V-type $\text{H}^+$-ATPase in the human eccrine sweat duct: immunolocalization and functional demonstration

D. GRANGER, M. MARSOLAI S, J. BURRY, and R. LAPRADE


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Granger, D., M. Marsolais, J. Burry, and R. Laprade. V-type $\text{H}^+$-ATPase in the human eccrine sweat duct: immunolocalization and functional demonstration. Am J Physiol Cell Physiol 282: C1454–C1460, 2002; 10.1152/ajpcell.00319.2001.—We investigated for the presence of a vacuolar-type $\text{H}^+$-ATPase (V-ATPase) in the human eccrine sweat duct (SD). With the use of immunocytochemistry, an anti-V-ATPase antibody showed a strong staining at the apical membrane and a weaker one in the cytoplasm. Cold preservation followed by rewarming did not alter this staining pattern. With the use of the pH-sensitive dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein on isolated and perfused straight SD under $\text{HCO}_3^-$-free conditions and in the absence of $\text{Na}^+$, proton extrusion was determined from the recovery rate of intracellular pH (dpH/dt) following an acid load. Oligomycin (25 $\mu$M), an inhibitor of F-type ATPases, decreased dpH/dt by 88 ± 6%, suggesting a role for an ATP-dependent process involved in pH recovery. Moreover, dpH/dt was inhibited at 95 ± 3% by 100 nM luminal concanamycin A, a specific inhibitor of V-ATPases, whereas 10 $\mu$M bafilomycin A1, another specific inhibitor of V-ATPases, was required to decrease dpH/dt by 73%. These results strongly suggest that a V-ATPase is involved in proton secretion in the human eccrine SD.

Address for reprint requests and other correspondence: R. Laprade, Université de Montréal, GRTM, C.P. 6128, Succursale Centre-ville, Montreal, Quebec, Canada H3C 3J7 (E-mail: Raynald.Laprade@umontreal.ca).

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The secretory portion of the eccrine sweat gland generates a fluid called the primary secretion. The composition of this isotonic fluid is similar to an ultrafiltrate of the plasma and has a pH of 7.4. However, as it flows through the ductal portion of the gland, this fluid is modified by the reabsorption of solutes such as $\text{Na}^+$, $\text{Cl}^-$, lactate, and $\text{HCO}_3^-$, leaving a hypotonic sweat. Current models of ionic transport in the human eccrine sweat duct propose the presence of luminally located $\text{Na}^+$ and $\text{Cl}^-$ channels and basolaterally located $\text{Na}^+-\text{K}^+$-ATPase, as well as $\text{K}^+$ and $\text{Cl}^-$ channels. The pH of final sweat is a function of sweat rate, being as acidic as pH 5 when the sweat rate is low and increasing as sweat rate increases. The acidity of sweat implies that sweat duct cells secrete protons across the apical membrane, and the presence of an electrogenic proton-secreting pump at this membrane has been proposed. Indeed, vacuolar-type $\text{H}^+$-ATPases (V-ATPases) have been shown to be responsible for the acidification in many organelles of eukaryotic cells, including clathrin-coated vesicles, lysosomes, endosomes, and vacuoles of plants and fungi. Moreover, V-ATPases are highly expressed in the apical membrane of specialized epithelial cells in the kidney, epididymis, and vas deferens, where they play a major role in the acidification of urine and luminal fluid of the reproductive tract. Recently, in the sweat duct, we have demonstrated by both immunolocalization and intracellular pH (pHi) measurements that both $\text{Na}^+/\text{H}^+$ exchanger isoforms 1 (NHE1) and 3 (NHE3) were absent from the luminal membrane, whereas NHE1 was present at the basolateral membrane. In addition, an immunolocalization study showed the presence of an apical V-ATPase.

Therefore, the aim of the present work was to further the recent immunolocalization studies and to demonstrate the functional activity of the apical V-ATPase in the sweat duct. For this purpose, we microperfused straight sweat ducts in vitro and measured the effect of oligomycin, an inhibitor of mitochondrial ATP synthase (FoF1) (39) and specific inhibitors of V-ATPases, bafilomycin A1 (8) and concanamycin A (19–21), on dpH/dt and on the pH recovery rate from an intracellular acid load. Both $\text{HCO}_3^-$ and $\text{Na}^+$ were absent from the solutions to eliminate the possible contributions of these ions to proton transport.

