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ATPase ACTIVITY AND ATP-DEPENDENT PROTON TRANSLOCATION IN PLASMA MEMBRANE VESICLES OF TURTLE BLADDER EPITHELIAL CELLS

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ATP-induced quenching of fluorescence of acridine orange (a pH probe) or Oxonol V (a potential difference probe) is evoked in turtle bladder membrane vesicles in suspending media of appropriate ionic composition and is insensitive to oligomycin, valinomycin, and ouabain. These effects are ascribed to a membrane-bound, ouabain-resistant ATPase which mediates an active electrogenic proton transport.

Electrogenic proton-translocating ATPase complexes have been found in the membranes of subcellular bodies such as mitochondria [1–3] and chloroplasts [4–6] as well as in the plasma membranes of bacterial [7–9], plant [10], and fungal cells such as *Neurospora* [11,12]. In addition, a non-electrogenic proton translocating ATPase, operating as a K^+–H^+ antiporter, has been found and well characterized in isolated plasma membrane vesicles of gastric parietal cells [13–15].

In relation to these studies, we have previously shown that an isolated plasma membrane fraction from turtle urinary bladder epithelial cells contains a ouabain-resistant (Mg^{2+}-dependent) ATPase activity [16] which was subsequently separated electrophoretically from the ouabain-inhibitable ($Na^+ + K^+$)-ATPase activity [17]. Since the epithelial cells acidify luminal fluids in the intact turtle bladder [18–20], we decided to determine whether the isolated plasma membrane fraction of these cells possesses an active, ATP-dependent, electrogenic proton translocating activity, which is catalyzed by its ouabain-resistant ATPase element. In the experiments described here, we present evidence for the presence of an ATP-driven, ouabain-resistant electrogenic transport of protons in suspensions of plasma membrane vesicles isolated from turtle bladder epithelial cells. In contrast to the isolated mitochondrial fraction of the same cells, neither the ATPase activity nor the proton translocating activity of the plasma membrane fraction is inhibited by oligomycin.

Epithelial cells were removed from groups of twenty or more bladders by the method of Lipman et al. [21] after which a light weight plasma membrane vesicle fraction, a heavier membrane fraction (so-called fluffy layer), and a mitochondrial fraction were isolated by differential centrifugation as described elsewhere [16,17]. The fractions were used fresh or were suspended in 1.0 M sucrose and stored for 1–2 weeks at −20°C until used. Membrane protein was measured using the method of Lowry et al. [22] and assays for ATPase activity were those described previously [23]. Gradients of

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Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DCCD, *N,N'*-dicyclohexylcarbodiimide; $\Delta\mu_{H^+}$, transmembrane H^+-electrochemical gradient; Oxonol-V, bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol; ΔpH, transmembrane pH gradient; $\Delta\Psi$, transmembrane potential difference.

pH (ΔpH) across vesicle membranes were detected by measuring the fluorescence intensity of the dyes, quinacrine or acridine orange, in suspensions of the vesicles at room temperature (22°C). The technique was that recommended for gastric epithelial cell membranes [14,15], in which a decrease in fluorescence intensity of acridine orange has been shown to indicate in the pH of the intravesicular fluid relative to that of the extravesicular fluid. In the present system, vesicles of the light plasma membrane fraction (50 to 1000 μg of protein) were suspended in a solution containing: 0–280 mM sucrose; 100 mM KCl or NaCl; 3 mM $MgCl_2$ or $MgSO_4$; 10 mM Tris-Hepes (pH, 7.3); 4.0 μM quinacrine or 0.5–1.0 μM acridine orange; and when indicated: 5–15 μg oligomycin per mg membrane protein; and/or 0.1 mM ouabain; and/or 2.0 uM valinomycin in a final volume of 0.6–0.7 ml. The cuvette containing this mixture was placed in a Perkin-Elmer 650–10S fluorescence spectrophotometer, from which the intensity of the emitted light beam (at 500 nm for quinacrine or 526 nm for acridine orange) was measured at 90° to the excitatory incident beam (420 nm for quinacrine or 491 nm for acridine orange) and monitored on a Perkin-Elmer 023 strip chart recorder. Light scattering measurements were made on the same fluorometer serving as a nephelometer. With the latter measurements, the vesicular form and the osmotically-responsive nature of the light plasma membrane fractions were established in a manner recommended elsewhere [15].

