Biochimica et Biophysica Acta 900 (1987) 88–102
Elsevier

BBA 73563

Vanadate inhibition of ATP-dependent H^+ transport in membrane vesicles from turtle bladder epithelial cells

Steven J. Youmans * and William A. Brodsky

Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York, NY (U.S.A.)

(Received 5 September 1986)
(Revised manuscript received 30 January 1987)

Key words: Proton transport; ATP dependence; Membrane vesicle; Vanadate inhibition; Epithelial cell; (Turtle bladder)

The ATP-dependent proton transport into vesicles of a mixed membrane fraction obtained from turtle bladder epithelial cells consists of at least two kinetically defined moieties: one, which is maximally inhibited by 25% with nanomolar levels of vanadate, but not inhibited at all with equimolar levels of *N*-ethylmaleimide, and another, which is maximally inhibited by 70% with micromolar levels of *N*-ethylmaleimide and by 25% with equimolar levels of vanadate. In contrast to the transport function, the associated enzymatic function (the ouabain-resistant ATPase activity) in these membranes, not inhibited by nanomolar levels of vanadate or *N*-ethylmaleimide, is maximally inhibited by 40% with micromolar levels of vanadate and by 13% with equimolar levels of *N*-ethylmaleimide. Independent of these kinetic differences between the enzyme and the transport functions, membranes containing the *N*-ethylmaleimide-sensitive proton transport function are electrophoretically separable from those containing the vanadate-sensitive transport function. For example, the kinetically defined, vanadate-sensitive proton transport function is recovered exclusively and kinetically identified in one of four electrophoretic membrane fractions, EF-II; while the *N*-ethylmaleimide-sensitive function is recovered in EF-III as well as in EF-II. Membranes of EF-IV, maximally enriched in ouabain-resistant ATPase activity, possess no proton transport function at all, even in the absence of *N*-ethylmaleimide or vanadate. Additional data under in vivo as well as under in vitro conditions are required to prove that the vanadate-sensitive proton transport in these vesicles is an in vitro manifestation of the mechanism responsible for generating the vanadate-sensitive luminal acidification process under in vivo conditions in the intact turtle bladder.

* Present address: Department of Medicine, School of Medicine, East Carolina University, 1720 Knollwood Drive, Greenville, NC 27834, U.S.A.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Correspondence: W.A. Brodsky, Department of Physiology and Biophysics, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York, NY 10029, U.S.A.

Introduction

Background

On the basis of previously obtained data from this [1] and other laboratories [2], it has been well established that the turtle urinary bladder must possess a discrete, metabolically energized, ion transport mechanism, the action of which drives a

primary active and electrogenic transport process of luminal * acidification.

In attempting to identify and localize this transport mechanism, subsequently obtained data have shown that: (i) isolated membrane fractions, obtained from turtle bladder epithelial cells contain a ouabain-resistant (Mg^{2+})-ATPase and a ouabain-sensitive ATPase activity [3,4]; (ii) membranes devoid of the ouabain-sensitive activity are electrophoretically separable from those containing ouabain-sensitive ATPase activity; and (iii) a Mg^{2+}-ATP-dependent proton transport process can be evoked in vesicles of these isolated membrane fractions [5].

On the basis of these and other data on transport-related enzyme activities such as carbonic anhydrase in turtle bladder cells [6,7], it has been assumed that: the proton pumping mechanism, responsible for luminal acidification in the intact bladder, is an integral part of the Mg^{2+}-ATPase enzyme complex; and that this complex is located in the apical membranes of carbonic anhydrase-rich cells of the turtle bladder epithelium. However, neither of these assumptions is backed by sufficient or unambiguous evidence, which gives rise to the following problems.

(i) The enzymatic (Mg^{2+}-ATPase) identification of the proton pump mechanism is limited by the lack of any reagent with quantitatively correlative, specific inhibitory effects on the enzymatic activity and on the concomitant ion transport function; i.e., with effects analogous to those of ouabain on the ($Na^+ + K^+$)-ATPase activity and the concomitant rate of sodium transport.

(ii) A primary active, electrogenic process of luminal alkalinization has recently been demonstrated in bladders from normal and alkalotic turtles [8,9]; and it is thought that the pump mechanism driving this ion transport process is located in the basal-lateral membranes of a second kind of carbonic anhydrase-rich cell [10,11]. Therefore at least some of the in vitro proton pumping in isolated membrane fractions could be due to the presence of inside-out basal-lateral vesicles containing the aforementioned luminal alkalinization mechanism.

(iii) The assumed apical membrane location of the luminal acidification mechanism may well be correct, but a direct experimental demonstration of this location has not as yet been obtained. This is because there are two major cell types in the bladder epithelium: granular-rich and carbonic anhydrase-rich; and two sub-types of the carbonic anhydrase-rich cells, alpha and beta [10,11]. Therefore at least four and possibly six different kinds of plasma membranes can be recovered in different kinds of membrane fractions obtained from the turtle bladder epithelial cell layer – even if one ignores the additional recovery of endosomes, endocytoplasmic reticular and other intracellular membrane structures in these membrane fractions.

Present approach to the problem

Studies reported here were initiated to determine whether orthovanadate could be used as an inhibitory marker for the ouabain-resistant, oligomycin-resistant Mg^{2+}-ATPase activity and the concomitant proton transport function in isolated membrane vesicles obtained from turtle bladder epithelial cells. This decision was prompted by the established inhibitory effects of vanadate on the proton transport and Mg^{2+}-ATPase functions in fungal plasma membranes [12] as well as on the luminal acidification process in the intact turtle bladder [13].

The rationale of the presently reported experiments, like that of Goffeau and Slayman [12], is that vanadate can be classified as a specific inhibitor of membrane-bound, Mg^{2+}-ATPase-coupled, proton-translocating operations. This rationale has been proved useful (at least in the fungal membrane systems), even though vanadate is not as absolutely specific an inhibitor of the ATPase-coupled proton transporting operations as is ouabain of the ATPase-coupled sodium and potassium transporting operations; and even though vanadate is a well-established inhibitor of the ouabain-sensitive ($Na^+ + K^+$)-ATPase-coupled sodium transport operations in several cell systems [14,15], including that of the turtle

* The demonstration of luminal acidification, along with its primary active and electrogenic characteristics, does not depend on whether that acidification process is due to the bicarbonate reabsorption or proton secretion. This has been a long-standing controversy [1,2] which is not approached in what follows here.

bladder epithelium [13]. Nevertheless, vanadate has no effect on the mitochondrial ATPase-coupled proton transport function and its effects on proton transport in the fungal membranes are elicited in the presence of ouabain and oligomycin [12].

Another reagent, *N*-ethylmaleimide, has recently been used by others [5,16,17] as a marker for the proton translocating, Mg^{2+}-ATPase complex in bovine kidney medulla and in other cells, but not in those of the turtle bladder. Like vanadate, *N*-ethylmaleimide is without effect on the mitochondrial ATPase-coupled proton transport function, and for this reason, it was pertinent to compare the effects of *N*-ethylmaleimide with those of vanadate in the present study.

Data from the presently reported experiments will demonstrate: (i) the extent to which the in vitro, ATP-dependent proton transport function can be correlated with the concomitant ouabain-resistant ATPase activity in isolated membrane fractions obtained from turtle bladder epithelial cells; (ii) the kinetic parameters of vanadate-induced and *N*-ethylmaleimide-induced inhibition of the in vitro proton transport and ATPase functions in mixed membranes as well as in electrophoretically separated membranes of this epithelium; and (iii) the extent to which in vitro changes in proton transport induced by vanadate or *N*-ethylmaleimide can be correlated with the in vivo changes of the luminal acidification process in the intact bladder.

Methods

Preparatory

Slightly modified from those reported previously [18].