MATERIALS AND METHODS

Immunocytochemistry

Tissue fixation. Specimens of human healthy skin (female) were taken, after esthetic surgery, with consent from patients and ethics committee approval. Skin was conserved at 4°C in Dulbecco’s phosphate-buffered saline containing $\text{CaCl}_2$ and $\text{MgCl}_2$ from Sigma Chemical (St. Louis, MO) until use. Sections of human skin were fixed for 1 h in 2% paraformaldehyde, 75 mM lysine, and 10 mM sodium periodate (PLP) at room temperature. Slices (3 mm) were cut with scissors and fixed overnight at 4°C in fresh PLP. The tissue was moved in phosphate buffer containing 1.9 mM $\text{NaH}_2\text{PO}_4$, 0.5 mM $\text{Na}_{2}\text{HPO}_4$, and 0.15 M $\text{NaCl}$.
8.1 mM Na2HPO4, 0.9% NaCl, and 0.02% NaN3, pH 7.4 (PBS), until the cryostat step. 

Effect of temperature. In another series of experiments, we fixed skin samples after a rewarming period of 0, 15, 30, and 60 min at 37°C in Dulbecco’s phosphate buffer. The fixation step for this experiment was done at 37°C in a PLP solution.

Cryostat sectioning. Sections infiltrated with 30% sucrose and frozen in liquid nitrogen were covered with a drop of Tissue-Tek embedding medium (Sakura, Torrance, CA) and cut at a thickness of 5 μm on a cryostat −25°C. Sections were picked up onto Fisher Superfrost Plus-charged glass slides and stored at −20°C.

Antibodies. The proton pump antibody is a rabbit anti-serum raised against the COOH-terminal 14 amino acids of the 31-kDa subunit (E subunit) of the V-ATPase (13) (pro-vided by Dr. Dennis Brown, Massachusetts General Hospital, Charlestown, MA), used at a 1:100 dilution. The secondary antibody, a goat anti-rabbit IgG-FITC (ImmunoResearch, West Grove, PA) was used at 7.5 μg/ml. Negative controls, including omission of primary antibodies, and preimmune serum were performed. Sections of rat kidney were used as positive control.

Incubation procedure. The incubation procedures were based on the protocol described by Brown et al. (15). The standard procedure and also the antigen unmasking procedure, in which an incubation with 1% SDS was performed, were completed (15). The incubation of sections with antibodies was carried out overnight at 4°C for the anti-proton pump antibody and for 2 h at room temperature for the secondary antibody.

Photography. Tissues were viewed at ×400 magnification with a Nikon Fluor 40/1.3 oil objective in a Nikon epifluorescence microscope with fluorescein filters (excitation 450–490 nm, barrier 510 nm). Sections were photographed in black and white with a Nikon FX-35DX camera on Kodak TMax 400 film at 1600 ASA.

Materials and reagents. Sodium nitrate was purchased from Anachemica Chemicals (Montreal, Canada). BDH Laboratories (Poole, England) supplied l-lysine monohydrochloride. All other chemical products were purchased from Sigma Chemical.

Measurements of pHi

Isolation and microperfusion of sweat duct. Eccrine sweat glands were isolated by using a previously described shearing technique (28). Portions of straight reabsorptive duct were dissected with sharpened forceps and microperfused in vitro, as previously described for kidney tubule (1). Visual criteria for luminal perfusion and duct viability were validated by using basolateral membrane potential measurements that were stable for >1 h and that responded to luminal Na+ and Cl− substitutions. Bath solution temperature was kept at 37°C, and the flow of the bath solution (flow rate >1 ml/min) was aimed directly at the sweat duct as described by Macrì et al. (29). The luminal perfusion rate was high enough to eliminate axial changes in the luminal fluid composition.

pH. pHi was measured with the fluorescent probe 2’,7’-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF). The acetoxyethyl ester form of the dye (BCECF-AM) was added to the bath solution at a final concentration of 5 μM, and loading was allowed in the nonperfused bath for 5–10 min at 25°C. After loading, the bath was perfused with control solution at 37°C for at least 5 min before an experiment was begun. The duct was alternately excited at 450 and 500 nm, and the emitted fluorescence was monitored at 530 nm with the photomultiplier-based spectrofluorimeter PTI D104 (Photon Technology International, London, Ontario) linked to a computer. The fluorescence ratio (F500/F450) corrected for autofluorescence was calculated and converted to pH, at the end of each experiment by calibration with the high-K+-nigericin method (38).

