J. Membrane Biol. 142, 21–34 (1994)

The Journal of
Membrane
Biology

Role of Vacuolar Adenosine Triphosphatase in the Regulation of Cytosolic pH in Hepatocytes

S.J. Wadsworth*, G.D.V. van Rossum
Department of Pharmacology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Received: 21 March 1994/Revised: 3 June 1994

Abstract. The responses of the cytosolic pH of hepatocytes in suspension to agents affecting the activity of vacuolar adenosine triphosphatase (V-ATPase) and Na/H exchange have been studied. Changes of cytosolic pH were determined both with dual-wavelength excitation (500/440 nm) of the fluorescence of 2′,7′-*bis*-(2-carboxyethyl)-5(and 6)-carboxyfluorescein and from the distribution of ^{14}C-dimethyloxazolidinedione; both methods gave very similar results. Changes of vesicular pH were determined by comparing the fluorescence of fluorescein isothiocyanate-dextran and rhodamine B isothiocyanate-dextran taken up by endocytosis. Nitrate, which inhibits V-ATPase in isolated organelles, induced a concentration-dependent acidification of the cytosol and alkalinization of vesicles, with maximal effects at 25–37.5 mM in each case, indicating that V-ATPase contributes to removal of cytosolic protons. On continued exposure to nitrate, the acidification underwent an amiloride-inhibitable reversal. At the higher concentrations of NO_3^-, both cytosolic acidification and vesicular alkalinization were reduced or absent. Bafilomycin A_1 caused alkalinization of vesicular pH; cytosolic acidification was not observed, possibly because of other ionic exchanges. Recovery of cytosolic pH from an acid load (2 min exposure to 5% CO_2) was sensitive to both 25 mM NO_3^- and to ouabain. The pH dependence of the nitrate effect was tested with media of different pH; the activity was negligible at cytosolic pH 6.2 and rose to a maximum at cytosolic pH 7.3. Treatment of hepatocytes with 0.5–1.0 mM ouabain resulted in an initial alkalinization (0.5–2 min duration) of the cytosol, followed by a spontaneous reversal and, on occasion, further acidification. The alkalinization was blocked by 25 mM NO_3^-, but not by 25 mM gluconate. The results suggest that the cytosolic alkalinization is caused by a stimulation of H^+ uptake by V-ATPase activity. We conclude that V-ATPases make an important contribution to the regulation of the cytosolic pH of hepatocytes.

Key words: Vacuolar ATPase — pH in hepatocytes — Nitrate-induced acidification — Ouabain-induced alkalinization — V-ATPase and cytosolic pH

Introduction

Regulation of cellular pH within a narrow range is crucial for the maintenance of normal metabolic activity, and small shifts of pH can act as intermediate signals in the initiation of a number of processes [16]. Control of cytosolic pH is considered to require the interaction between a number of transport processes and ionic gradients at the plasma membrane. In the hepatocyte, a primary role is believed to be played by the amiloride-sensitive Na^+/H^+ exchanger [11], which becomes activated to extrude protons when cytosolic pH falls [23]. While current work emphasizes events at the plasma membrane, many cytoplasmic organelles participating in the intracellular membrane traffic maintain characteristic internal pH values more acidic than the cytosol. They do so by active accumulation of H^+ by the vacuolar adenosine triphosphatases (V-ATPases) of their membranes [7, 18, 26]. In the intact cell, the protons so taken up must be derived from the cytosol and it would therefore seem that the V-ATPases must contribute in some measure to the maintenance of cytosolic pH. There is indirect evidence for this in that Hep 2 cells subjected to hypotonic shock [17] and cultured hepatocytes depleted of ATP [4] showed acidification of the cytosol concomitantly with alkalinization of organellar contents. The latter case, in particular, suggests that inhibition of V-ATPase for lack of its substrate could have been the cause.

---

* *Present address:* Department of Pediatrics, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Correspondence to: S.J. Wadsworth

That V-ATPases do contribute to regulation of cytosolic pH has been demonstrated in cells where these enzymes occur in the plasma membranes, namely proton-secreting epithelia [2, 13, 20, 24, 28] and macrophages [14, 29]. However, the role of the V-ATPases has not been directly studied in the hepatocyte, a cell which does not appear to have V-ATPase in the plasma membrane [26] so that any effect would therefore most likely be due to an action at the organellar membranes.

The work described here examined the possible role of H^+ transport by V-ATPase in the regulation of cytosolic pH in hepatocytes by making use of two of its characteristics: (i) modulation of H^+ transport by Cl^-, which is cotransported *via* a specific channel [9, 32, 40, 42]; (ii) inhibition by NO_3^-, which acts competitively with Cl^- [32, 41]. Possible interaction with the Na/H exchanger was examined by its inhibition with amiloride and ouabain, while contributions from HCO_3^--dependent systems were eliminated by using media lacking this anion. The results are consistent with a contribution of the V-ATPases to pH control and suggest that over a certain range of intracellular pH the V-ATPase and Na/H exchanger activities are complementary. Some of this work has been published in abstract form [35, 36].

Materials and Methods

MATERIALS

Ouabain, amiloride, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), Na-gluconate, nigericin, gramicidin, collagenase (Type IV), 5,5-dimethyl-2,4-oxazolidinedione (DMO), fluorescein isothiocyanate-dextran (average mol. wt. 71,200; FITC-dextran) and rhodamine B isothiocyanate-dextran (RBI-dextran) were purchased from Sigma Chemical (St. Louis, MO). The acetoxymethyl ester of 2′,7′-*bis*-(2-carboxyethyl)-5(and 6)-carboxyfluorescein (BCECF) and Oxonol V were purchased from Molecular Probes (Eugene, OR). ^{14}C-DMO and ^{36}Cl were from Amersham (Arlington Heights, IL). Bafilomycin A_1 was purchased from Professor K. Altendorf, Universität Osnabrück, Germany. Concentrated stock solutions were prepared in dimethyl sulfoxide as follows: ouabain (400 mM), amiloride (400 mM), bafilomycin (1 mM), Oxonol V (5 mM), and BCECF (5 mM). Nigericin was prepared as a 10 mM stock in ethanol.

CELL PREPARATION

Male Sprague-Dawley rats (Zivic-Miller, Allison Park, PA), 150–200 gm, were anesthetized with pentobarbital (35 mg/kg) and hepatocytes were isolated by collagenase perfusion [27]. The livers were perfused successively with: (1) a Ca^{2+}-free buffer (mM: 137 NaCl, 4.7 KCl, 2.0 Na-phosphate, 0.6 $MgSO_4$, 10.0 HEPES, 1.0 EGTA, pH 7.4) and (2) a collagenase-containing buffer (mM: 126 NaCl, 6.7 KCl, 4.8 $CaCl_2$, 40 HEPES, pH 7.6, collagenase 130 U/ml). The cells were finally suspended in (3) "HEPES-Ringer" (mM: 125 NaCl, 5 KCl, 1.2 $CaCl_2$, 1.0 $MgSO_4$, 2.0 Na-phosphate, 20 HEPES pH 7.4) to give an average density of 15–20 mg cell protein/ml, depending on the size of the rat. Initial cell viability, as determined by trypan-blue exclusion, was >90%. The medium used for experimental incubation of the cells was HEPES-Ringer or modifications of it where varying amounts of Cl^- were replaced by equimolar amounts of NO_3^- or gluconate. Where appropriate, control incubation media contained solvents at the same concentrations as media containing test agents.

pH MEASUREMENTS

Cytosolic pH

This was usually measured by BCECF fluorescence changes, but in some experiments both ^{14}C-DMO and BCECF were used to check the findings; both indicators gave comparable values for cytosolic pH and for pH changes (*see* Results). In each case, 2.5 ml cell suspension plus 2.5 ml HEPES-Ringer (or a modification of it, *see above*) were incubated in a 50 ml plastic Erlenmeyer flask which was gassed lightly with O_2 and gently agitated in a water bath at 37°C; at intervals over a period of up to 3 hr, aliquots were taken for experimental studies. Trypan blue exclusion was checked periodically to ensure a minimum of 85% viability.