Isolation of the cell homogenate. The source of membrane vesicles from each set of the present experiments was a pool of epithelial cells which had been obtained from 25–30 freshly excised turtle urinary bladders. Following excision, each bladder was filled with and incubated in calcium-free, EDTA-supplemented sodium Ringer solution for 1 h, during which time, the epithelial cells were separated from the sub-mucosal layer. The cell-containing mucosal fluids were removed, pooled, and the entire suspension centrifuged at $5000 \times g$ for 10 min, to yield a pellet of packed cells (approximate volume, 11–12 ml). The pellet was then pulverized and the resulting cell homogenate suspended in 30–40 ml of a solution consisting of 200 mM sucrose/1.0 mM EDTA/10 mM Tris-HCl at a final pH of 7.4.

Isolation of mixed membrane fraction. Membranes were separated from other sub-cellular components of the cell homogenate by a sequence of conventional ultracentrifugation steps. After centrifugation of the crude homogenate at $5000 \times g$ for 10 min, nuclei and unbroken cells were found in the pellet (P-1) which was discarded, leaving a supernatant (S-1) containing other sub-cellular components (mainly mitochondria and a mixture of membranes). The next centrifugation (that of S-1 at $20\,000 \times g$ for 30 min) yielded a two-layered pellet (P-2) and a supernatant (S-2). The lower layer of P-2 (a closely packed salmon-colored mass) was found to be maximally enriched in oligomycin-sensitive ATPase activity and devoid of ouabain-sensitive ATPase activity. The upper layer of this pellet (a white fluffy mass) was found to contain equal levels of oligomycin-sensitive and ouabain-sensitive $(Na^+ + K^+)$-ATPase activities [4,19]. Finally, centrifugation of the supernatant, S-2, at $100\,000 \times g$ for 1 h, yielded the third supernatant (S-3), which was discarded, and a pellet (P-3). This pellet, containing 25–30 mg of membrane protein, was operationally defined as the low-density, mixed membrane fraction.

Characteristics of mixed membranes in P-3. (a) Enzymatic. With a previously described assay for ATPase activity [18] and with that of Michell et al. [20] for β-glucuronidase activity, these membranes were found to be maximally enriched in ouabain-sensitive $(Na^+ + K^+)$-ATPase activity, devoid of oligomycin-sensitive ATPase activity, and devoid of β-glucuronidase activity. It can therefore be said that the presently prepared mixed membrane fraction (P-3) was rich in sodium pump-containing plasma membranes and devoid of lysosomal and mitochondrial membranes.

(b) Osmotic reactivity. Using the methods of Sachs et al. [21], the 90° scattering intensity of monochromatic light, initially passed through particles of P-3 in a Tris-buffered, (KCl + valinomycin) -containing medium of specified osmolality, was found to increase with an imposed increase in osmolality of the medium (indicating

particle shrinkage), and to decrease with an imposed decrease in osmolality of the suspending medium (indicating particle swelling). It can therefore be said that the membranes of P-3 are vesicular in form, and consequently suitable for assays of solute and water transport. In fact, the mixed membrane pellet (P-3) was the source of vesicles for most of the presently reported experiments.

Electrophoretic separation of mixed membranes. For the remaining experiments, the membranes in P-3 were partially separated from each other by free-flow electrophoresis. In this procedure, P-3 (containing 25–30 mg of membrane protein) was suspended in 5–10 ml of electrophoresis solution (280 mM sucrose/1.0 mM EDTA/10 mM Tris-acetate, final pH 7.4) and injected at a rate of 0.2 ml/min for 50 min, into the free flowing solution between the electrode plates of the electrophoretic chamber. During this period, the suspended membranes were continuously exposed to a horizontally oriented electric field of 90 volts/cm and current of 100 mA at 6 ° C. The individual effluent fractions (2.5–3.0 ml each), collected in 90 test tubes, were pooled in four major fractions, designated F-I, to F-IV, in order of decreasing electrophoretic mobility toward the anode.

Assay of proton transport in vesicles

Parameters of active proton transport were operationally defined by the ATP-induced rate and maximal magnitude of the decrements in Acridine orange fluorescence emitted from membrane vesicles in an incubation mixture, which in turn was kept within the cuvette of a Perkin-Elmer spectrofluorometer (model 650-10S). The Acridine orange fluorescence, initiated by a 493 nanometer incident light beam entering the cuvette, was determined from the intensity of the 526 nanometer light beam emerging from the cuvette at an angle of 90 ° from the incident beam. In each assay, an aliquot of vesicles (100–150 μg of membrane protein) was initially suspended in a medium of the following composition: 100 mM KCl/3.0 mM $MgSO_4$/15 mM sucrose/10 mM Tris-Hepes/0.1 mm EDTA/1.0 μM Acridine orange, and unless otherwise indicated, 10^{-4} M ouabain/5–10 μg/ml oligomycin; final pH, 7.4; final volume, 700 μl. The vesicles were allowed to pre-incubate in this medium for 5 min or until the Acridine orange fluorescence signal reached a maximal steady state level, at which time Tris-ATP was added to the medium to a final conc of 1.0 mM. The usual response to this addition was a rapidly developing decrease (quench) in the Acridine orange fluorescence to a new steady-state level, amounting to 40–50% of the pre-ATP level. Finally the fluorescence signal (at the low level) was restored to the pre-ATP level by the addition of 2–3 μM nigericin.

The same assay was carried out on a paired aliquot of membrane vesicles suspended in the aforementioned incubation medium with a specified concentration of vanadate or with a specified concentration of *N*-ethylmaleimide.

Precautions with the handling of vanadate. In some of the early experiments (not reported here), we found a significant vanadate-induced inhibition of the ATP-dependent proton transport in the first vesicle aliquot from a single batch of membranes. But despite repetitive washouts (as many as ten for each cuvette and pipette), this vanadate-induced inhibition could not be reproduced in the second, third or fourth vesicle aliquots from the same batch of membranes. This problem disappeared when we initiated the use of disposable plastic instead of non-disposable glass cuvettes, pipettes, and syringes.

Other assays

The specific activities of ouabain-resistant (Mg^{2+})-ATPase and ouabain-sensitive ($Na^+ + K^+$)-ATPase were determined from the rate of release of $^{32}P_i$ following the addition of [γ-^{32}P]ATP to the aforementioned vesicle suspensions, using previously described methods [18].

The concentration of protein in each aliquot of membrane (or other sub-cellular component) was determined by the method of Lowry et al. [22] in some cases, or by that of Bradford [23] in others.

Results

I. Mixed membrane vesicles

Experiments on ATP-dependent proton transport and Mg^{2+}-ATPase activity were carried out in paired vesicle-containing suspensions incubated in the presence and absence of vanadate or *N*-ethylmaleimide.

Active proton transport

The time-dependent (triphasic) response to ATP (Fig. 1). (a) In control vesicles (lower most curve). The addition of ATP- to vesicle-containing media, devoid of vanadate and *N*-ethylmaleimide, induced a triphasic pattern of proton transport, the characteristics of which were as follows.

Phase one. During first min after addition of ATP, the initial (and the maximal) rate of intravesicular acidification, indirectly determined from the rate of quenching of relative Acridine orange fluorescence (FQ/dt), amounted to 19.0 FQ units/min. This rate rapidly decreased during the 2nd to 4th min of the post-ATP period, presumably because of the progressively increasing magnitude of the unfavorably oriented, transmembrane gradient of pH which developed.

Phase two. Within 5 min after the addition of ATP, there was no further change in the magnitude of this pH gradient, which reached and remained constant at a maximal level (49 FQ units) for 4–5 min. During this time, the rate of proton pumping into vesicles was presumably equal to that of proton diffusion out of the vesicles.

Phase three. From the 10th to the 40th min of the post-ATP period, the magnitude of the Acridine orange fluorescence quench decreased gradually from the maximal level to the near-zero (pre-ATP) level, indicating that the rate of proton pumping into the vesicles was less than that of proton diffusion out of the vesicles. This can be ascribed to the ongoing, ATPase catalyzed consumption of ATP, with a progressive decrease in the ATP concentration in the medium.