Acid loading. Sweat ducts were H+ loaded with the ammonia prepulse technique. NH4Cl (20 mM) was added to the bath solution for 30–60 s and then removed, resulting in abrupt intracellular acidification.

Solutions and chemicals products. The HCO3−-free control solution used to perfuse and bathe the sweat ducts contained (in mM) 114 NaCl, 25 sodium gluconate, 2.5 K2HPO4, 1 MgCl2, 1 CaCl2, 5 glucose, and 4 sodium lactate. Osmolality was adjusted to 300 mosmol/kgH2O with mannitol and pH adjusted to 7.4 with Tris-HEPES. For experiments done in the absence of Na+, sodium lactate and NaCl were replaced by N-methyl-D-glucamine (NMDG)-lactate and -chloride, respectively.

BCECF-AM was obtained from Molecular Probes (Eugene, OR) and stored in a freezer as a 1 mM stock in 95% ethanol. NH4Cl was purchased from Fisher Scientific (Fair Lawn, NJ). Bafilomycin A1, diluted in dimethyl sulfoxide (DMSO) and stored for 4°C, was the gift of Dr. S. Pathak (SmithKline Beecham). Concanamycin A (also known as Folinycin) was from Sigma Chemical and was diluted in DMSO and stored at −20°C. The oligomycin used was a mixture of oligomycins A, B, and C (~65% oligomycin A) diluted in ethanol (Sigma Chemical). The final concentration of DMSO or ethanol in any solution did not exceed 0.1% and had no effect on pHi.

All other chemical products were purchased from Sigma Chemical.

Statistics. Results are presented as means ± SE. Statistical significance was analyzed by using the paired or unpaired Student t-test. Significance was accepted at P ≤ 0.05.

RESULTS

Distribution of V-ATPase in the Sweat Duct

Immunostaining. To determine the cellular localization of proton pump in the human eccrine sweat duct, we used a rabbit anti-V-ATPase antibody on cryosections (5 μm) of PLP-fixed human skin. Comparison between phase-contrast and fluorescent images (Fig. 1, A and B) shows that the luminal cells of the sweat duct are heavily labeled at their apical pole. Also, cytoplasmic staining is shown, suggesting a redistribution of V-ATPase into cytoplasmic vesicles. In the secretory coil, we obtained a discontinued and weaker apical staining compared with that shown in the reabsorptive sweat duct (Fig. 1D). Moreover, no difference appeared in V-ATPase distribution when we used an SDS unmasking technique for antigen retrieval epitope (15), although the intensity of staining was slightly increased (not shown). Control incubation with the use of preimmune rabbit serum gave no detectable staining (not shown). Sections of rat kidney were used as positive control and gave results identical to those previously published by Brown and coworkers (12, 13).

Effect of temperature. In kidney-intercalated cells, it has been shown that cold preservation followed by rewarming to 37°C induces a marked redistribution of proton pumps into endocytotic vesicles (10). However, no significant difference in the distribution of proton
pumps was identified on secretory coil and sweat duct cells after a period of rewarming preceding tissue fixation, compared with control (without rewarming). In all samples (rewarming during 0, 15, 30, and 60 min), a strong V-ATPase staining, identical to that shown in Fig. 1B, was always present at the apical membrane of sweat ducts.

**Functional Activity of the Proton Pump in the Sweat Duct**

**Acid load and pH$_i$ recovery.** To investigate the functional activity of protons pumps, we monitored the rate of pH$_i$ recovery, following an acute acid load induced by a 20 mM NH$_4^+$ pulse, on perfused sweat ducts. We measured the recovery rate of pH$_i$ (dpH/dt) in the absence of HCO$_3^-$ to eliminate the contribution of potential bicarbonate transporters. Under control conditions, we measured a baseline pH$_i$ of 7.33 ± 0.03 (n = 41). Addition of 20 mM bath NH$_4$Cl followed by its withdrawal produced a sharp fall of pH$_i$ with subsequent recovery within 3 min (Fig. 2, A and B, first pulse). Na$^+$ was then replaced by NMDG, in both bath and lumen, to inhibit all potential Na$^+$/H$^+$ exchangers, such as the basolateral NHE1 that we recently identified in this tissue (25). Under these conditions, after a strong decrease of pH$_i$ due to the reversal of the basolateral Na$^+$/H$^+$ exchanger, a second NH$_4$Cl pulse was applied. Two types of response were obtained following the acid load: 1) a slower pH$_i$ recovery, decreased by 65 ± 6% (n = 14) with respect to control, which occurred in most of the sweat ducts (14 of 21 tested) (see Figs. 2A and 6A); and 2) no significant pH$_i$ recovery following the acid load, which occurred in about one-third of the ducts tested (Fig. 2B).