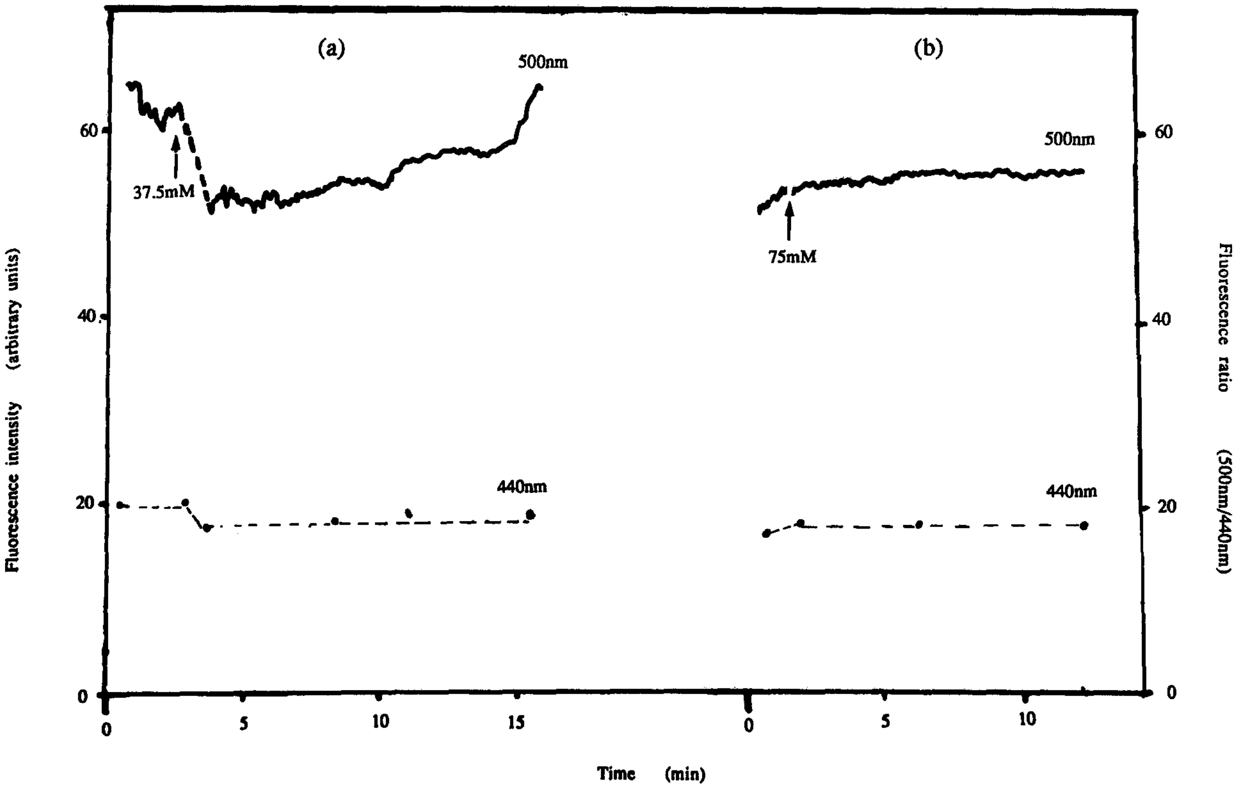
For observations with DMO, cells in the flask were equilibrated for 20 min with ^{14}C-DMO (0.5 mM, 0.5 μ Ci/ml) after which test agents were added and incubation continued as indicated in Results. Samples of the suspension (0.5 ml; approx. 10–15 mg protein) were centrifuged at 1,500 × *g* for 5 sec and aliquots of supernatant and cell pellets were then used for liquid scintillation counting of β emission, with corrections for quenching and background. Total pellet water was obtained as the difference between wet and dry weights (after drying at 105°C) and extracellular water was determined with inulin assayed chemically [12]; values for intracellular water were obtained by difference. Cytosolic pH was calculated following Pollock [21].

For measurements with BCECF, cells in the flasks (*see above*) were equilibrated with 5–10 μM BCECF. A loading time of 30 min gave the maximum fluorescence signal. Samples of the cell suspension (0.5 ml) were transferred to a cuvette in the fluorimeter (Perkin-Elmer, model 203). Fluorescence emission at 530 nm was monitored upon excitation at 500 nm (pH-dependent wavelength) with periodic, brief (5–10 sec) measurements at 440 nm (pH independent). After stable baseline values were obtained (5–10 min), the chosen test conditions were initiated and measurements continued. pH was determined by comparing the ratio of fluorescence emitted upon excitation at the two wavelengths, 500/440, with a calibration curve prepared by adding cells to a high K^+ modification of the HEPES-Ringer (120 mM K^+ replacing Na^+) with varying pH and containing 1 μM nigericin [30, 31]. pH was adjusted with 10 N NaOH or 1 N HCl to give pH 6.5, 7.0, 7.2 and 7.4. Acid loading of cells equilibrated with BCECF was done by gassing the incubation flask with 5% CO_2 for 2 min. Cells were then centrifuged (1,500 × *g* for 5 sec) and returned either to control HEPES-Ringer or its modifications as noted in Results. This transfer procedure was completed in 20–30 sec.

Vesicular pH

Hepatocytes were loaded with FITC-dextran and RBI-dextran (0.5 mg/ml each), following Geisow [8]. Cells were incubated in HEPES-Ringer with the two markers for 40 min at 25°C followed by a 10 min chase. This was sufficient time for the measured pH to reach 4.5–5.5 which is representative of the pH reported for endosomes [5]. FITC-dextran demonstrates pH-dependent fluorescence changes using the wavelengths, excitation 490 nm, emission 530 nm; rhodamine shows pH-independent fluorescence at excitation 550 nm/emission 580 nm. FITC-dextran fluorescence decreased as pH decreased while RBI-fluorescence remained virtually constant during the experiment; the excitation ratio 490/550 nm therefore provided a measure of pH changes. A calibration curve for this ratio was prepared by incubating loaded cells for 10 min in HEPES buffers of varying pH (4.5–7.4) containing 1 μM monensin to allow equilibration of vesicular pH with the extracellular buffer [8].

In control experiments, cells were added to a HEPES buffer of pH 6.5 to check that the FITC-dextran was located inside the vesicles and not in the cytosol or extracellularly. The absence of a rapid decrease in the fluorescence ratio, 490/550 nm, upon transfer from pH 7.4 to 6.5, suggests a specific, intravesicular localization for this dye.



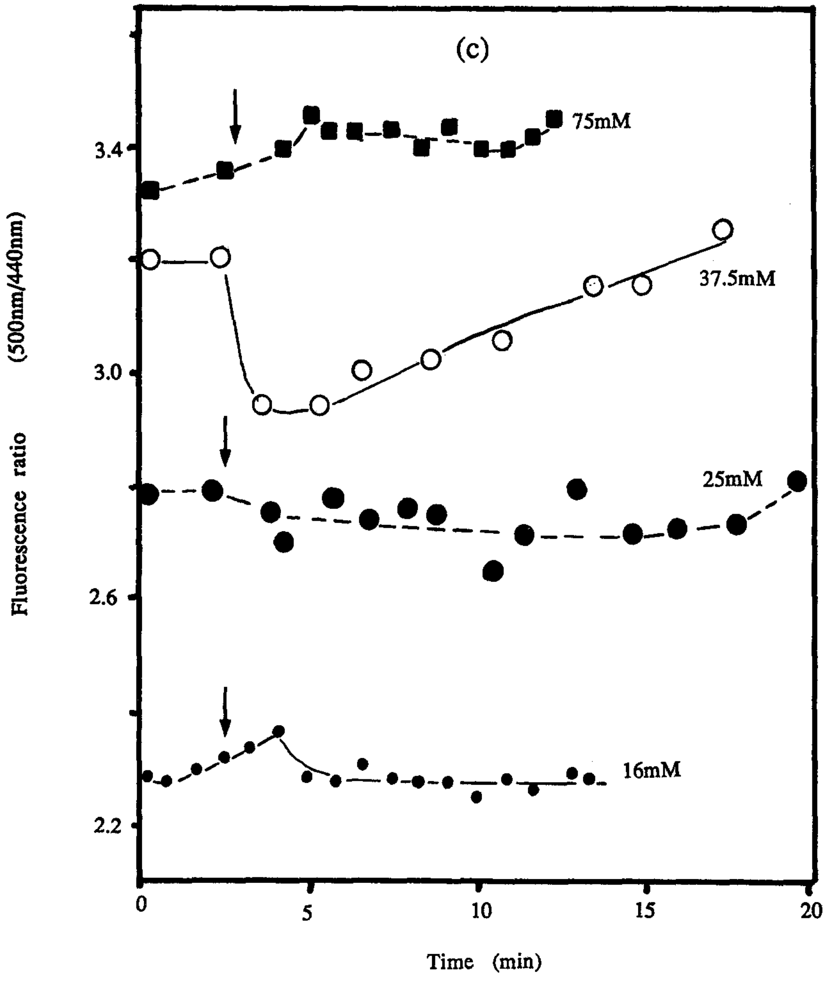


Fig. 1. Concentration-dependent nitrate-induced acidification measured by BCECF fluorescence in a series of assays from a single preparation of hepatocytes; (*a*) 37.5 mM NO_3^- (*b*) 75 mM NO_3^-. The fluorescence emitted at 530 nm was recorded continuously upon excitation at 500 nm (upper trace), with occasional brief readings at 440 nm (points noted on lower line). (*c*) Ratio of fluorescence intensity, 500/440 nm, at each of four incubations with different concentrations of NO_3^-. For further details of the procedure and calibration method, *see text.* The cells were incubated initially in the HEPES-Ringer, to establish control readings. At the arrows, the cells were introduced into media containing NO_3^- at the concentrations indicated.

Table 1. Concentration dependence of the nitrate-induced cytosolic acidification as determined by (A) BCECF fluorescence and (B) ^{14}C-DMO distribution

Control pH	Nitrate (mM)	Minimum pH after nitrate added	ΔpH	*N*	*P*[a]
(A) BCECF measurements[b]					
6.67 ± 0.11	12.5	6.62 ± 0.12	−0.04 ± 0.02	11	>0.05
7.01 ± 0.15	16.0	6.90 ± 0.17	−0.11 ± 0.04	17	<0.05
7.06 ± 0.09	25.0	6.83 ± 0.08	−0.23 ± 0.03	39	<0.001
6.91 ± 0.12	37.5	6.70 ± 0.13	−0.20 ± 0.09	13	0.05
6.93 ± 0.15	50.0	6.93 ± 0.14	−0.01 ± 0.05	17	>0.05
6.72 ± 0.13	75.0	6.82 ± 0.15	0.05 ± 0.04	13	>0.05
(B) DMO measurements[c]					
7.29 ± 0.046	12.5	7.36 ± 0.09	0.078 ± 0.12	5	>0.05
	16.0	7.18 ± 0.13	−0.112 ± 0.075	5	>0.05
	25.0	6.95 ± 0.19	−0.382 ± 0.15	5	<0.05
	75.0	7.50 ± 0.12	0.167 ± 0.064	5	>0.05

[a] Probability of significance of difference between control and nitrate-treated cells, determined by paired *t*-test.