(b) In vanadate-treated vesicles (middle curve). Parameters of the ATP-induced development of intravesicular acidification, in terms of Acridine orange fluorescence quenching (FQ), were determined in six batches of membrane vesicles, which had been pre-incubating in a vanadate-supplemented (10^{-5} M) medium for 5 min prior to the addition of ATP. The mean value for the ATP-induced, initial rate of proton transport into

TABLE I

EFFECT OF VANADATE ON PARAMETERS OF ATP-DRIVEN PROTON TRANSPORT INTO VESICLES OF THE MIXED MEMBRANE FRACTION OBTAINED FROM SIX DIFFERENT POOLS OF TURTLE BLADDER EPITHELIAL CELLS

FQ, fluorescence quench units. Results for incubation with or without vanadate are given as mean values ± S.E. for each transport parameter under specified incubation conditions. Results for the absolute or the percentage decrease are given as mean values ± S.E. for the individual differences of transport parameter in pairs of control and vanadate-treated vesicles from each membrane fraction.

Incubation conditions	Proton transport parameters	
	Initial rate (FQ/min)	Max. pH gradient (FQ max)
No vanadate	18.8 ±0.6	49.0±1.5
Vanadate (10^{-5} M)	13.9 ±1.3	36.4±4.6
Absolute decrease	4.92±0.88	12.6±2.0
Percentage decrease	26.5 ±5.0	26.3±4.6

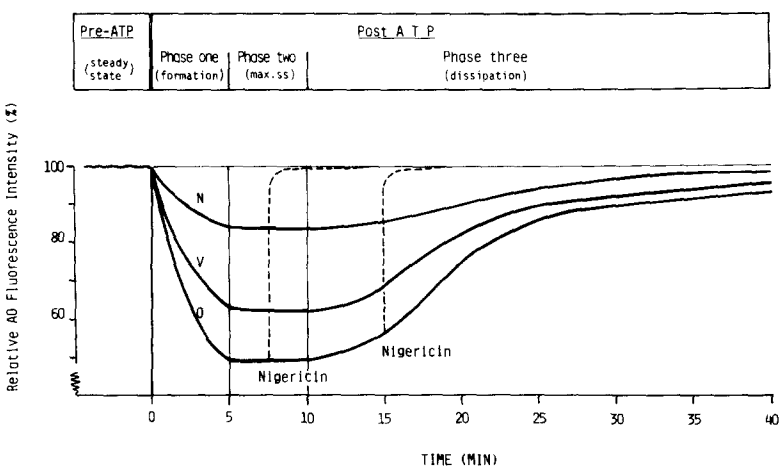

Fig. 1. Inhibition of the rate and magnitude of pH gradient formation (estimated from Acridine orange (AO) fluorescence quenching) in experimental vesicle suspensions where the addition of vanadate (V) or *N*-ethylmaleimide (N) preceded that of ATP, and the uninhibited rate and magnitude of gradient formation in control vesicles suspensions (O) where no inhibitor had been added before ATP. The dashed lines show how pH gradients in all three vesicle sets are obliterated by nigericin, at different times of the post-ATP period. Experimental and control vesicles were obtained from a single batch of the mixed membrane fraction isolated from turtle bladder epithelial cell.

these vesicles, 13.9 FQ units/min, and that for the magnitude of the maximal steady state gradients of pH formed, 36.4 FQ units, were each 26% less than the corresponding parameters in a set of paired vesicles incubating in a vanadate-free medium (Table I).

(c) In *N*-ethylmaleimide-treated vesicles (upper curve). Using *N*-ethylmaleimide (10^{-5} M) instead of vanadate, the same kind of experiment was carried out on vesicles from three of the six batches of mixed membranes. It was found that: (i) the ATP-induced, initial rate of pH gradient formation ranged from 5.7 to 6.5 FQ units/min, an inhibition of 70–65% relative to the control rate, and that (ii) the magnitude of the maximal pH gradient (formed 5 min after addition of ATP) ranged from 12 to 15 FQ units, an inhibition of 75–70% relative to the control value for the maximal pH gradient.

(d) Summary. The ATP-induced, initial rate of formation of a pH gradient and the magnitude of the subsequently formed, maximal pH gradient in *N*-ethylmaleimide-treated vesicles were each two-thirds less than the corresponding parameters in untreated (control) vesicles, while those in vanadate-treated vesicles were one-fourth less than the corresponding parameters in the control vesicles. Apparently 10^{-5} M levels of *N*-ethylmaleimide inhibit more of the in vitro proton transport function than equimolar levels of vanadate, as has been previously reported by Gluck et al. and Al-Awqati [16].

Accepting these data at face value leaves unanswered the question of how or if vanadate or *N*-ethylmaleimide modifies the rate of dissipation of a preformed maximal pH gradient. This is because the addition of vanadate or *N*-ethylmaleimide prior to ATP decreases the magnitude of the ATP-induced, maximal steady-state gradient of pH (as well as the initial rate of pH gradient formation), and the subsequent rate of dissipation of the formed pH gradient is of necessity a function of the magnitude of that gradient as well as a function of the inhibitor concentration. Therefore, in the next set of experiments, the rate of pH gradient dissipation was determined when the inhibitor was added after a pH gradient of standardized magnitude had been formed.

Rate of dissipation of a pre-formed pH gradient (Fig. 2). (a) Effect of vanadate. When vanadate was added to the vesicle-containing medium (final conc., 10^{-5} M) during phase two of the post-ATP period, there followed a rapidly developing decrease (half-time, 1.8 min) in the preformed, maximal, steady-state gradient of pH, the magnitude of which was reduced (in 5 min) to the level reached during the same post-ATP period of the experiments on vanadate-pretreated vesicles (see Fig. 1). During the next 30 min (phase three), the magnitude of the pH gradient decreased slowly at about the same rate as that which occurred in the vanadate-free (control) system. This was not an isolated phenomenon, having been reproduced in 25 similar tests on five different batches of turtle bladder membranes.

(b) Lack of effect of *N*-ethylmaleimide. After

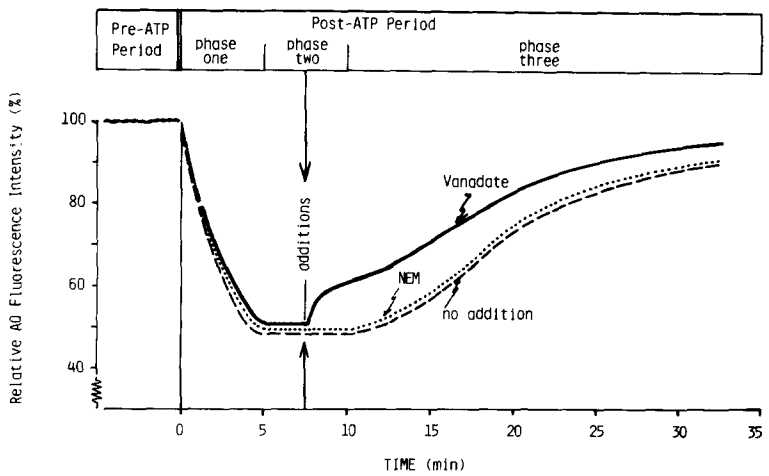

Fig. 2. Decrease in the magnitude of a preformed, steady-state gradient of pH in vesicle suspensions where the additional of vanadate followed that of ATP, and the lack of such a decrease in vesicles where the addition of *N*-ethylmaleimide (NEM) also followed that of ATP, or in vesicles where neither inhibitor was added. Source of vesicles and method for pH gradient determinations, the same as indicated under Fig. 1 and described in Methods.

the addition of *N*-ethylmaleimide (10^{-5} M) to another aliquot of these vesicles during phase two of the post-ATP period, there was no change in the maintained level of the pre-formed pH gradient or in the subsequent rate of dissipation of that gradient. In fact, the post-ATP parameters in the presence of *N*-ethylmaleimide were indistinguishable from those in vesicle-containing suspensions devoid of both *N*-ethylmaleimide and vanadate.