**ATPase inhibitors.** To investigate whether the pH$_i$ recovery in response to the acid load in the absence of Na$^+$ and HCO$_3^-$ is ATP dependent, we tested the effect of oligomycin, an inhibitor of mitochondrial ATP-synthase (F-type ATPases) (39). Addition of oligomycin (25 μM) to bath and luminal solutions had no effect on basal pH$_i$ but strongly inhibited pH$_i$ recovery after the acid load (Figs. 3 and 6B). A rapid recovery of pH$_i$ was observed when control solutions (containing Na$^+$) were reperfused in lumen and bath, likely due to the activity of the basolateral Na$^+$/H$^+$ exchanger.

Because the previous result suggests that pH$_i$ recovery is ATP dependent, we tested different inhibitors of V-ATPases. Figure 4 shows a typical tracing where addition of luminal baflomycin A$_1$, a specific inhibitor of V-ATPases (8), inhibits dpH/dt. On average, 1 μM baflomycin A$_1$ produced an inhibition of 28% (n = 3), whereas with 10 μM baflomycin A$_1$, a 73% inhibition (n = 4) was observed (see Fig. 6B). Additional experiments at 0.1 μM led to inhibition of 28 and 40%. The effect of 10 μM baflomycin A$_1$ observed in Fig. 4 is the same when it is applied immediately after the control NH$_4^+$ pulse (not shown) and eliminates the possibility of a run-down effect. No change of steady-state pH$_i$ occurred when baflomycin A$_1$ was added to the luminal solution.

A third inhibitor tested was concanamycin A, another specific inhibitor of V-ATPases (19–21). As
shown in Fig. 5, addition of 100 nM concanamycin A in the luminal solution led to a slow acidification of 0.16 pH units within 5 min. Moreover, no pHi recovery was observed (n = 4) following the acid load, suggesting total inhibition of the V-ATPase by concanamycin A (Figs. 5 and 6B). As in Fig. 3, a recovery of pHi was observed with the reintroduction of Na⁺ in bath and luminal solutions.

DISCUSSION

Distribution of V-ATPase

Using an antibody against the 31-kDa subunit of the V-ATPase, we confirmed the presence of a V-ATPase in human eccrine sweat gland. In the duct, our results show that V-ATPases are more concentrated at the apical membrane but are also present within distinct structures into the cytoplasm, possibly acidic organelles (Fig. 1B). In addition, secretory coil cells...
In the presence of luminal bafilomycin A1 (1 and 10 nM), observed on 14 of 21 tested sweat ducts, a weak, discontinued apical staining (Fig. 1D) was present. In contrast to kidney intercalated cells, cold preservation followed by rewarming to 37°C does not lead to any difference in the distribution of V-ATPases in sweat duct compared with ducts fixed without rewarming. Indeed, a complete disappearance of apical proton pump staining in intercalated cells was shown following this treatment, which induced a marked redistribution of the V-ATPases into endocytotic vesicles (10). In the kidney, it was demonstrated that the cytoskeleton is disrupted by cold while the proton pumps remain at the apical membrane. However, when kidney collecting ducts are rewarmed, the pumps are temporarily internalized into vesicles until the cytoskeleton is regenerated, within ~1 h. It therefore appears that V-ATPases of the sweat ducts are not internalized following rewarming at 37°C and remain at the apical membrane.