[b] For BCECF measurements, the pH of each sample of cells was first determined in control (135 mM Cl^-) medium. The cells were then transferred to medium in which one of the concentrations of NO_3^- replaced an equal amount of Cl^-. The fluorescence traces were then followed continuously, and the minimum value of pH was determined; the minimum was always attained within 2 min (*cf.* Fig. 1).

[c] For DMO measurements, all cells were first incubated in control medium, and a sample was taken for determination of control pH. From the suspension remaining in the control flask, a sample of cells was transferred to media at each of the four concentrations of NO_3^- indicated, and aliquots were then taken at intervals for determination of the minimum pH value. Thus, in this protocol, a single control sample was obtained for each set of determinations with NO_3^- concentrations.

Membrane Potential

Cells were equilibrated with the fluorescent dye, Oxonol V, at 5 μM [10]. Fluorescence was measured at 620 nm upon excitation at 580 nm. The potential (E_m) was determined from a calibration curve prepared by increasing the medium K^+ in steps of 15 mM until a plateau of fluorescence was reached, indicating complete depolarization. Gramicidin was present to allow K^+ to equilibrate across the plasma membrane. The membrane potential was then calculated from the Nernst equation.

Other Assays

Influx of Cl^- was determined by adding 0.5 μCi ^{36}Cl to 5 ml cell suspension. Ouabain or nitrate was introduced at the same time as the tracer to determine their initial effects on influx rate. Cells were then processed as described in the section on DMO. Cells were routinely assayed for K^+ by emission flame photometry and for ATP enzymically [38].

Results

Effects of Nitrate on Cytosolic and Vesicular pH

To determine whether V-ATPases contribute to cytosolic pH regulation, the effects of different concentrations of NO_3^-, an inhibitor of V-ATPase in isolated organelles, were tested in the intact hepatocytes, Figure 1 shows representative responses of BCECF fluorescence, all from the same preparation of cells. Figure 1*a* and *b* show the pen tracings (upper line) of the fluorescence emitted at 530 nm in response to excitation at the pH-sensitive wavelength, 500 nm; the periodic breaks in the trace are points at which the excitation wavelength was briefly (approx. 10 sec) altered to the pH-insensitive wavelength, 440 nm (shown as points on the lower line). The tracings show a short section of the initial, control fluorescence when the cells were in the HEPES-Ringer solution with 132.4 mM Cl^-. The cells were then transferred to media with some of the Cl^- replaced by NO_3^-, 37.5 mM (Fig. 1*a*) or 75 mM (Fig. 1*b*), and recording was started again within 20–30 sec. Fluorescence excited at 440 nm showed little response to the presence of NO_3^- but the pH-sensitive fluorescence trace at 37.5 mM NO_3^- showed a profound, initial decrease of pH which was followed by a slower recovery to the baseline (Fig. 1*a*). By contrast, at 75 mM NO_3^- there was little change of fluorescence (Fig. 1*b*). The ratio of fluorescence excited at 500 nm to that at 440 nm in these two experiments, together with others at 25 and 16 mM NO_3^-, are shown in Fig. 1*c*. With this particular preparation of cells, the maximum change of pH was seen with 37.5 mM NO_3^-, but the concentration for peak acidification was somewhat variable between preparations. In an extensive series of experiments the initial acidification was maximal at 25–37.5 mM NO_3^- and absent at higher concentrations (Table 1A). The time to maximal acidification varied somewhat in different experiments but was always completed within 2 min. Analogous experiments in which cytosolic pH was determined from the distribution of

Table 2. Vesicular pH after addition of nitrate[a]

Nitrate (mM)	pH before nitrate	ΔpH after addition of nitrate	*N*	*P*[b]
0	5.06 ± 0.26 (control)		4	
25	5.14 ± 0.26	0.077 ± 0.011	4	<0.01
37.5	5.21 ± 0.28	0.15 ± 0.024	4	<0.01
50	5.25 ± 0.23	0.18 ± 0.043	4	<0.05
75	5.09 ± 0.23	0.035 ± 0.052	4	>0.05

[a] pH was measured with FITC-dextran. Cells were preincubated in HEPES-Ringer (132.4 mM Cl^-) before being transferred to media in which HEPES-Ringer was modified by substituting the indicated concentrations of nitrate for equal concentrations of Cl^-.

[b] Significance of differences compared with control, determined from paired *t*-test.

^{14}C-DMO showed a similar pattern of results, although no measurements were made at 37.5 and 50 mM NO_3^- (Table 1B).

The initial cytosolic acidification at the lower concentrations of NO_3^- is consistent with an inhibition of V-ATPase and consequent reduction of proton removal from the cytosol. This conclusion is corroborated by measurements of vesicular pH, using FITC-dextran (Table 2). Nitrate caused a statistically significant alkalinization of the vesicles at 16–50 mM, with maximal effect at the same concentration range (25–37.5 mM NO_3^-) as that giving maximal acidification of the cytosol. Furthermore, NO_3^- had no effect on vesicular pH at 75 mM, again corroborating the observations on cytosolic pH.

Some experiments were done to test whether NO_3^- had effects other than the inhibition of vesicular proton transport. Levels of ATP one minute after exposure to 25 mM NO_3^- were equal to those of control cells and tended to be higher than controls after longer times (Table 3). This is consistent with the inhibition of ATP consumption by an ATPase, as seen also when ouabain inhibited Na-K transport (Table 3). Nitrate, however, did not inhibit the Na-K transport for it failed to reduce K^+ content at 25 or 75 mM (*not shown*). The mean cellular water content 1 min after transfer to 25 mM NO_3^- was 5.49 ± 0.60 μl/mg dry wt ($n = 6$), a value somewhat (but not significantly) higher than that of control cells, 3.49 ± 0.28 μl/mg (6). However, the former value remained constant for at least the first 10 min treatment with NO_3^-, during which the initial cytosolic acidification and subsequent recovery were completed, so that volume changes appear not to account for the pH changes. Only after 30 min with NO_3^- was there a substantial increase of cell volume, to a value of 9.88 ± 3.50 μl/mg, compared to 3.80 ± 0.51 μl/mg in control cells. Lastly, neither 25 nor 50 mM NO_3^- affected the plasma membrane potential after 5 min (*see below*). There is, therefore, little or no indication that the pH changes produced by NO_3^- are due to anything but inhibition of vesicular H^+ accumulation.

The slower (10–15 min) recovery of the cytosol from the initial acidification (Fig. 1, *a,c*), in the continued presence of NO_3^-, was markedly sensitive to amiloride. In the experiments of Table 4, recovery from the acidification caused by 16 mM NO_3^- was completely absent while at 25 mM recovery occurred, but more slowly. Thus, the removal of the excess protons retained in the presence of NO_3^- was at least largely due to the Na/H exchange at the plasma membrane. Moreover, amiloride enhanced the initial, nitrate-induced acidification (Table 4, ΔpH), suggesting that in control conditions both V-ATPase and Na/H exchange contribute to removal of protons from the cytosol.

The reason for the absence of an initial cytosolic acidification and of vesicular alkalinization in the presence of higher concentrations (50–75 mM) of nitrate is less obvious. It is probably due to the ability of NO_3^- to substitute for Cl^- as a counter-ion for H^+ transport (*see below*).

EFFECTS OF BAFILOMYCIN

At concentrations up to 1 μM, bafilomycin A_1 is a specific inhibitor of the V-ATPase of isolated organelles [3], phagosomes in intact macrophages [15, 29] and plasma membranes of renal and alveolar epithelial cells [13, 20]. However, at concentrations of 5 μM and above it also inhibits Na-K ATPase in isolated plasma membranes [3]. It was tested on the intact hepatocytes at concentrations of 1.0–50.0 μM and in no case did it have a significant effect on cytosolic pH (Table 5A). By contrast, at 4 and 8 μM it increased vesicular pH by 0.12–0.5 units (Table 5B), a result consistent with the expected inhibition of endosomal V-ATPase. However, already at 1 μM there were indications that it affected cell K^+ content and at 10 μM and above it caused a significant loss of K^+ (Table 5A), consistent with inhibition of Na-K ATPase activity. These latter findings show that, at least in intact hepatocytes, the concentrations of bafilomycin required to inhibit vesicular V-ATPase have a less specific effect than NO_3^- does. Its failure to acidify the cytosol may therefore be a consequence of secondary ionic exchanges.