(c) In summary, vanadate (at 10^{-5} M) is as effective a dissipator of a preformed pH gradient in the presence of ATP (Fig. 2) as it is as an inhibitor of the formation of an ATP-dependent pH gradient in these vesicles (Fig. 1). In contrast, *N*-ethylmaleimide (at 10^{-5} M), a more potent inhibitor of pH gradient formation than vanadate, fails to alter the pH gradient dissipation in the prior presence of ATP. Evidently, the ATP-activated form of the proton carrier becomes resistant to *N*-ethylmaleimide, but not to vanadate. Because all of the aforementioned effects had been elicited with supra-maximal inhibitory levels of each inhibitor, it was decided to determine the kinetic parameters of proton transport inhibition in order to gain additional insight into how each inhibitor interacts with the proton carrier system in these vesicles.

Dosage response. Inhibition of proton transport (Fig. 3). In order to quantitate the inhibitory effect of vanadate and of *N*-ethylmaleimide on the proton transport function, vesicles were incubated in media devoid of and supplemented with these inhibitors at concentrations between 10^{-9} M and 10^{-3} M, prior to the addition of ATP.

(a) Vanadate-treated vesicles (circles). Each parameter of the ATP-dependent proton transport function (initial rate or magnitude of pH gradient formation) was found to be an inverse logarithmic function of the vanadate concentration. The minimal detectable effect of vanadate, at a concentration of 20 nM, was an 8% decrease in the magnitude of each transport parameter. The maximal (saturating) effect, at vanadate concentrations in excess of 100 nM, was a 35% decrease in each parameter; and the I_{50} level of vanadate (that required for inducing a half-maximal inhibition of proton transport) was 47 nM for the initial rate parameter (closed circles) and 40 nM for the steady-state gradient parameter (open circles).

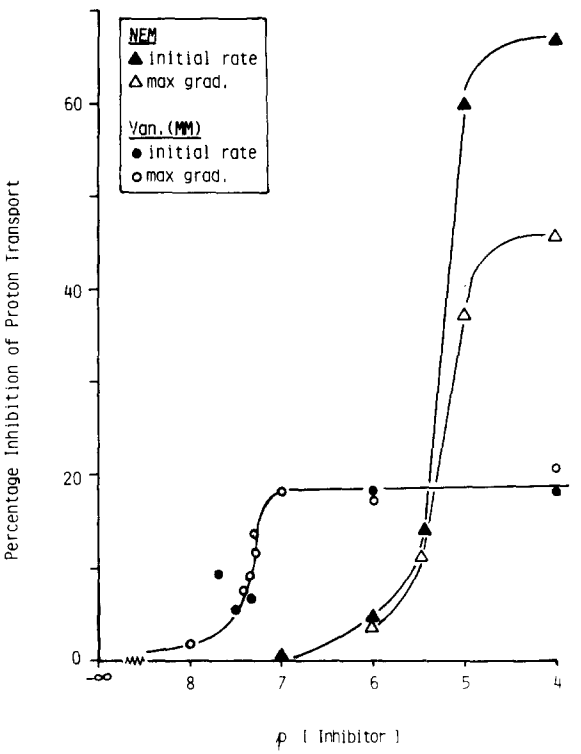

Fig. 3. Dosage response. Percentage inhibition of the rate of pH gradient formation (closed symbols) and of the magnitude of the subsequently formed, steady-state pH gradient (open symbols) in the presence of vanadate (circles) or *N*-ethylmaleimide (triangles) versus the logarithm of the reciprocal molar concentration of each inhibitor (i.e., versus p*I*).

(b) *N*-Ethylmaleimide-treated vesicles (triangles). In contrast to vanadate, 10–100 nM concentrations of *N*-ethylmaleimide in the vesicle-containing medium had no detectable inhibitory effect on the rate or magnitude of pH gradient formation. However, at increasing concentrations (from 6 μM to 0.5 mM), the inhibitory effect of *N*-ethylmaleimide increased to become over 3-fold greater than that induced by equimolar concentrations of vanadate. The initial rate (closed triangles) and magnitude (open triangles) of pH gradient formation in the presence of 0.5 mM *N*-ethylmaleimide were, respectively, 75% and 65% less than the corresponding control levels (the maximal inhibition), and half this degree of inhibition was reached in the presence of *N*-ethylmaleimide at 6.3 and 7.8 μM, respectively (the I_{50} values).

On the basis of these data, the following conclusions were drawn. In the micromolar range,

N-ethylmaleimide is a more potent inhibitor of proton transport than vanadate. But in the nanomolar range, vanadate is the more potent inhibitor. Moreover, the inhibitory affinity of vanadate for a significant part of the proton transport function is over 100-fold greater than that of *N*-ethylmaleimide.

Associated ATPase activity

Because of the known presence of ouabain-resistant (Mg^{2+})-ATPase activity, along with the ATP dependence of the proton transport function in these membranes, the next set of experiments was designed to determine how the enzymatic activity is related to the transport function.

Concomitant rates of ATP hydrolysis and proton transport (Fig. 4). Each of two vesicle-containing aliquots (from a single batch of mixed membranes) was suspended in a separate volume (1 ml) of an identical Acridine organe-supplemented, Tris-buffered, KCl-containing incubation mixture, devoid of vanadate or *N*-ethylmaleimide. After a 5 min pre-incubation period, [γ-^{32}P]ATP was added separately to each mixture. In one mixture, the formation and dissipation of a transmembrane

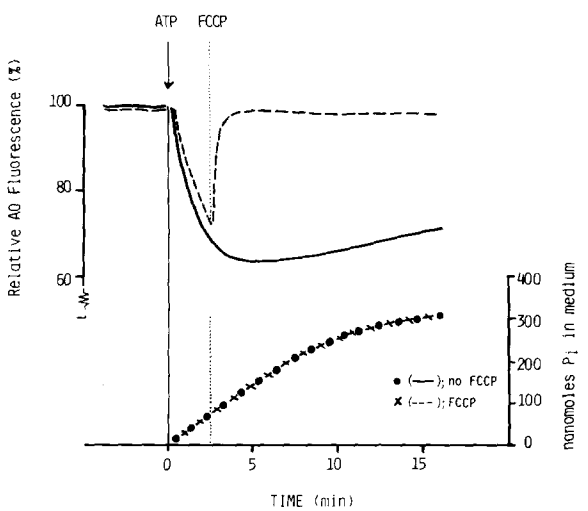

Fig. 4. Concomitant formation of pH gradient (upper panel) and inorganic phosphate (lower panel) formation following addition of ATP to paired aliquots of membrane vesicles. In one pair of aliquots (without FCCP), the proton-transport function is designated by the solid line (upper panel) and the ATPase function by closed circles (lower panel). In the other pair, the proton-transport function is designated by the dashed line, and the ATPase function by crosses.

gradient of pH was continuously monitored from the changes in Acridine orange fluorescence. In the other (paired) mixture, the quantity of inorganic phosphate (P_i) released (from the catalyzed hydrolysis of ATP) was determined during the same periods of formation and dissipation of the pH gradient. The concomitantly occurring proton transport and ATPase activity were as follows.

(i) During the first 4 min of the post-ATP period (phase one), the intravesicular pH decreased rapidly (half-time, 1.0 min) with the formation of a transmembrane pH gradient which reached a maximal steady-state level (upper panel). Concomitantly, the rate of P_i formation remained constant at 37.5 nmol/min with an accumulation of 150 nmol of P_i in the medium (lower panel). (ii) During the next 1.5 min (phase two), the magnitude of the pH gradient and rate of P_i release remained constant, while 56 additional nanomoles of P_i accumulated in the medium. (iii) During the remaining 20 min (phase three), the magnitude of the pH gradient steadily decreased along with the rate of P_i production (20 nmol/min) and 300 additional nanomoles of P_i accumulated in the medium.

In another pair of vesicles, FCCP was added 2 min after ATP in the presence of a partially formed pH gradient. There followed an immediate and rapid elimination of the gradient (15 s) to the near-zero (pre-ATP) level, in which state the net acidification rate was presumably nullified (upper panel, Fig. 4). However, the rate of P_i release associated with the zero net acidification after FCCP remained the same as that associated with the non-zero acidification before FCCP (lower panel, Fig. 4).

