most fundamental ion pumps in nature. *J. Exp. Biol.* **172:**19–27

Onken, H., Putzenlechner, M. 1995. A V-ATPase drives active, electrogenic and Na^+-independent Cl^- absorption across the gills of *Eriocheir sinensis*. *J. Exp. Biol.* **198:**767–774

Pedersen, P.L., Carafoli, E. 1987. Ion motive ATPases. I. Ubiquity, properties, and significance to cell function. *Trends Biochem.* **12:**146–150

Rick, R. 1992. Intracellular ion concentrations in the isolated frog skin epithelium: Evidence for different types of mitochondria-rich cells. *J. Membrane Biol.* **127:**227–236

Schwartz, G.J., Barasch, J., Al-Awqati, Q. 1985. Plasticity of functional epithelial polarity. *Nature* **318:**368–371

Schweikl, H., Klein, U., Schindlbeck, M., Wieczorek, H. 1989. A vacuolar-type ATPase, partially purified from potassium transporting plasma membranes of tobacco hornworm midgut. *J. Biol. Chem.* **264:**11136–11142

Sumner, J.P., Dow, J.A.T., Earley, F.G.P., Klein, U., Jäger, D., Wieczorek, H. 1995. Regulation of plasma membrane V-ATPase activity by dissociation of peripheral subunits. *J. Biol. Chem.* **270:**5649–5653.

Whitear, M. 1975. Flask cells and epidermal dynamics in frog skin. *J. Zool. Lond.* **175:**107–149

Wieczorek, H., Putzenlechner, M., Zeiske, W., Klein, U. 1991. A vacuolar-type H^+ pump energizes K^+/H^+-antiport in an animal plasma membrane. *J. Biol. Chem.* **266:**15340–15347

Wieczorek, H., Harvey, W.R. 1995. Energization of animal plasma membranes by the proton-motive force. *Physiol. Zool.* **68:**15–23

Zhang, J.M., Feng, Y., Forgac, M. 1994. Proton conduction and bafilomycin binding by the V_0 domain of the coated vesicle V-ATPase. *J. Biol. Chem.* **269:**23518–23523