EFFECTS OF OUABAIN ON CYTOSOLIC pH

Ouabain could affect pH regulation by either of two effects. Its primary action, to inhibit the coupled transport of Na^+ and K^+ (Na-K transport), is expected to reduce the activity of the Na/H exchanger, so causing cytosolic acidification. A secondary consequence of the inhibition of Na-K transport in liver cells is a net retention of Cl^- [25,34]. The consequent increase of cytosolic Cl^- could

Table 3. Effects of incubation time and various agents on the ATP content of hepatocytes[a]

Agents	$[Cl^-]_o$ mM	*t* in presence of agents (min)			
		1	10	30	120
Control	132	2.5 ± 0.3	1.6 ± 0.4	2.1 ± 0.5	1.74; 3.18[b]
Ouabain 0.5 mM	132	2.3 ± 0.9	1.9 ± 0.3	3.2 ± 1.0	
Nitrate 25 mM	107	2.4 ± 0.6	2.4 ± 0.6	3.2 ± 0.8	
Gluconate 25 mM	107	2.4 ± 0.8	3.0 ± 0.9	2.0 ± 0.6	
NO_3^- + Ouabain	107	2.7 ± 0.5	2.0 ± 0.3	3.3[b]	
Amiloride 1 mM	132		3.5[b]	3.8[b]	

[a] Cells were preincubated in HEPES-Ringer (132 mM Cl^-) and then transferred to Erlenmeyer flasks under the conditions noted. Samples were taken for ATP analysis at intervals. For further details of incubation conditions and ATP analysis, *see* Materials and Methods. Each value is the mean ± SEM of four samples, except as noted below.

[b] Single samples.

Table 4. Effect of amiloride on nitrate-induced acidification[a]

Preincubation	pH after preincubation[b]	Nitrate (mM)	ΔpH after[c] nitrate	Final pH[d]	(*n*)
Control	6.75 ± 0.28	16.0	−0.09 ± 0.08	6.76 ± 0.27	(4)
Amiloride[e]	6.71 ± 0.22	16.0	−0.22 ± 0.02	6.40 ± 0.26	(4)
Control	6.83 ± 0.24	25.0	−0.15 ± 0.04	6.81 ± 0.22	(8)
Amiloride[e]	6.84 ± 0.21	25.0	−0.25 ± 0.05	6.81 ± 0.20	(8)

[a] Cells were preincubated for 15 min in HEPES-Ringer either without (control) or with 0.25 mM amiloride; during this period, any acidification due to amiloride was completed. Cells were then transferred to Ringer with 16 or 25 mM nitrate substituted for Cl^- (*see* Materials and Methods) either without or with amiloride and incubation continued for a further 15 min. pH values are given as mean ± SEM. [b]pH of cells at completion of preincubation. [c]Maximum change of pH after transfer to nitrate medium (attained within 2 min of transfer). [d]pH 15 min after transfer to nitrate medium. [e]Amiloride was present throughout preincubation and subsequent incubation in nitrate medium.

stimulate the V-ATPases, resulting in enhanced uptake of cytosolic H^+ into organelles. The initial consequence of treatment with ouabain was indeed a concentration-dependent, transient alkalinization of the cytosol. Figure 2 shows results from a single preparation of cells; Fig. 2*a* is the tracing of a response to 0.5 mM ouabain while Fig. 2*b* shows the changes of fluorescence ratio (500 nm excitation/440 nm) at 0.1, 0.25 and 0.5 mM; Table 6A summarizes results from a large series of experiments. The time to maximal alkalinization varied from approximately 20 sec (the time required to obtain the first reading after ouabain addition) to 2 min after addition. The peak alkalinization was always followed by a return to the baseline pH within 2–3 min. These rapid changes were not accompanied by significant changes of ATP content compared to control cells (Table 3). Similar results were obtained when cytosolic pH was determined with DMO (Table 6B). Support for an increased V-ATPase activity as the mechanism underlying the ouabain-induced alkalinization comes from two observations. First, addition of 1 mM ouabain and ^{36}Cl simultaneously to the cells showed that ouabain stimulated the influx of the Cl^- by approximately 50% ($P < 0.05$) in the first minute, a time coinciding with the transient alkalinization, but not after 5 and 10 min (*not shown*). Second, 25 mM NO_3^- eliminated the ouabain-induced alkalinization measured both by BCECF (Fig. 2*c;* Table 6B) and DMO (Table 6C). This was the case whether the 25 mM nitrate was substituted for an equal amount of Cl^- (leaving 107.4 mM) or added to the full HEPES-Ringer (*not illustrated*).

However, a number of observations indicate that ouabain also caused the expected inhibition of H^+ extrusion by the Na-H exchanger. (i) The cytosolic alkalinization was rather rapidly reversed (Fig. 2). (ii) In a number of the experiments of Table 6, the highest concentrations of ouabain caused some acidification (by 0.02–0.25 pH units) rather than alkalinization. This was the case in 5 of the 37 experiments with 0.5 mM ouabain and 5 of the 12 with 1 mM ouabain. Both points (i) and (ii) suggest that a more complete inactivation of Na-H exchange (either by more prolonged exposure to, or higher concentrations of, ouabain) led to a retention of H^+ which exceeded its removal by V-ATPase. (iii) In the presence of 25 mM NO_3^-, ouabain not only failed to cause the alkalinization (Fig. 3*c*) but sometimes led to a sustained acidification which was not reversed (*not shown*). It is concluded that ouabain had both the effects suggested above but that, at least immediately after addition of ouabain, the stimulated activity of V-ATPases

Table 5. Effects of bafilomycin A_1 on cytosolic pH, vesicular pH and cellular K^+ content[a]

Bafilomycin (μM)	(A) Cytosolic pH and cellular K^+ content[a]			(*n*)
	K^+ content (mmol/kg. dry wt)	pH before bafilomycin	pH with bafilomycin	
Control	210 ± 56			(8)
1.0	186 ± 11	7.20 ± 0.14	7.26 ± 0.12	(3)
5.0	216 ± 27	7.10 ± 0.26	7.41 ± 0.22	(3)
10.0	165 ± 12	6.71 ± 0.42	6.76 ± 0.47	(5)
25.0	145 ± 42	7.04 ± 0.22	7.06 ± 0.26	(5)
50.0	87 ± 20	7.18 ± 0.20	7.22 ± 0.16	(5)

(B) Vesicular pH[b]

Control pH	pH with bafilomycin	
	4 μM	8 μM
4.51[c]	4.62	4.67
5.74	5.76	5.86
5.85	6.35	

[a] Cells were loaded with BCECF during incubation in flasks. Samples were transferred to fluorimeter cuvettes, and pH was determined before and after addition of bafilomycin A_1 to the final concentrations indicated. Bafilomycin was added to other flasks incubated in parallel with those used for fluorimetry; 20 min after the addition, cell samples were taken for K^+ analysis. [b]Procedures as for A, except that cells were loaded with FITC-dextran and RBI-dextran instead of BCECF. [c]Results are single observations from three separate experiments.

was great enough to counteract the accumulation of H^+ resulting from inhibition of the Na-H exchanger.

Comparison of Nitrate and Gluconate

In many of the experiments described so far, NO_3^- was introduced as a substitute for an equimolar amount of Cl^-. It is possible that the observed effects could have been due in part to the effect of the reduced Cl^- concentration on V-ATPase activity. To test this, and also to determine whether the ability of NO_3^- to permeate membranes [39] was a factor, a comparison was made of NO_3^- and the nonpermeant anion, gluconate. At 25 mM, both nitrate and gluconate substituted for chloride produced a cytosolic acidification of similar magnitude, but whereas (as described above) the nitrate-treated cells recovered from this acidification within 10–15 min, those treated with gluconate did not (Table 7). Moreover, gluconate produced a marked acidification at all concentrations up to 75 mM (Table 7), in contrast to NO_3^- (Tables 1 and 7). This suggests that, at high concentrations, NO_3^- may effectively substitute for Cl^- as the counter-ion for H^+ transport, so relieving its own inhibition of the V-ATPase, whereas the membrane-impermeant gluconate ion cannot.

Gluconate also differed from NO_3^- in three other respects: (i) At 25 mM, gluconate failed to eliminate the ouabain-induced alkalinization (Table 8). (ii) Gluconate at 25 mM did not conserve cellular ATP levels (Table 3). These two observations point to a direct inhibition of the V-ATPase by NO_3^- which is not due to reduced Cl^-. (iii) In measurements using the fluorescent dye, Oxonol V, plasma membrane potential was not significantly affected 5 min after transfer from the full HEPES-Ringer to media in which 25 or 50 mM Cl^- was replaced by NO_3^- or gluconate. However, 75 mM gluconate induced a threefold greater increase of fluorescence intensity (+17 ± 3%) than 75 mM NO_3^- (+5.5 ± 2.2%), indicative of a more marked depolarization in gluconate-containing medium. This is to be expected when intracellular chloride leaks from the cell in response to the 50–60% reduction of medium Cl^- and cannot be replaced by entry of the impermeant gluconate. By contrast, equilibration of the permeant NO_3^- would compensate for any Cl^- loss.