These data can tentatively be interpreted as follows. Given that the concentration of Tris-HCl in the intravesicular compartment becomes equal to that in the extravesicular compartment (i.e., 10 mM), it follows that the total quantity of Tris-HCl in an assumed intravesicular volume of 1 μl would be 10 nmol. This quantity of buffer, the pK of which is 8.3 [24], would be titrated from pH 7.3 to pH 4.3 with 0.9 nequiv. of protons, which amounts to 0.6% of the quantity of P_i released (150 nmol) during the first 4 min after addition of ATP. Therefore, one cannot readily estimate the degree

of coupling between the ATPase-catalyzed reaction and the proton transport process. Nevertheless, decrements in ATPase activity might be correlated with those in proton transport, and this was looked for next.

Dosage-response. Inhibition of ATPase activity (Fig. 5). Vanadate-induced (open circles). In these experiments, aliquots of membrane fraction (10–15 μg) were pre-incubated for 5 min in separate flasks, each containing 100 μl of the standard incubation fluid (see Methods) supplemented with different concentrations of vanadate, and paired aliquots were similarly pre-incubated in the same standard fluid, devoid of vanadate. After addition of [γ-^{32}P]ATP, the ouabain-resistant (Mg^{2+})-ATPase activity in the presence of 0.5 mM vanadate (the highest concentration used) was 15.4 μmol/mg per h or 38% less than that found (24.7 μmol/mg per h) in the absence of vanadate, and half this degree of inhibition (19%) was found in the presence of 30 μM vanadate. However, there was no saturation of this inhibition, which continued to increase up to 38% as the vanadate levels were increased from 10 to 500 μM. This means that the 30 μM level was less than the 'true' I_{50} level and that the inhibitory affinity of vanadate for the ATPase function was at least

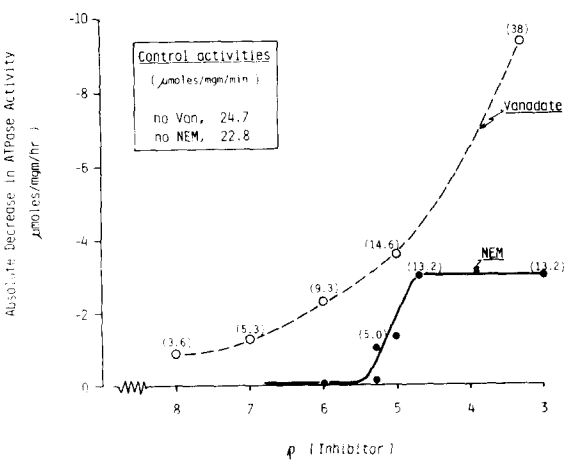

Fig. 5. Dosage response inhibition of the enzymatic ATPase function. Absolute decrement of ATPase activity in the presence of vanadate (○) and in the presence of *N*-ethylmaleimide (●) as a logarithmic function of the reciprocal molar concentration of each inhibitor. Numbers in parentheses denote the percentage inhibition of ATPase activity at each concentration of vanadate and *N*-ethylmaleimide (NEM).

three orders of magnitude less than its inhibitory affinity for the proton transport function (Figs. 5 and 3).

N-Ethylmaleimide-induced (closed circles). In the presence of *N*-ethylmaleimide, the minimal detectable inhibition of ouabain-resistant ATPase activity, 5%, was found at a concentration of 5.0 μM. The maximal (saturating) degree of inhibition, 13%, reached at 20 μM, remained unchanged after increasing this *N*-ethylmaleimide concentration to 1.0 mM and half this maximal inhibition (6.5%) was evoked in the presence of 8.0 μM *N*-ethylmaleimide. Although the inhibitory affinity of *N*-ethylmaleimide for the ATPase function was not significantly different from its affinity for the proton transport function (compare Fig. 5 with Fig. 3) the maximal *N*-ethylmaleimide-induced inhibition of the transport function (70%) was over 5-fold greater than that of its inhibition of the enzyme function.

In summary, the inhibitory affinity of vanadate for the proton transport function was 175-times greater than that of *N*-ethylmaleimide, but the maximal inhibitory effect of vanadate on this transport was one third to one half that of *N*-ethylmaleimide. In contrast, the inhibitory affinity of vanadate for the ATPase function was about half that of *N*-ethylmaleimide, but the maximal inhibitory effect of vanadate on the ATPase activity was more than 3-fold greater than that of *N*-ethylmaleimide. In order to determine whether there was any correlation between the proton transport and enzymatic functions, it was decided to determine whether and to what extent the vanadate-sensitive functions could be separated from the *N*-ethylmaleimide-sensitive functions.

Electrophoretically isolated vesicles

As described in the Methods section, 60 electrophoretically separated, membrane-containing effluent fluids were pooled into four separate sets of electrophoretically isolated membranes (F-I, II, III, and IV), and after other aliquots of the same mixed membranes had been passed through the chamber in the absence of an imposed electric field, ten membrane-containing effluent fluids were pooled into a single mixed membrane fraction. Aliquots of the mixed membrane fraction and FI

to F-IV were then suspended for 5 min in the standard incubation mixture: devoid of vanadate and *N*-ethylmaleimide (O); supplemented with vanadate (V) or with *N*-ethylmaleimide (N); and assayed for proton transport and ATPase activity.

Distribution of functions

Proton transport (Fig. 6). In what follows, the proton transport function is represented in terms of the maximal steady state gradient of pH (ΔpH), not in terms of the initial rate of pH gradient formation (ΔpH/min), because induced changes in the former were essentially the same as those in the latter parameter. In these terms, the proton transport functions were found in the membranes of three electrophoretic effluent fractions: the mixed membrane fraction (MME), F-II, and F-III. Membranes of F-IV were devoid of any ATP-dependent proton transport function whatsoever and those of F-I possessed minimal, but detectable levels of this function.

(a) Under control incubation conditions (in the absence of vanadate or *N*-ethylmaleimide), ΔpH_{max} in the mixed membrane fraction amounted to 41 FQ units, that in membranes of F-II to 46 FQ units, that in F-III to 17 FQ units; and that in F-I to 6.0 FQ units.

(b) In the presence of vanadate (10^{-5} M), ΔpH_{max} in the mixed membrane fraction, 30 FQ units, was 26% less than that found under control conditions in this fraction; while Δ pH_{max} in membranes of F-II, 30 FQ units, was 32% less than the ΔpH_{max} in this fraction under control conditions. Therefore the vanadate-sensitive moiety of ΔpH_{max}, isolated exclusively in membranes of F-II, amounted to 14.0 FQ units, which was 40% greater than the vanadate-sensitive moiety in the mixed membrane fraction. Vanadate had no detectable effect on ΔpH_{max} in vesicles of F-I, F-III or F-IV.

(c) In the presence of *N*-ethylmaleimide, ΔpH_{max} was found to be 10.5 FQ units in vesicles of the mixed membrane fraction; 18.8 FQ units in those of F-II; and 8 FQ units in the vesicles of F-III. Therefore, the *N*-ethylmaleimide-sensitive moiety of ΔpH_{max} amounted to 30.5 FQ units, or 74% of the uninhibited transport function in the mixed membranes 26.2 FQ units or 57% of the uninhibited function in F-II, and 9.0 units or 53% of that transport function in vesicles of F-III.