**Functional Characterization of V-ATPase**

**Effect of Na⁺ removal.** In the absence of Na⁺, dpHᵢ/dt decreased by 65 ± 5% in most sweat ducts (14 of 21 tested), in agreement with the presence of a basolateral Na⁺/H⁺ exchanger (Fig. 2A) (25). However, for the other 7 ducts, we measured little or no pHᵢ recovery (Fig. 2B). It is unlikely that these ducts were damaged, because a pHᵢ recovery was observed as soon as Na⁺ was added to the bath solution. The most probable hypothesis for the different responses upon an acid load is that an axial heterogeneity may exist through the ductal portion of the sweat gland, as in the kidney tubule (9), although no clear differences in staining patterns for V-ATPases appear in sections of sweat ducts tested with immunochemistry.

**Effect of oligomycin.** The nearly complete inhibition of pHᵢ recovery by oligomycin demonstrates that the transporter involved in pHᵢ recovery after the acid load in the absence of Na⁺ and HCO₃⁻ requires ATP (Fig. 3). Indeed, oligomycin very rapidly decreases the level of cellular ATP. The inhibitory effect of oligomycin on pHᵢ recovery does not exclude the possibility that an H⁺-K⁺-ATPase, also requiring ATP and playing a role in luminal acidification such as in the gastrointestinal tract (5) and kidney (40), could be involved in this response. However, a previous study of Reddy and Quinton (36) based on electrophysiology and intracellular K⁺ measurements concluded that neither a K⁺ conductance nor a K⁺-dependent carrier transport system exists at the apical membrane of the sweat duct.

**Effect of bafilomycin A₁.** It has been shown that inhibition of V-ATPases occurs at nanomolar concentrations of bafilomycin A₁. However, it also has been reported that 10 μM bafilomycin A₁ is necessary to obtain half-maximal inhibition of the V-ATPase in the Malpighian tubules of an ant (18). It has been proposed that accessibility to the target sites of the H⁺-ATPase may be responsible for the different affinities observed in different preparations. Also, one cannot exclude the possibility of slightly different binding sites to explain these differences. Our results already show an inhibition at 100 nM that reaches 73% at 10 μM. Therefore, together with the evidence reported above (36), the fact that 50 μM bafilomycin is necessary to inhibit 50% of

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**Fig. 6. Summary of effects of inhibitors on pHᵢ recovery, following an acid load, in HCO₃⁻-free solutions. A: recovery rate in presence (control) and absence of Na⁺ (0 Na⁺), observed on 14 of 21 tested sweat ducts. B: relative recovery rate with respect to 0 Na⁺ condition in the presence of luminal bafilomycin A₁ (1 and 10 μM), oligomycin (25 μM), and concanamycin A (100 nM). Data are presented as means ± SE; n = no. of experiments. Results are statistically very significant (**P < 0.01) or significant (*P < 0.05) with respect to 0 Na⁺ condition.**
the gastric H⁺-K⁺-ATPase (30) makes it unlikely that the effect of baflomycin is on an H⁺-K⁺-ATPase.

**Effect of concanamycin A.** Compared with baflomycin A₁, concanamycin A showed a much stronger inhibition of 95% of pHᵢ recovery at 100 nM (Figs. 5 and 6B). In addition, concanamycin A is the only tested inhibitor that produced an effect on steady-state pHᵢ, with an acidification of 0.16 pH units in 5 min. This result demonstrates that the apical V-ATPase in sweat duct is active at resting cell pHᵢ.

Dröse et al. (20, 21) were the first to demonstrate that concanamycins are even more potent inhibitors than the baflomycins A₁ of the activity of the Kdp-ATPase from *Escherichia coli* and the V-ATPase from *Neurospora crassa*, with an IC₅₀ between two and five times lower and with a slightly different active site. Similarly, concanamycin A requires one-tenth the dose of baflomycin A₁ for inhibiting lysosome acidification and V-ATPase activity (37). It has been proposed that the increased sensitivity of V-ATPases for concanamycin is on an H⁺-ATPase vs. baflomycin A₁. Our results with all tested inhibitors demonstrated an apical proton secretion activity that is much more sensitive to concanamycin A than to baflomycin A₁. Our results with all tested inhibitors strongly suggest that the inhibition of pHᵢ recovery is due to the inhibition of a V-ATPase. Because no other proton transporter activity has yet been identified at the apical membrane of the human eccrine sweat duct, and particularly because V-ATPase expression is very low in the secretory portion of the gland, we suggest that the apical V-ATPase is probably involved in active luminal acidification of sweat in the duct.

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