It was found that the relative fluorescence intensity of acridine orange or quinacrine decreased following the addition of ATP to suspensions of isolated membrane vesicles; and that this effect was reversed, returning fluorescence to baseline, after subsequent addition of the protonophore, FCCP, or after the addition of nigericin (Figs. 1a and 1b). Additional observations (Fig. 1b) were the following: (i) In the presence of the highly reactive and non-specific inhibitor, DCCD (at 300 uM), the ATP-induced fluorescence quenching response was reduced to one third of control and the subsequent addition of FCCP was followed by a small but transient increase in the fluorescence. The lack of FCCP-induced dequenching of quinacrine fluorescence, for which there is no ready explanation, was not found when acridine orange

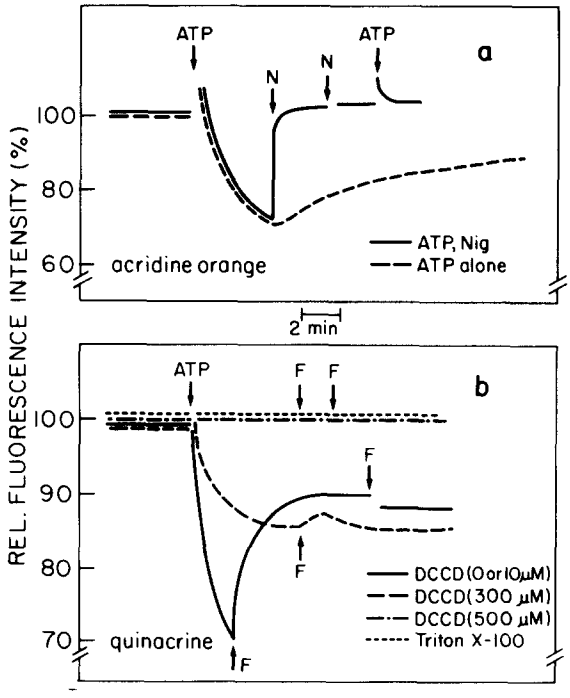

Fig. 1. (a) Relative fluorescence intensity of acridine orange (λ_{ex} = 491 nm, λ_{em} = 526 nm) versus time in a suspension of turtle bladder epithelial cell plasma membrane vesicles before and after 1 mM ATP addition with (———) and without (- - - - -) subsequent additions of 1.0 μM nigericin (N). The medium contained: 400 μg membrane protein; 100 mM KCl; 10 mM Tris-Hepes (pH 7.3); 3 mM $MgCl_2$; 0.1 mM ouabain; 12 μg per mg membrane protein oligomycin; and 1.0 μM acridine orange; final volume, 660 μl. Relative fluorescence intensity of quinacrine (λ_{ex} = 420 nm, λ_{em} = 500 nm) versus time in a plasma membrane vesicle suspension similar to that described in panel (a), but containing 900 μg membrane protein and 4.0 μM quinacrine replacing acridine orange, in the presence of 0.5% Triton X-100 (· · · · ·) or 500 μM DCCD (· – · – ·); or 300 μM DCCD (- - - - -); or no additions, the control state (———); before and after 1 mM ATP and with subsequent additions of 2.0 μM FCCP (F).

was used as the fluorescent ΔpH probe. (ii) The ATP-induced fluorescence decrease and its reversal by FCCP were completely blocked in the presence of 500 μM DCCD or in the presence of the membrane-disruptive detergent, Triton X-100 (0.5%). (iii) However, these parameters were not altered in the presence of oligomycin, at levels far greater than those needed for inhibition of the turtle bladder mitochondrial ATPase and at levels 3–10-fold greater than those required for inhibition of the ATP-driven proton translocation in

bovine heart sub-mitochondrial particles [24]. Nor were these fluorescence changes altered in the presence of ouabain at levels greater than those needed for inhibition of the $(Na^+ + K^+)$-ATPase [16,23]. Thus, the ATP-induced fluorescence changes were not due to the mitochondrial ATPase or to the $(Na^+ + K^+)$-ATPase activity. (iv) In a set of parallel experiments on the ouabain-resistant ATPase activity of these vesicles, it was found that the DCCD-induced inhibition of this enzyme activity was roughly parallel to the DCCD-induced blockade of the ATP-dependent proton translocation (see below).