(d) Summary. Vesicles containing the *N*-ethylmaleimide-sensitive moiety of ΔpH_{max} migrated electrophoretically with membranes of F-III as well as with those of F-II which means that the *N*-ethylmaleimide-sensitive moiety of proton transport in membranes of F-III was completely separated from the vanadate-sensitive moiety. Although the magnitude of *N*-ethylmaleimide-sensitive proton transport in membranes of the mixed membrane fraction, F-II, and F-III was greater than that of the vanadate-sensitive transport function, the electrophoretic mobility range of mem-

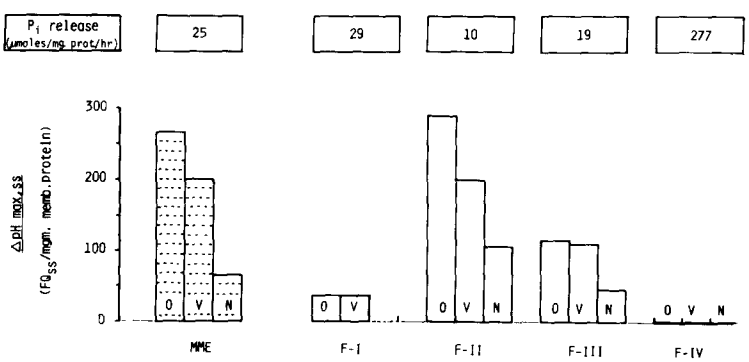

Fig. 6. Free flow electrophoresis. Magnitude of the ATP-induced maximal pH gradient in the presence of vanadate (V) or *N*-ethylmaleimide (N) and in the absence of these inhibitors (O) as recovered in: (i) a set of vesicles (MME) from the mixed membrane fraction, which had passed through the free-flow chamber in the absence of an imposed electric field; and in (ii) four subsets of these vesicles (F-I to F-IV) from another aliquot of the same mixed membrane fraction which had passed through the free-flow chamber in the presence of an imposed electric field. For each transport assay, the quantity of membrane protein was 150 μg. Concomitant Mg^{2+}-ATPase activity (rate of P_i release) for each fraction is shown in boxes in upper panel.

branes enriched in the vanadate-sensitive component was narrower than that of membranes carrying the *N*-ethylmaleimide-sensitive proton transport component.

One is therefore forced to conclude that there are at least two discrete, qualitatively different, active proton transport mechanisms in at least two different (electrophoretically defined) membrane types, which have been isolated from the two major classes of turtle bladder epithelial cells. Since it is generally assumed that any such proton transport mechanism is an integral part of a specified (ouabain-resistant) ATPase complex, it was decided to determine whether the electrophoretic distribution of membranes containing this enzyme activity was the same as that of membranes containing the proton transport activity.

2. ATPase activity (Table II). In the absence of vanadate or *N*-ethylmaleimide, the ouabain-resistant (Mg^{2+})-ATPase activity was maximal in membranes of F-IV, yet there was no proton transport activity whatsoever in these membranes. In contrast, the Mg^{2+}-ATPase activity in membranes of F-II was minimal, yet these membranes were maximally enriched in proton transport activity.

In the presence of vanadate (10^{-5} M), ATPase activity was decreased by 15% in membranes of the mixed membrane fraction by 17% in those of F-II, by 13% in membranes of F-III, by 6% in membranes of F-I, and by less than 2% in those of F-IV. It should also be noted that the magnitude of the vanadate-sensitive moiety of ATPase activity in F-II was 59% less than that in membranes of the mixed membrane fraction.

In order to determine the extent to which the vanadate-sensitive proton transport activity depends on the vanadate-sensitive ATPase activity, it was necessary to evaluate each activity as a function of vanadate concentration.

Dosage-response patterns

Proton transport (Figs. 7 and 8). In the following dosage-response tests, carried out in vesicles of F-II and in those of the mixed membrane effluent, it was found that the initial rates of pH gradient formation (15 FQ/min in F-II and 22 FQ/min in the mixed membrane effluent) were maximally inhibited by 23% and 18.5% respectively, in the presence of vanadate between levels of 0.1 and 10.0 μM, and that half-maximal inhibition was reached in the presence of 45 and 50 nM vanadate in F-II and the mixed membrane fraction, respectively (Fig. 7). The magnitude of the subsequently formed steady-state pH gradient in the presence of vanadate betwen 0.1 and 10.0 μM was maximally reduced by 27.5% in F-II and by 16.5% in the mixed membrane fraction, while half of this maximal inhibition was reached in the presence of 47 and 40 nm levels of vanadate in F-II and the

TABLE II

ELECTROPHORETIC DISTRIBUTION OF THE OUABAIN-SENSITIVE ($Na^+ + K^+$)-ATPASE ACTIVITY IN ALIQUOTS OF MIXED MEMBRANE FRACTIONS (MME) AND FOUR ELECTROPHORETICALLY LOCALIZED, MEMBRANE SUB-FRACTIONS (F-I TO F-IV) DURING INCUBATION IN TRIS-BUFFERED, ($Mg^{2+} + Na^+ + K^+$)-CONTAINING MEDIA IN THE PRESENCE AND ABSENCE OF OUABAIN AND/OR VANADATE, AT THE MOLAR CONCENTRATIONS ([M]) DESIGNATED.

Additions to media		ATPase activity (μmol/mg per h)				
ouabain [M]	vanadate [M]	MME	F-I	F-II	F-III	F-IV
0	0	90.2	60.8	36.1	44.9	285
10^{-4}	0	63.4	32.0	14.7	27.4	277
10^{-4}	10^{-5}	53.9	26.6	12.2	23.9	273

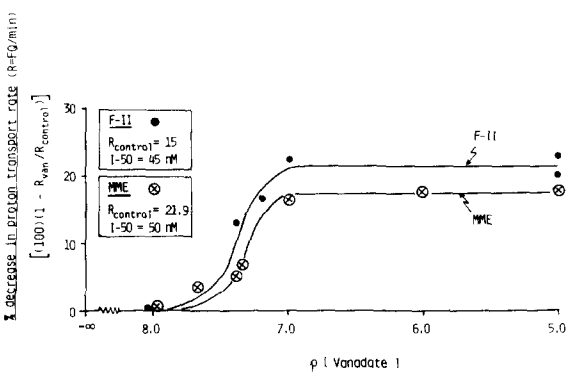

Fig. 7. Dosage response (rate parameter of proton transport function). Percentage decrease of ATP-induced initial rate of pH gradient formation as a logarithmic function of the reciprocal molar concentration of vanadate in vesicles of unseparated membranes (mixed membranes, MME) and in those in electrophoretically isolated membranes of F-II.

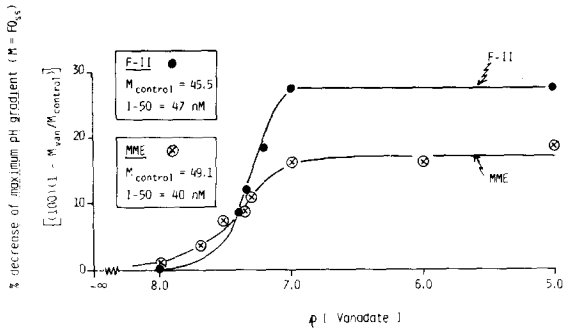

Fig. 8. Dosage response (gradient parameter of proton transport function). Percentage decrease in magnitude of the maximal, steady-state gradient of pH formed versus logarithm of the reciprocal molar concentration of vanadate. These data, obtained during phase two of the post-ATP period, are from the same membrane vesicles (mixed membrane fraction, MME and F-II) as those depicted in Fig. 7.

mixed membrane fraction, respectively (Fig. 8).

Ouabain-resistant (Mg^{2+})-ATPase activity (Fig. 9). The dosage-response pattern for the vanadate-induced inhibition of ATPase activity in F-II was essentially the same as that in the mixed membrane fraction (e.g., I_{50} levels of 12 and 14 μM in F-II and the mixed membrane fraction, respectively). But, in contrast to the vanadate-sensitive proton transport function, found only in F-II, the vanadate-sensitive ATPase functions were found in all of the electrophoretic fractions. The I_{50} levels of vanadate for the ATPase function in these fractions (in micromolar units) were 6.3 in F-I, 12.0 in F-II, 4.2 in F-III, and 7.9 in F-IV.

In summary, there was no systematic relation between the vanadate-sensitive enzymatic and proton transport functions in the electrophoretically separated or in the mixed membrane fractions. This fits the fact that the vanadate-sensitive enzymatic function migrated with membranes of all electrophoretic fractions, while the vanadate-sensitive transport function migrated exclusively with those of F-II. Moreover, the inhibitory affinity of vanadate for the transport function was 1000-fold greater than its affinity for the enzymatic function, a fact which underscores the lack of any kinetically detectable dependence of the proton transport functions on the enzymatic (ATPase) functions in these membrane vesicle fractions.