These data appear to indicate that in experiments substituting NO_3^- for an equivalent amount of Cl^-, the effects of NO_3^- are only partially attributable to the corresponding reduction of medium Cl^-, while most are due to a direct inhibition at the V-ATPase. Two other findings support this. First, 25 mM NO_3^- substituted for Cl^- did not reduce the initial influx of ^{36}Cl across the plasma membrane (*not shown*). Second, a series of experiments in which 25 mM NO_3^- or gluconate were added to the full HEPES-Ringer (132.4 mM Cl^-) caused acidifications similar to those noted above; the cytosolic pH fell by 0.33 ± 0.08 (4) pH units in response to nitrate and by 0.23 ± 0.10 (4) when gluconate was added. In addition, cell water contents in gluconate (e.g., after 1 min, 5.05 ± 0.60 μl/mg dry wt) did not differ significantly from those of nitrate-treated cells (mean 5.49 μl/mg, *see above*).

pH Dependence of the V-ATPase

The sensitivity of the V-ATPase to intracellular pH was examined by adding cells to media of differing pH (pH_e ranging from 6.0–8.0), with and without 25 mM nitrate, using BCECF. The effect of 25 mM NO_3^- on the change of cytosolic pH (pH_i) at each level of pH_e was determined. In each individual assay, the pH_i was determined first in the standard Ringer medium with pH_e 7.4 (Table 9, columns 1 and 4) and then after transfer to an experimental medium of different pH_e without (column 3) or with (column 5) 25 mM NO_3^-; in either case the change of pH_i was noted (i.e., for controls, column 1 *minus* column 3; for nitrate, 4–5). The transfer to media with or without nitrate was made alternately in consecutive assays with cells of the same preparation. The difference

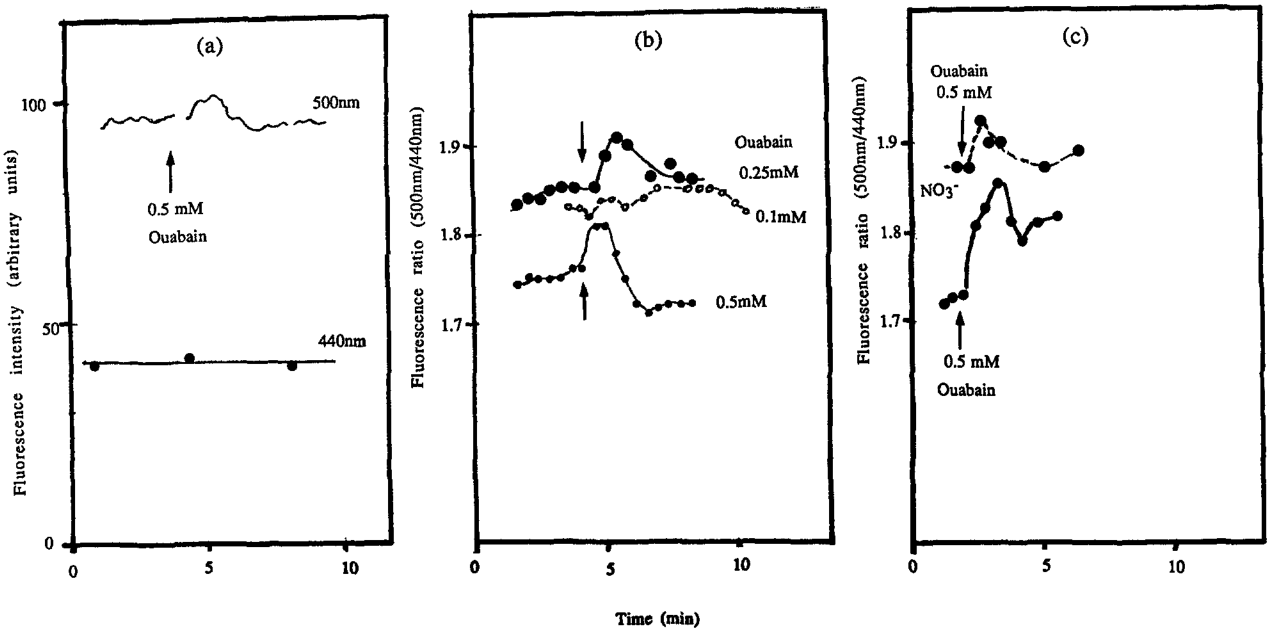


Fig. 2. Concentration-dependent ouabain-induced alkalinization and effect of nitrate. (*a*) Representative tracing showing effects of 0.5 mM ouabain, added at arrow. (*b*) Effects of different concentrations of ouabain on three samples from the same preparation of hepatocytes, showing the ratio of fluorescence intensities upon excitation at 500 and 440 nm. (*c*) Fluorescence ratios upon addition of 0.5 mM ouabain in the presence of 25 mM NO_3^- (upper trace) and its absence (lower trace). Addition of ouabain indicated by the arrows.

Table 6. Concentration dependence of ouabain-induced alkalinization and effects of nitrate[a]

Control pH	Ouabain (mM)	Maximum pH	ΔpH	*P*	*n*
(A) Ouabain titration; BCECF measurements					
6.84 ± 0.27	0.10	6.98 ± 0.25	0.14 ± 0.06	>0.05	6
7.19 ± 0.17	0.25	7.30 ± 0.14	0.11 ± 0.07	>0.05	9
6.93 ± 0.09	0.50	7.14 ± 0.07	0.20 ± 0.04	<0.001	37
6.69 ± 0.11	1.0	7.03 ± 0.20	0.34 ± 0.13	<0.05	12
(B) Effect of nitrate; BCECF measurements					
7.11 ± 0.15	0.5	7.43 ± 0.17	0.32 ± 0.09	<0.01	9
6.99 ± 0.11	0.5 + NO_3[b]	6.87 ± 0.10	−0.13 ± 0.04	<0.05	9
(C) DMO measurements					
7.27 ± 0.03	1.0	7.46 ± 0.04	0.19 ± 0.05	0.01	6
7.29 ± 0.05	1.0 + NO_3[b]	7.14 ± 0.11	−0.15 ± 0.09	>0.05	6
7.23 ± 0.07	1.0 + NO_3[c]	7.20 ± 0.08	−0.034 ± 0.10	>0.05	5

[a] For measurements with BCECF, pH values were followed for 5–10 min in HEPES-Ringer. After addition of ouabain, the maximum pH was attained between 0.5 and 2 min. For measurements with DMO, cells were loaded for 20 min either in HEPES-Ringer or in the medium with 25 mM NO_3^- substituted for the same concentration of Cl^- (*see* Materials and Methods); samples were then assayed for the "control" pH. Ouabain was then added, and samples were taken 1 min later; time course studies with DMO showed that this was the mean time for maximum alkalinization (*not shown*). [b] 25 mM nitrate present throughout incubation. [c] 25 mM present during last 60 sec of the preincubation.

between these values, (1–3) – (4–5) = ΔpH_V, was taken to indicate the contribution of V-ATPase to the maintenance of pH_i. When the medium pH was 6.0–6.5, the cytosol in control cells was slightly more alkaline than the medium (pH_i 6.6–6.7; Table 9, column 3) and 25 mM nitrate did not cause a significant change in this (column 5), i.e., V-ATPase showed little activity (column 6). At pH_e 7.0 ($pH_i = 6.89 \pm 0.15$), the difference of pH_i in cells with and without nitrate became more pronounced (ΔpH_V 0.059 ± 0.042; Table 9, column 6) and at pH_e 7.4 (pH_i 7.3 ± 0.11) the difference between control and nitrate-treated cells was maximal (ΔpH_V 0.095 ± 0.040).

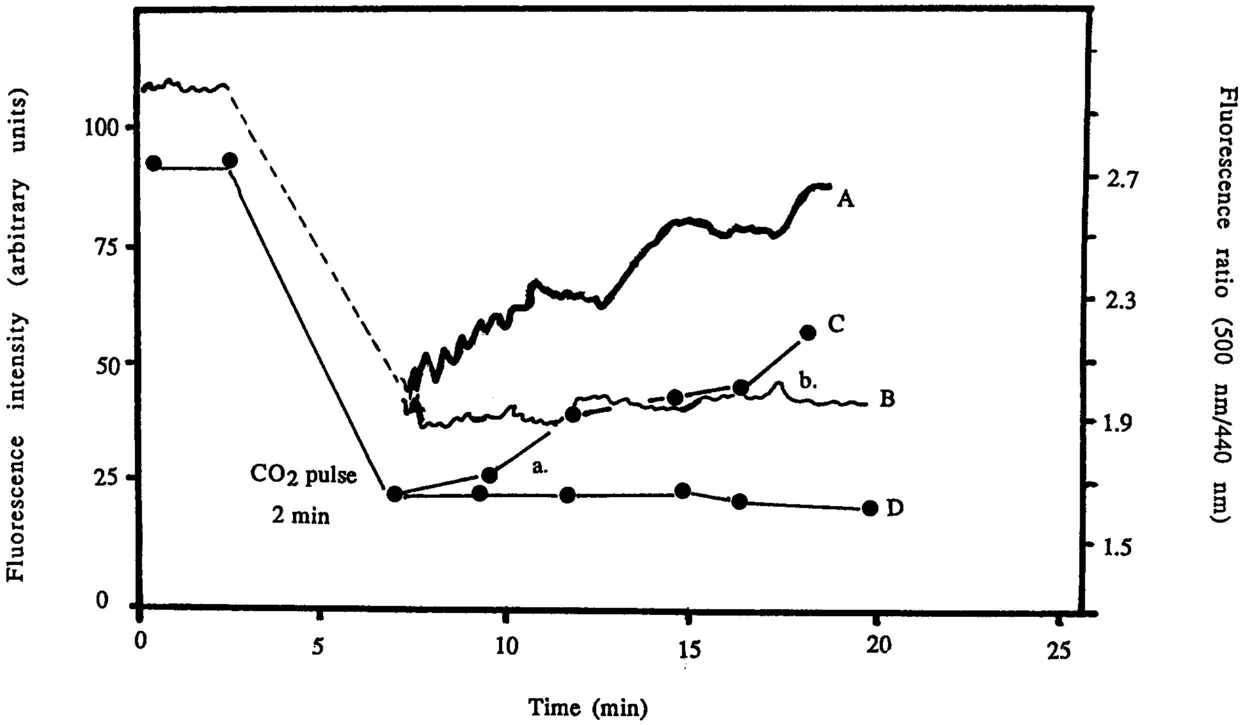


Fig. 3. Representative BCECF tracings of acid loading followed by recovery in either control or nitrate (25 mM) media. The figure illustrates two samples of cells from the same preparation of hepatocytes. Lines *A* and *B* are fluorescence tracings at 500 nm excitation from cells in the Cl^- medium or 25 mM NO_3^- medium, respectively. Lines *C* (control) and *D* (nitrate) show the ratio of fluorescence intensities at 500 and 440 nm. The first part of each line illustrates the initial pH for both the control and nitrate (25 mM) treated cells. The latter were pretreated with 25 mM nitrate for 15–20 min to allow the nitrate-induced acidification as well as recovery from this acidification to be completed. Acid loading was then carried out by gassing with 5% CO_2 for 2 min, with consequent fall both of the fluorescence intensity at 500 nm and of the ratio. The recovery was then followed for 15 min after restoration to HEPES-Ringer with or without nitrate. Rates of recovery for five such experiments are presented in Table 11. The figure suggests possible regions in which the Na/H exchanger (*a.*) and V-ATPase predominate (*b.*). *See* text for further explanation.