Without effect on the ATP- and protonophore-induced changes in proton tranlocation (fluorescence changes) were (i) the removal of K^+ by substituting NaCl or choline chloride for KCl in the medium; or (ii) the removal Cl^- by substituting KNO_3 and $MgSO_4$ for KCl and $MgCl_2$. On the other hand, these fluorescence changes were markedly diminished when the plasma membrane vesicles were suspended in a potassium gluconate-containing incubation mixture devoid of both Cl^- and NO_3^-; and completely abolished after suspending the vesicles in an incubation mixture devoid of all ions (sucrose replacement) except for Tris-Hepes and $MgSO_4$. With vesicles that had been pre-incubated overnight at 0°C in 100 mM potassium gluconate, the ATP-induced fluorescence quenching at 25°C was stimulated by valinomycin. Valinomycin had no effect on the ATP-induced fluorescence changes when the vesicles were incubated in KCl or KNO_3 media. The electrogenic nature of this proton translocation was directly established in preliminary experiments by virtue of an observed ATP-dependent quenching of fluorescence of the potential difference-detecting anionic probe, Oxonol V.

These data can be explained by assuming that (i) an ATP-driven, electrogenic proton translocating mechanism resides in the vesicular membranes; that (ii) in parallel with this pump mechanism, are Cl^--selective (or NO_3^--selective) conductance paths; and that (iii) there is little or no conductance for K^+ or H^+ in the absence of the appropriate ionophore.

These transport changed could be correlated, in a semi-quantitative manner, with the Mg^{2+}-dependent, ouabain-resistant ATPase activity in the light weight plasma membrane fraction, but not with the ATPase activity in paired mitochondrial fractions obtained from the same batches of turtle bladder epithelial cells (Table I). The mitochondrial ATPase was maximally inhibited by oligomycin (at concentrations as low as 5 μg/mg membrane protein) or by DCCD (at concentrations as low as 10 μM). In contrast, the plasma membrane ATPase was not affected by 10 μm DCCD and was resistant to oligomycin at a concentration of 1100 μg/mg protein, which is over 200-times that needed for inhibition of the

TABLE I

MEAN VALUES (±S.E.) FOR OUABAIN-RESISTANT ATPase ACTIVITY IN PLASMA MEMBRANE AND MITOCHONDRIAL FRACTIONS POOLED FROM THE ISOLATED EPITHELIAL CELL HOMOGENATE OF 20 TURTLE BLADDERS WITH AND WITHOUT ADDITION OF OLIGOMYCIN AND DCCD

Composition of assay medium: 85 mM NaCl; 15 mM KCl; 3 mM $MgCl_2$; 50 mM Tris-HCl (pH 7.3); 3 mM MgATP (with [γ-^{32}P]ATP at $3.3 \cdot 10^5$ cpm/μmol); 1.0 mM ouabain; and 5–10 μg membrane protein; final volume, 100 μl. Oligomycin and DCCD were added in ethanol to a final ethanol concentration of 1.0% by volume. In controls (without oligomycin or DCCD) the ethanol concentration was the same. Reactions were run at 38°C for 10 min; were triggered by ATP and were terminated with 25 μl of 25% trichloroacetic acid. P_i was extracted as molybdate complex into isobutanol. Numbers within parentheses denote the number of experiments (not assays) under each of the designated conditions. The number of assays was four for each experiment.

Inhibitor	Quantity added	Ouabain-resistant ATPase activity	
		Plasma membranes (μmol/mg/per h)	Mitochondria
Control	None	33.1 ± 2.3 (3)	24.2 ± 2.3 (3)
Oligomycin (μg/mg protein)	5	31.4 ± 3.2 (3)	9.7 ± 1.2 (3) [a]
	1100	28.2 ± 1.1 (3)	8.2 ± 1.4 (3) [a]
Control	None	31.9 ± 3.2 (4)	24.2 ± 2.3 (3)
DCCD (μM)	10	33.0 ± 3.2 (4)	8.2 ± 0.42 (3) [a]
	100	26.0 ± 2.2 (4) [a]	10.7 ± 1.3 (3) [a]
	500	16.6 ± 2.2 (4) [a]	8.8 ± 1.5 (3) [a]

[a] These values are significantly less than control values ($P < 0.01$; one-tailed t test). Remaining values, statistically indistinguishable from control.

mitochondrial ATPase. Correspondingly, neither oligomycin nor 10 μM DCCD had any effect on ATP-induced quinacrine or acridine orange fluorescence changes (Fig. 1). Sufficient increases in DCCD concentration (> 100 uM) did however inhibit both the plasma membrane ATPase and the protein translocating activities. Much larger increases in DCCD concentration (500 μM) were required for complete inhibition of the ATP-dependent proton transport (Fig. 1b) and for 65–75% inhibition of the ATPase activity (Table I). Thus, the greater the inhibition of ouabain-resistant ATPase activity, the greater the blockade of proton translocation into the vesicles; which suggests that the ouabain-resistant ATPase element could be is the proton translocator in these membranes.