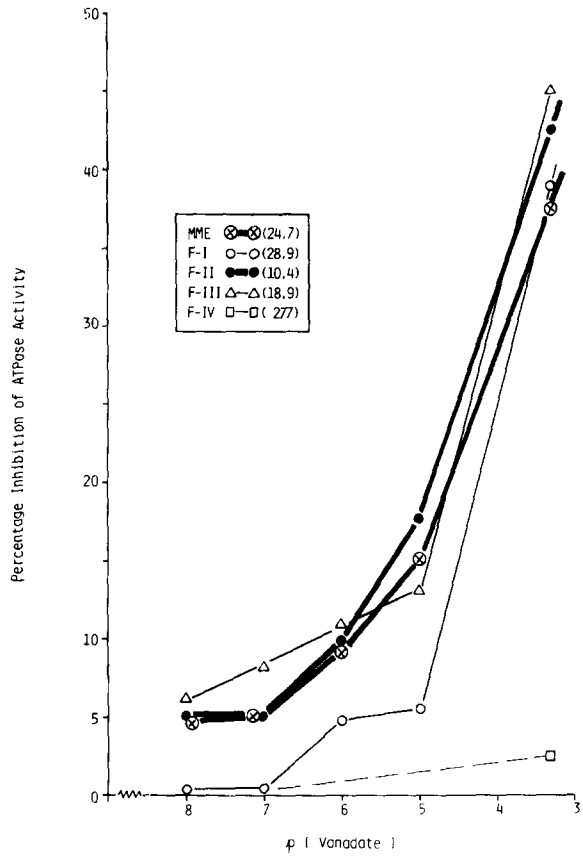

Fig. 9. Dosage response (ATPase function) Percentage inhibition of ouabain-resistant ATPase activity versus the negative logarithm of vanadate concentration in electrophoretically separated membranes (F-I to F-IV) and in unseparated (mixed) membranes (MME). Thick line plots denote the enzyme function of those vesicles (F-II and MME) in which the vanadate-sensitive proton transport function resides. The thin line denote the enzyme function of vesicles devoid of the vanadate-sensitive moiety of the proton transport function (those in F-I and III) and that of vesicles devoid of any proton-transport function whatsoever (those in F-IV).

Discussion

Proton pump-containing membranes

In order to account for the electrophoretic separation of membranes containing the *N*-ethylmaleimide-sensitive proton transport function from those containing the vanadate-sensitive function (Fig. 6) as well as to account for for the different patterns of proton transport inhibition induced by *N*-ethylmaleimide and vanadate (Fig.

3), we have been forced to invoke a tentative hypothesis consisting of the following assumptions. (i) A kinetically defined, vanadate-sensitive proton transport process, generated by a single kind of pump mechanism, is uniquely located in one of four electrophoretically isolated membrane fractions. (ii) Another kinetically defined process, the *N*-ethylmaleimide-sensitive proton transport, generated by a second kind of pump mechanism, is located in two of the four electrophoretically isolated membrane fractions.

Although this hypothesis is consistent with presently published data on proton transport in membrane fractions obtained from turtle bladder cells (in our laboratory [4,5,19], and in that of Lubansky and Arruda [17], it has no specified requirements for: (i) determining which of the two proton pump mechanisms is the one responsible for the luminal acidification process in the intact turtle bladder; for (ii) identifying the specific plasma membrane or cell type in which the luminal acidification mechanism is located; or for (iii) determining how the associated membrane-bound ATPase reaction delivers metabolic energy into the proton pump mechanism. These problems are discussed in what follows.

Pump-specific identification

A minimal requirement for equating the in vitro vanadate-sensitive or *N*-ethylmaleimide-sensitive proton transport mechanism with the in vivo mechanism responsible for urinary acidification in the intact turtle bladder would be a demonstrated correlation between the in vitro and in vivo effects of vanadate or *N*-ethylmaleimide. Whereas both of these agents inhibit the in vitro proton transport process in isolated membrane vesicles (Figs. 1–3), vanadate inhibits the in vivo process of luminal acidification in the intact turtle bladder [13]; but *N*-ethylmaleimide does not (Durham, Matons and Brodsky, unpublished data). It can therefore be postulated that the vanadate-sensitive, proton-transporting mechanism in isolated membrane fractions is part or all of the mechanism responsible for luminal acidification in the intact turtle bladder. On the other hand, the *N*-ethylmaleimide-sensitive, proton-transporting mechanism cannot be identified as that responsible for in vivo luminal acidification, unless one invokes ad hoc assumptions (not yet experimentally supported) concerning possible reactions between *N*-ethylmaleimide and other sub-cellular structures or metabolites in the intact, acidifying bladder cell.

Membrane specific identification

On the one hand, the in vivo effects of vanadate [13] provide the minimal physiological evidence required for an identification of the in vitro acidification function in membrane vesicles with the in vivo mechanism responsible for urinary acidification in the intact bladder. On the other hand, there is not as yet any direct structural or biochemical evidence with which any specifically isolated vesicular membrane can be identified as the apical membrane in which the in vivo luminal acidification mechanism resides. The lack of this membrane specificity is underscored by the following considerations.

(i) Like other epithelial cells [25,26] those of urinary epithelia such as the toad bladder [27] and turtle bladder [28] possess intracytosolic proton pump containing membranes, some of which participate in the exocytotic and endocytotic phases of an apical membrane recycling process. (ii) Over and above surface membrane recycling, Steinman et al. [25] have reviewed the literature showing ample evidence for a bi-directional traffic of intracytosolic membranes, which fuse with each other as well as with the surface membranes. These movements, fusions, and changes in membrane characteristics apparently involve internalized (endocytosed) segments of the plasma membrane, along with endosomes, secretory vesicles, endocytoplasmic reticulum, lysosomes, Golgi bodies, and exocytotically formed, new segments of the plasma membrane. (iii) With respect to quantity as well as composition of cell membranes, Alberts et al. [26] have cited evidence showing that the plasma membranes of certain epithelial cells (liver and pancreas) account for less than 10% of the total mass of membrane structures (even after discounting the contributions of nuclei, mitochondria, and lysosomes), while the other intracytosolic membranes (endosomes, endocytoplasmic reticulum, Golgi bodies) account for the remaining 90% of this mass.

Therefore it can be said that (i) different kinds

of intracytosolic membranes as well as different kinds of plasma membranes are recovered in isolated membrane fractions or subfractions from the turtle bladder epithelial cells; that (ii) ATP-dependent proton-transporting elements of at least two kinds (vanadate sensitive and *N*-ethylmaleimide sensitive) are distributed among vesicles of these intracytosolic and plasma membranes in each of the electrophoretically isolated membrane fractions; and that (iii) the in vitro vanadate-sensitive proton-pumping process might be a physiological manifestation of the in vivo luminal acidification process.

What remains to be done is to isolate the vanadate-sensitive proton-transporting function in a set of identified apical membrane vesicles along with or separate from other membrane vesicles which are devoid of any proton-pumping functions; and to obtain this set of membranes from an original homogenate of isolated carbonic anhydrase cells rather than from one of both carbonic anhydrase and granular cells.

Associated ATPase activity

Although additions of both ATP and magnesium are required for activating proton transport as well as ATPase activity in membrane fractions of turtle bladder cells, there is no quantitative index of the extent to which such enzymatic functions are coupled to the proton-transporting functions, as has been shown by the following results.