Table 7. Comparison of effects of nitrate and gluconate on cytosolic pH[a]

Experimental anion (mM)	ΔpH in response to experimental anion	
	NO_3^-	Gluconate
25	−0.28 ± 0.05 (14)[b]	−0.28 ± 0.04 (14)
37.5	−0.10 (2)[b]	−0.45 ± 0.12 (3)
50	−0.10 ± 0.08 (7)	−0.17 ± 0.10 (7)
75	+0.16 (2)	−0.47 (2)

[a] pH was measured with BCECF. Cells were preincubated in HEPES-Ringer (132.4 mM Cl^-) before being transferred to media in which HEPES-Ringer was modified by substituting the indicated concentrations of anion for equal concentrations of Cl^-. Values are mean ± SEM (*n*) for the maximum change of pH (ΔpH) following the introduction of the experimental anion. [b] This initial acidification upon NO_3^- addition was followed by a return to the initial pH pertaining in the original HEPES-Ringer, in contrast to the unreversed acidification noted with all concentrations of gluconate (*see text*).

Thus, as the cytosolic pH decreased below 7.3, the nitrate-inhibitable V-ATPase became progressively less active. A similar pH dependence of V-ATPase activity has been noted with isolated organelles [1, 39].

Table 8. Initial effect of ouabain on cytosolic pH in media with 25 mM NO_3^- or gluconate

Experimental anion (25 mM)	pH before ouabain addition	pH after ouabain (1 mM)	ΔpH
Control			
(132.4 mM Cl^-)	7.20 ± 0.34	7.34 ± 0.28	0.14 ± 0.06
Nitrate (25 mM)	6.97 ± 0.25	6.71 ± 0.26	−0.23 ± 0.012
Gluconate (25 mM)	7.07 ± 0.32	7.20 ± 0.31	0.13 ± 0.017

pH measured with BCECF. Cells were preincubated for 10 min either in the HEPES-Ringer or in media in which 25 mM NO_3^- or gluconate were substituted for an equal concentration of Cl^-. The pH values shown are those noted before, and 1–2 min after, addition of 1 mM ouabain. (*n* = 3). Values are mean ± SEM (*n* = 3 in each case).

ACID LOADING

Cells were subjected to an acid load by gassing with CO_2 (*see* Materials and Methods) and then returned to a "recovery medium" of the control HEPES-Ringer with or without ouabain or 25 mM NO_3^-. In the last case, the cells were pretreated with nitrate and allowed to recover from the nitrate-induced acidification (*cf.* Fig. 1), before

Table 9. pH dependence of the nitrate-inhibitable V-ATPase[a]

(1) pH_i at pH_e 7.4 (control)	(2) pH_e	(3) pH_i at pH_e	(4) pH_i at pH_e 7.4 (control before NO_3)	(5) Min. pH_i + nitrate at pH_e	(6) Nitrate-inhibited ΔpH_v	*P*	(*n*)
7.39 ± 0.16	6.0	6.58 ± 0.13	7.38 ± 0.15	6.61 ± 0.16	−0.032 ± 0.044	>0.05	4
7.30 ± 0.16	6.5	6.74 ± 0.21	7.25 ± 0.18	6.70 ± 0.21	0.020 ± 0.031	>0.05	7
7.19 ± 0.11	7.0	6.89 ± 0.15	7.26 ± 0.14	6.89 ± 0.16	0.059 ± 0.042	>0.05	8
7.31 ± 0.11	7.4	7.30 ± 0.11	7.42 ± 0.09	7.23 ± 0.11	0.095 ± 0.04	<0.05	10
7.58 ± 0.21	8.0	7.55 ± 0.22	7.68 ± 0.21	7.55 ± 0.19	0.056 ± 0.055	>0.05	7

For description of the experimental procedure, *see text.* Columns (1) and (4) give the pH_i values at the control pH_e of 7.4. Column (4) is the value before addition of nitrate. Column (2) is the pH_e for columns (3) and (5). Column (3) shows the pH_i the cells can attain at the given pH_e without nitrate. Column (5) shows the minimum pH obtained after addition of nitrate. Values for column (6) were obtained as follows:
ΔpH_v (nitrate-inhibitable V-ATPase) = [pH_i (at pH_e 7.4) − pH_i (at pH_e)] − [pH_i (at pH_e 7.4 before NO_3) − min. pH_i (at pH_e + NO_3)];
i.e., as columns: 6 = [1 − 3] − [4 − 5].

Table 10. Effects of nitrate and ouabain on recovery from acid loading[a]

Medium before CO_2 pulse	ΔpH after CO_2 pulse	Recovery medium	ΔpH during recovery for 15 min	% Recovery	(*n*)
HEPES	−1.14 ± 0.13	HEPES	0.59 ± 0.08	68.0 ± 10.3	23
HEPES	−0.62 ± 0.09	HEPES + ouabain	0.06 ± 0.03	13.4 ± 6.4	6
Nitrate (25 mM)	−1.20 ± 0.19	Nitrate (25 mM)	0.14 ± 0.05	13.6 ± 5.9	16

Measurements of pH were made with BCECF. Experiments followed the pattern of Fig. 3. Cells were subjected to a pulse of CO_2 (5% in 95% O_2 for 2 min) and then transferred to fresh HEPES-Ringer at pH 7.4 modified where indicated. Ouabain was used at 0.5 mM. Values of pH were determined just before gassing, immediately after transfer to fresh medium and 15 min later; the differences between these values are given as mean ± SEM.

Table 11. Effects of nitrate on rates of recovery from acid loading

	Rate of recovery (ΔpH/min)	
	First phase of recovery	Second phase of recovery
HEPES (control)	0.18 ± 0.03	0.22 ± 0.04
Nitrate (25 mM)	0.048 ± 0.03	0.01 ± 0.01

Recoveries from acid loading (increase in pH) for five experiments with control and corresponding nitrate-treated cells were used to calculate rates of recoveries for the two rapid phases of recovery (*see* Fig. 3 and text for further details.) The methods followed those used in Table 10.

the CO_2 pulse was given, to ensure separation of the recoveries from the two different causes of acidification. Fifteen minutes was chosen as the standard time for which to monitor recovery of pH. Results of a typical experiment are shown in Fig. 3 and summarized in Tables 10 and 11. The gassing with CO_2 caused a mean decrease of 0.6–1.2 pH units in different experiments and control cells showed an average of 68% reversal of this change, expressed as percent pH units (Table 10). When ouabain (0.5 mM) was present in the recovery medium, the recovery rate expressed as the change in pH units (ΔpH) was only 10% of that in controls, with the net recovery amounting to only 13% of the acidification. This indicates the considerable importance of the Na-H^+ exchanger for proton expulsion upon acid-loading. Nitrate (25 mM) also inhibited the recovery, but the mean recovery rate as ΔpH in 15 min, presumably due to Na/H exchange, was double that in the presence of ouabain. Table 11 expresses the recovery from an acid load as the rate of pH increase for five control and corresponding nitrate-treated cells. Figure 3 and Table 11 show an indication of a biphasic recovery. Initially, a rapid rate of recovery occurred (presumably indicating rapid activation of the Na/H exchanger), followed by a slowing of the rate of recovery (possibly due to the fall of Na/H exchanger activity as pH_i rises). This phase was then followed by a second increase in pH (possibly mediated by activation of the V-ATPase). In most experiments nitrate reduced and ouabain eliminated the first phase of recovery with no evidence of a second phase. These five experiments in Table 11 illustrate such an inhibitory effect of nitrate on the first phase of recovery, although the degree to which nitrate inhibited the first phase of the recovery was greater than predicted from the pH sensitivity of the V-ATPase (Table 9). Clearly, each of the two processes is important in the recovery, although the degree of interaction between the Na/H exchanger and V-ATPase appears to be more complex than expected.