Not shown in the table are comparable data for the ouabain-resistant ATPase activity of the heavier weight plasma membrane in the fluffy layer fraction from the same epithelial cell batch. Thus either oligomycin (at 5 μg/mg) or DCCD (at μM) was found to inhibit 30% of the ouabain-resistant ATPase in the fluffy layer, but none of the activity in the light weight plasma membrane fraction, indicating mitochondrial contamination of the former but not the latter.

When subjected to free-flow electrophoresis, the light weight plasma membrane fraction was separated into membranes of different surface charge density and electric mobility. As previously reported [17], membrane sub-fractions in the present experiments were recovered in effluent tubes at various loci along the electric field. Membranes containing ouabain-sensitive, $(Na^+ + K^+)$-ATPase migrated into ten effluent tubes close to the anode; and twenty tubes away, membranes enriched in ouabain-resistant ATPase (and devoid of ouabain-sensitive ATPase) migrated into ten effluent tubes closer to the cathode. Oligomycin had no detectable effect on the ATPase activity in any of these electrophoretically-separated membrane fractions. With this method, basal lateral membranes are operationally defined as those enriched in ouabain-sensitive ATPase; apical membranes, as those enriched in ouabain-resistant ATPase activity; and mitochondrial membranes (which were not found), as those enriched in oligomycin-sensitive ATPase. Therefore, it is not unreasonable to assume that the presently observed ATP-induced proton translocation in a non-electrophoresed suspension of light-weight plasma membrane vesicles is catalyzed by a ouabain-resistant ATPase, which is probably the same element as that recovered in those plasma membranes emerging near the cathodal pole of the free flow electrophoresis apparatus.

In other studies of the ATP-dependent proton translocating activity, Dixon and Al-Awqati [25] showed that the forced flow of protons from the luminal to the serosal fluid of the intact bladder is followed by an increment in the concentration of intracellular ATP. However, this increment was abolished in the presence of luminal oligomycin, a well established specific inhibitor of mitochondrial ATPase, but not of plasma membrane ATPase [26]. In another study, Gluck et al. [27] used a sucrose density fraction of the fluffy layer membranes, found to be enriched in ouabain-sensitive $(Na^+ + K^+)$-ATPase as well as in DCCD-sensitive ATPase, ouabain-resistant ATPase, and FITC-Con A (a fluorescein-labelled dextran isothiocyanate-Concanavalin A complex to which the luminal surface of the intact bladder had been exposed prior to cell fractionation). Despite the failure to separate ouabain-sensitive ATPase from ouabain-resistant ATPase, the ATP-dependent translocating activity in this fluffy layer fraction was attributed by these authors [27] to its content of apical membranes. Gluck et al. [28] also showed that luminally applied fluorescent isothiocyanate-labelled dextran (FITC) is apparently endocytosed only by mitochondrial-rich cells and extruded from these cells into the luminal fluid after stimulation of the luminal acidification process. Although this evidence is sufficient to localize the acidification function in the mitochondrial-rich cells, it is not a direct demonstration of the apical membrane location of this function.

On the basis of available evidence at this time, the ATP-dependent proton translocation has not yet been directly demonstrated in purified apical membrane vesicles of the turtle bladder epithelial cell. What can be said, however, on the basis of the present study, is that plasma membrane vesicles from turtle bladder epithelial cells contain a ouabain-resistant ATPase, the activity of which parallels that of the concomitant ATP-dependent, electrogenic translocation of protons; and that the

properties of this ATPase are pharmacologically different from those of the F_1-F_o-ATPase complex in the mitochondrial fraction of these cells. Although inferential, the data from free-flow electrophoresis together with the remainder of the evidence shown here support the hypothesis which holds that the ouabain-resistant ATPase element in the mixed membranes is probably a proton translocator residing in the apical membrane components of this plasma membrane suspension. Finally the role of this enzyme in urinary acidification has not yet been established. This is in part because the presently available data on urinary acidification are not sufficient to establish proton secretion as the mechanism of urinary acidification [29,30].

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