(i) In the absence of vanadate or *N*-ethylmaleimide, the quantity of P_i released (from the ATPase-catalyzed hydrolysis of ATP) is at least 250-times greater than the quantity of protons required for lowering the intravesicular pH by as much as 3.0 units, and in fact remains unchanged even when the proton transport is nullified (Fig. 4). (ii) The kinetic parameters of vanadate-sensitive ATPase activity are neither quantitatively nor qualitatively related to those of the corresponding vanadate-sensitive proton transport function (Figs. 7–9). (iii) the maximal degree of *N*-ethylmaleimide-induced inhibition of the ATPase activity is much less than that of its half-maximal inhibition of the proton transport function (Figs. 3 and 5). (iv) Among the electrophoretically separated membrane fractions (F-I to F-IV inclusive), those possessing maximal proton transport functions do not comigrate with those possessing maximal ATPase functions (Figs. 6 and 9).

At present, one can only speculate on how a fraction of the energy released from the ATPase-catalyzed hydrolysis of ATP is coupled to and activates the proton-transporting mechanism. As a matter of fact, there are three ATP-reactive enzymes in membrane fractions or sub-fractions of bladder epithelial cells: ATPase, adenylate cyclase, and protein kinase [18]. The reactions catalyzed by these enzymes (phosphorylation, adenosylation or other) could be combined in some way to provide a stepwise path for the transfer of chemical energy into the luminal acidification mechanism of the intact bladder as well as into the proton-pumping mechanism in isolated membrane vesicles obtained from such a bladder.

Differences in reported results

In comparing the present results with those obtained in other laboratories, it is pertinent to cite an apparent discrepancy in the reported effects of vanadate on the proton-transport function in isolated membrane vesicles of turtle bladder epithelial cells. On the one hand, Gluck and his colleagues failed to find any inhibitory effect of vanadate on the proton transport function in membrane vesicles of turtle bladder cells [3]. On the other hand, data from this [4,5,19] and another laboratory [17] demonstrate that vanadate does inhibit this proton-transport function. Moreover, the vanadate-sensitive proton-transport process in vesicles of the present study was found to be a uniformly reproducible function with well-defined kinetic parameters (see Figs. 3, 7–9), which is more than sufficient to establish the existence of vanadate-sensitive, proton-transporting element in turtle bladder membranes. In order to account for any failure to find such a function, one can invoke a role for each of two factors which have been shown to attenuate the inhibitory effect of vanadate.

First, in the experiments of Gluck et al. [3], as well as in some of the earlier experiments of the present study (see Methods section), vanadate could have been adsorbed onto the surfaces of non-disposable, glass cuvettes, pipettes, or other glassware; and desorbed from such surfaces in

quantities sufficient to reduce the proton-transport rate by vesicles suspended in media which are nominally devoid of vanadate. Secondly, the membranes isolated by Gluck et al. [3] were prepared and stored in a solution containing 1 mM dithiothreitol prior to the transfer of these membranes into the final incubation medium for assay of their proton-transporting function (or functions). The quantity of dithiothreitol transferred with these membranes into such a medium, supplemented with vanadate, could have been sufficient to reduce the vanadium from the plus V to the plus IV form and, if so, its inhibitory action would be attenuated [14,15]. Yet, other than vanadate sensitivity, the properties of the proton-transporting vesicles reported by Gluck et al. [3] are similar in some respects to those reported here [4,5,19] and elsewhere [17].

An additional problem

Further purification of these vesicles will probably yield a single isolated, functionally competent proton pump-containing complex. When incorporated into synthetically made phospholipid membranes and exposed to ATP, such a complex should pump protons into the liposomes. However, unless the synthetically contrived proton-umping operation replicates all of the characteristics of the in vivo luminal acidification process (including its vanadate inhibitability), there would remain a lack of unambiguous evidence for having obtained the in vitro manifestation of the in vivo pump responsible for urinary acidification.

Acknowledgements

This study has been supported in part by funds from the National Science Foundation (PCM83-02680); and in part by funds from National Institutes of Health (2 R01-DK16928). The authors (S.J.Y. and W.A.B.) are pleased to acknowledge the valuable help they have received from: Dr. George Sachs (UCLA), Dr. Gaetano Saccomani (Univ. Alabama); Dr. John H. Durham (this Department); and Dr. Kathy Barry (East Carolina University) for their scientific advice and incisive criticism; as well as from Ms. Cristina Matons for skillful and systematically controlled enzymatic analyses; and Ms. Maureen Reeck for conscientious secretarial assistance.

References

1 Brodsky, W.A. and Schilb, T.P. (1974) Current Topics in Membrane Transport. (Bronner, F. and Kleinzeller, A., eds.), Vol. 5, pp. 161—24, Academic Press, Inc., New York
2 Steinmetz, P.R. (1974) Physiol. Rev. 54, 890–956
3 Gluck, S., Kelly, S. and Al-Awqati, Q. (1982) J. Biol. Chem. 257, 9230–9233
4 Youmans, S.J., Worman, H.J. and Brodsky, W.A. (1983) Biochim. Biophys. Acta 730, 173–177
5 Youmans, S.J., Durham, J.H. and Brodsky, W.A. (1984) Fed. Proc. 43, 629
6 Scott, W.N., Shamoo, Y.E. and Brodsky, W.A. (1970) Biochim. Biophys. Acta 219, 248–250
7 Rosen, S. (1972) J. Histochem. Cytochem. 20, 696–702
8 Satake, N., Durham, J.H. and Brodsky, W.A. (1981) in Structure and Function in Epithelia and Membrane Biophysics. (Dinno, M., ed.), pp. 13–24, Alan Liss, Inc., New York
9 Satake, N., Durham, J.H., Ehrenspeck, G. and Brodsky, W.A. (1983) Am. J. Physiol. 244, C259–C269
10 Durham, J.H. and Matons, C. (1984) Biochim. Biophys. Acta 769, 297–310
11 Stetson, D.L., Beauwens, R., Palmisano, J., Mitchell, P.P. and Steinmetz, P.R. (1985) Am. J. Physiol. 249, F546–F552
12 Goffeau, A. and Slayman, C.W. (1981) Biochim. Biophys. Acta 639, 197–223
13 Ehrenspeck, G. (1980) Biochim. Biophys. Acta 601, 427–432
14 Nechay, B.R., Nanninga, L.B., Nechay, P.S., Post, R.L., Grantham, J.J., Mcara, I.G., Kubena, L.F., Phillips, T.D. and Nielsen, F.H. (1986) Fed. Proc. 45, 123–132
15 North, P. and Post, R.L. (1984) J. Biol. Chem. 259, 4971–4978
16 Gluck, S. and Al-Awqati, Q. (1984) J. Clin. Invest. 73, 1704–1710
17 Lubansky, H.J. and Arruda, J.A.L. (1985) J. Biol. Chem. 260, 4035–4040
18 Brodsky, W.A., Cabantchik, Z.I., Davidson, N., Ehrenspeck, G., Kinne-Saffron, E.M. and Kinne, R. (1979) Biochim. Biophys. Acta 556, 490–508
19 Youmans, S.J., Worman, H.J. and Brodsky, W.A. (1983) in Membrane Biophysics II: Physical Methods in the Study of Epithelia. (Dinno, M.A., Callahan, A.B. and Rozzell, T.C., eds.), Vol. 126, pp. 159–171, Alan Liss, Inc., New York
20 Michel, R., Karnovsky, M.J. and Karnovsky, M.L. (1970) Biochem. J. 116, 207–216
21 Sachs, G., Jackson, R.S. and Rabon, E.C. (1980) Am. J. Physiol. 238, G151–G164
22 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
23 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254
24 Izatt, R. and Christensen, J.J. (1968) in Handbook of Biochemistry (selected data for Molecular Biology) (Sober, H.A., eds.), p. J-193, Chemical Rubber Co., Cleveland
25 Steinman, R.M., Melman, I.S., Muller, W.A. and Cohn, Z.A. (1983) J. Cell Biol. 96, 1–27
26 Albers, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1983) in Molecular Biology of the Cell, Ch. 7, Garland Publishing Inc., New York
27 Masur, S.K., Holtzman, E., Schwartz, I.L. and Walter, R. (1971) J. Cell Biol. 49, 582–594
28 Gluck, S., Cannon, C. and Al-Awqati, Q. (1982) Proc. Natl. Acad. Sci. USA 79. 4237–4331