Discussion

Most studies of the regulation of cytosolic pH in hepatocytes have emphasized the importance of transport systems of the plasma membrane. Bicarbonate-dependent systems are believed to be of little importance in pH regulation in hepatocytes and were eliminated as a factor in our experiments by the use of HCO_3^--free media. Rather, the Na/H exchanger has been shown to account for 70% of the pH regulation in hepatocyte couplets [11], becoming active especially when cytosolic pH falls [23]. Results described above are in agreement with the importance of this system for they show that recovery from an acid load, whether as a presumed endogenous load by release of vacuolar protons (i.e., in the presence of nitrate) or as an exogenous load (following acidification of the medium with CO_2), is inhibited by amiloride or ouabain. However, the principal inference from our results is that V-ATPase-dependent proton transport also plays a considerable role in these cells. This is indicated by the cytosolic acidification and vesicular alkalinization caused by moderate concentrations (16–37 mM) of nitrate and the effects on cytosolic pH of conditions that alter Cl^- content. The contribution of V-ATPases to pH regulation increased as the pH of the cytosol increased towards neutrality i.e., in the contrary fashion to the contribution of the Na/H exchanger.

Measurements of cytosolic pH were made by two widely used methods which depend on different principles, namely the pH-dependent changes of the fluorescence of BCECF and the altered distribution of the weak acid, DMO, between cells and medium. In each case the marker compound entering the cells can be expected to be predominantly confined to the cytosol, rather than entering acidic organelles. Both methods gave very similar results when directly compared (Tables 1 and 6). This was true both for the steady-state pH levels under different conditions and for the changes of pH in response to test agents. Measurements of vesicular pH were made with a widely used marker, FITC-dextran, which is confined to acidic vesicles. The results indicate changes of vesicular pH opposite to those seen with BCECF and DMO and so further substantiate a distinct compartmentalization of the presumed vesicular and cytosolic pH indicators.

EVIDENCE FOR V-ATPase INVOLVEMENT

Nitrate as Inhibitor

Our evidence that V-ATPases play a role in pH regulation is to a large extent based on the universal effects of NO_3^- as an inhibitor and of Cl^- as an activator of these systems in a wide range of organelles in animal and plant cells [1, 9, 19, 22, 32, 34]. The kinetics of NO_3^- inhibition studied in liver Golgi membranes show it to act competitively against the activating effects of Cl^-, with K_i of 2–6 mM, depending on whether proton accumulation or ATPase was the activity studied [41]. Considering that the present work was done with intact cells, the requirement of 16 mM NO_3^- for a significant effect on cytosolic pH and 25 mM for maximal effect, is in reasonable agreement with the K_i values. Lukacs et al. [14] found an apparent K_i of 25 mM for nitrate inhibition of phagosomal acidification in permeabilized macrophages. That NO_3^- induced cytosolic acidification in hepatocytes by inhibiting vesicular V-ATPase is strongly supported by the concomitant increase in vacuolar pH measured with FITC-dextran. We are not aware of any other effect of NO_3^- on animal cells at these concentrations. Control experiments measuring other parameters showed no significant effects of 25 mM NO_3^- on cell viability, membrane potential or K^+ and ATP contents, except for an increase of the last that is itself possibly attributable to inhibition of ATP consumption by an ATPase.

In summary, three types of experiments were carried out with NO_3^-, all of which were consistent with the suggestion that it inhibited a system that was responsible for removing H^+ from the cytosol: (i) it caused cytosolic acidification and vesicular acidification in cells that were not subjected to other treatments; (ii) it prevented or greatly slowed recovery of pH after acidification by a pulse of CO_2; (iii) it prevented the transient alkalinization of the cytosol upon treatment with ouabain. Accordingly, we propose that V-ATPases are involved in each case.

Activation by Cl^-

The concentration of Cl^- required for half-maximal activation of V-ATPase in isolated Golgi vesicles was 27 mM for proton accumulation [41] and 16 mM for Cl^- uptake [33]. The cytosolic Cl^- concentration reported for isolated hepatocytes is 25–32 mM [6] and therefore in the range for small changes to exert a measure of control on V-ATPase activity in the intact cell. That such effects can occur is shown by experiments in which reduction of medium Cl^- from 132 to 107 mM, by replacement with 25 mM gluconate, led to an initial cytosolic acidification not significantly different from that due to NO_3^-. However, it does not follow that the acidification seen due to NO_3^- was solely due to reduction of Cl^-, for similar effects were obtained when NO_3^- was added to the full Ringer medium (i.e., 132.4 mM Cl^-) instead of being substituted for Cl^-.

A different indication of the activating effect of cytosolic Cl^- is suggested by the transient, ouabain-induced alkalinization. This effect of ouabain cannot be explained by activation of Na-dependent mechanisms of the plasma membrane, such as the Na/H exchanger, for their inhibition would be expected from a reduction of the electrochemical gradient of Na^+. It is, however, explicable if the increase of cytosolic Cl^- induced by ouabain, secondarily to inhibition of Na-K ATPase, activates the V-ATPases to remove protons from the cytosol. The importance of V-ATPase is shown by the fact that the cytosolic alkalinization is abolished by 25 mM nitrate, but not by 25 mM gluconate. Upon more complete inhibition of Na-H exchange activity, by higher concentrations of, or longer exposure to ouabain, the expected inhibition of Na-coupled processes became evident as the early return of pH to its baseline (pre-ouabain) level and subsequent acidification.

POSSIBLE CONTRADICTING RESULTS

High Concentrations of Nitrate

While the above results appear to be mutually consistent in suggesting a role for V-ATPase in cytosolic pH regulation, the failure of higher concentrations of NO_3^- (50–75 mM) to cause acidification requires explanation. That it is due to a release of the inhibition of V-ATPase is shown by the simultaneous absence of vesicular alkalinization at these concentrations of nitrate. This observation is similar to the finding with isolated Golgi vesicles that partial substitution of Cl^- by 25 mM NO_3^- (leaving 125 mM Cl^- present) caused greater inhibition of proton accumulation than did complete replacement with 150 mM nitrate [41]. In lysosomes, 100 mM nitrate had no significant effect on proton pumping activity [1]. Sulfite and SO_4^{2-} also show concentration-dependent, biphasic effects on lysosomal V-ATPase activity, although in the reverse sense to NO_3^- [1]. Thus, biphasic effects of anions are well recognized in isolated organelles and there is no reason to suppose that they will not also be reflected as changes in whole-cell cytosolic pH.

That the inhibition by NO_3^- is competitive with Cl^- is usually taken to mean that the former prevents the charge-compensating uptake of Cl^-, probably by blocking the Cl^- channel. However, nitrate is relatively well able to pass biological membranes [39]. It is therefore proposed that when the electrochemical gradient for NO_3^- across the V-ATPase-containing membranes is great enough, nitrate is itself able to pass either directly through the membrane or through a Cl^- channel (separate from the ATPase) in sufficient quantity to replace Cl^- as a counter-ion to H^+, so restoring H^+ transport. The failure of correspondingly high concentrations of the much less permeant anion, gluconate, to show a similar restoration of pH control is consistent with this proposal.

Bafilomycin

Like moderate concentrations of NO_3^-, bafilomycin A_1 caused alkalinization of the hepatocyte compartments containing FITC-dextran, confirming the role of V-ATPase in the action of NO_3^-. On the other hand, bafilomycin did not induce acidification of the cytosol at any concentration used (1–50 μM). However, the rather high specificity of bafilomycin as an inhibitor of V-ATPase rather than of Na-K ATPase is based on experiments with isolated organelles and is dependent on its concentration [3]. The situation is less clear in intact cells where both of these ATPases are active and the relative concentrations of bafilomycin pertaining in the immediate environment of each is likely to be modified by permeability barriers e.g., the concentration at the plasma membrane Na-K ATPase is likely to be higher than that attained at the organellar V-ATPases. The present findings (Table 5) suggest that there is a considerable overlap between the concentration of bafilomycin inhibiting vesicular V-ATPase and Na-K ATPase (as indicated by K^+ content) in the whole hepatocytes. This dual effect would result in several direct and indirect alterations of ionic homeostasis and membrane depolarization that could override the cytosolic pH changes immediately caused by inhibition of V-ATPase.

INTERACTION OF V-ATPase AND Na/H EXCHANGE IN pH REGULATION

Our results show that the activity of V-ATPase in determining cytosolic pH is sensitive to pH changes in the opposite manner to the Na/H exchanger. In hepatocytes, the latter is known to have minimal activity at pH_i 7.2 and maximal activity at pH_i 6.4 [23]. By contrast, the acidification caused by nitrate, reflecting the role of V-ATPase, is low at pH_i 6.6, but increases significantly when pH_i is 6.8 and is maximal at 7.3 and above. Similar results were obtained in studies of proton accumulation by isolated Golgi membranes [41] and lysosomes [1]. Thus, in principle, the two activities could readily complement each other as pH_i changes from acidic to neutral and *vice versa,* the one becoming more active as the other declines. Our results indicate that such an important interaction occurs; however, the degree of interaction between the two systems appears to be more substantial than predicted from the pH sensitivities of both systems.

One possible indication of the interaction between the Na/H exchanger and V-ATPase is the inhibition by amiloride of recovery from the nitrate-induced acidification. The recovery appears to be due to the amiloride-sensitive Na/H exchanger, which would normally be active at pH 6.8 (nitrate-induced). Secondly, the recovery from the externally introduced acid load appears to be sensitive to inhibitors (nitrate and ouabain) of both systems but faster when the Na/H exchanger was active ($\Delta pH = 0.14 \pm 0.05$ pH units) than without it ($\Delta pH = 0.06 \pm 0.03$ pH units). That the interaction between the two systems is more complex than indicated by the pH sensitivities is demonstrated by the degree of inhibition by nitrate of the first part of the recovery. All of the data, however, indicate that the interaction between the two systems is necessary for regulation of cytosolic pH.

SITE OF THE V-ATPase REGULATING CYTOSOLIC pH IN HEPATOCYTES

Although the V-ATPases are characteristically associated with the membranes of intracellular organelles, they have also been found in the plasma membranes of renal and alveolar epthelia [13] and of macrophages [14, 15]. In these instances, the plasma membrane V-ATPases contribute to regulation of cytosolic pH, but this does not preclude the V-ATPases of the intracellular organelles of these cells having a contributory role, although this has not been directly suggested, to date.

In the case of hepatocytes, there is no evidence for the presence of V-ATPase in the plasma membrane [26]. Our failure to find an effect of bafilomycin on cytosolic pH, while inhibiting K^+ accumulation, seems to be consistent with the lack of a contribution of plasma membrane V-ATPase to pH control. By contrast, the V-ATPases of intracellular organelles were inhibited both by nitrate and bafilomycin. We therefore conclude that it is these which contribute to removal of H^+ from the cytosol. Others have shown that leakage of H^+ from vesicular contents can acidify the cytosol [4, 17] although they did not explicitly postulate a role for the V-ATPases in pH control.

Whether the total organellar V-ATPase activity and buffering capacity are sufficient to play a role of significant magnitude in normal conditions, in comparison to the other recognized systems at the plasma membrane, will require further information. However, it is significant that morphometric estimates of vesicular surface area in the hepatocyte, under the heading of ''smooth endoplasmic reticulum'' (but including Golgi apparatus and a range of other vesicular membranes) is at least 16 times the surface area of the plasma membrane [37]. This suggests the possibility that the total organellar V-ATPase activity may be at least of a similar order to that of plasma membrane pH-regulating systems. Consistent with this Bronk and Gores [4] estimated the total volume of acidic vesicles in cultured hepatocytes to be approximately 4% of the cell volume and the immediate release of their acid contents to contribute 20% of the pH change resulting from ATP depletion.

This work was supported in part by National Institutes of Health B.R.S. Grant 507 RR05417 to Temple University.

References

1. Arizio, K., Shimaya, A., Hiratani, N., Ohkuma, S. 1991. Purification and characterization of lysosomal H^+-ATPase. An anion-sensitive v-type H^+-ATPase from rat liver lysosomes. *J. Biol. Chem.* **268:**5649–5660
2. Bidani, A., Brown, E.S. 1990. ATP-dependent pH_i recovery in lung macrophages: evidence for a plasma membrane H^+ ATPase. *Am. J. Physiol.* **259:**C586–C598
3. Bowman, E.J., Siebers, A., Altendorf, K. 1988. Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells and plant cells. *Proc. Natl. Acad. Sci. USA* **85:**7972–7976
4. Bronk, S.F., Gores, G.J. 1991. Efflux of protons from acidic vesicles contributes to cytosolic acidification of hepatocytes during ATP depletion. *Hepatology* **14:**626–633
5. Clague, M.J., Urbé, S., Aniento, F., Gruenberg, J. 1994. Vacuolar ATPase activity is required for endosomal carrier vesicle formation. *J. Biol. Chem.* **269:**21–24
6. Fitz, G.J., Scharschmidt, B.F. 1987. Intracellular chloride activity in intact rat liver: relationship to membrane potential and bile flow. *Am. J. Physiol. 252:*G699–G706
7. Forgac, M. 1989. Structure and function of vacuolar class of ATP-driven proton pumps. *Physiol. Rev.* **69:**765–796
8. Geisow, M.J. 1984. Fluorescein conjugates as indicators of subcellular pH. A critical evaluation. *Exp. Cell Res.* **150:**29–35
9. Glickman, J., Croen, K., Kelly, S., Al-Awqati, Q. 1983. Golgi membranes contain an electrogenic H^+ pump in parallel to a chloride conductance. *J. Cell Biol.* **97:**1303–1308
10. Gross, D., Loew, L.M. 1989. Fluorescent indicators of membrane potential: microspectrofluorometry and imaging. *Methods Cell Biol.* **30:**193–218
11. Henderson, R.M., Graf, J., Boyer, J.L. 1987. Na-H exchange regulates intracellular pH in isolated rat hepatocyte couplets. *Am. J. Physiol.* **252:**G109–G113
12. Kulka, R.G. 1956. Colorimetric estimation of ketopentoses and ketohexoses. *Biochem. J.* **63:**542–548
13. Lubman, R.L., Danto, S.I., Crandall, E.D. 1989. Evidence for active H^+ secretion by rat alveolar epithelial cells. *Am. J. Physiol.* **257:**L438–L-445
14. Lukacs, G.L., Rotstein, O.D., Grinstein, S. 1990. Phagosomal acidification is mediated by a vacuolar-type H^+-ATPase in murine macrophages. *J. Biol. Chem.* **265:**21099–21107
15. Lukacs, G.L., Rotstein, O.D., Grinstein, S. 1991. Determinants of phagosomal pH in macrophages. *J. Biol. Chem.* **266:**24540–24548
16. Madshus, I.H. 1988. Regulation of intracellular pH in eukaryotic cells. *Biochem. J.* **250:**1–8
17. Madshus, I.H., Tonnessen, T.I., Olsnes, S., Sandvig, K. 1987. Effect of potassium depletion of Hep 2 cells on intracellular pH and on chloride uptake by anion antiport. *J. Cell Physiol.* **131:**6–13
18. Mellman, I., Fuchs, R., Melenius, A. 1986. Acidification of the endocytic and exocytic pathways. *Annu. Rev. Biochem.* **55:**663–700
19. Moriyama, Y., Nelson, N. 1989. H^+-translocating ATPase in Golgi apparatus. Characterization as vacuolar H^+-ATPase and its subunit structures. *J. Biol. Chem.* **264:**18445–18450
20. Noel, J., Laprade, R., Vinay, P., Berteloot, A. 1992. The bafilomycin (Baf) sensitive H^+-ATPase requires 30% of the cell ATP turnover in dog renal proximal tubules (PT), but 10% in rabbit PT. *FASEB J.* **6:**A1460
21. Pollock, A.S. 1984. Intracellular pH of hepatocytes in primary monolayer culture. *Am. J. Physiol.* **246:**F738–F744
22. Randall, S.K., Sze, H. 1986. Properties of the partially purified tonoplast H^+-pumping ATPase from oat roots. *J. Biol. Chem.* **261:**1364–1371
23. Renner, E.L., Lake, J.L., Persico, M., Scharschmidt, B.F. 1989. Na^+-H^+ exchange activity in rat hepatocytes: role in regulation of intracellular pH. *Am. J. Physiol.* **256:**G44–G52

24. Ruiz, O.S., Arruda, J.A.L. 1992. ATP-dependent renal H^+ translocation: regional localization, kinetic characteristics and chloride dependence. *Proc. Soc. Exp. Biol. Med.* **200:**562–570
25. Russo, M.A., van Rossum, G.D.V., Galeotti, T. 1977. Observations on the regulation of cell volume and metabolic control in vitro: Changes in composition and ultrastructure of liver slices under conditions of varying metabolic and transport activity. *J. Membrane Biol.* **31:**267–299
26. Scharschmidt, B., Van Dyke, R.W. 1977. Proton transport by hepatocyte organelles and isolated membrane vesicles. *Annu. Rev. Physiol.* **49:**69–85
27. Seglen, P.O. 1976. Preparation of isolated rat liver cells. *Methods Cell Biol.* **8:**39–81
28. Simon, B.J., Kulanthaivel, P., Burckhardt, G., Ramamoorthy, S., Leibach, F.H., Ganapathy, V. 1992. Characterization of an ATP-driven H^+ pump in human placental brush-border membrane vesicles. *Biochem. J.* **287:**423–430
29. Swallow, C.J., Grinstein, S., Rotstein, O. 1990. A vacuolar type H^+-ATPase regulates cytoplasmic pH in murine macrophages. *J. Biol. Chem.* **265:**7645–7654
30. Thomas, J.A., Buchsbaum, R.N., Zimniak, A., Racker, E. 1979. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry* **18:**2210–2219
31. Tsien, R.Y. 1989. Fluorescent indicators of ion concentrations. *Methods Cell Biol.* **30:**127–156
32. Van Dyke, R.W. 1986. Anion inhibition of the proton pump in rat liver multivesicular bodies. *J. Biol. Chem.* **261:**15941–15948
33. van Rossum, G.D.V., Yeh, H.-I., Schisselbauer, J.C. 1991. Properties and possible functions of the vacuolar ATPase of liver Golgi apparatus. *Acta Med. Romana* **29:**7–18
34. van Rossum, G.D.V., Russo, M.A., Schisselbauer, J.C. 1987. Role of cytoplasmic vesicles in volume maintenance. *Curr. Top. Membr. Transp.* **30:**45–75
35. Wadsworth, S.J., van Rossum, G.D.V. 1990. Effect of ouabain on pH and isoosmotic volume changes in hepatocytes. *Eur. J. Pharmacol.* **183:**1218
36. Wadsworth, S.J., van Rossum, G.D.V. 1992. Possible role of the vacuolar ATPases in cytosolic pH regulation. *FASEB J.* **6:**A118
37. Weibel, E.R., Staubli, W., Gnagi, H.R., Hess, F.A. 1969. Correlated morphometric and biochemcial studies on the liver cell. *J. Cell Biol.* **42:**68–91
38. Williamson, J.R., Corkey, B.E. 1969. Assays of the citric acid cycle and related compounds by fluorometric enzyme methods. *Methods Enzymol.* **13:**434–513
39. Wright, E.M., Diamond, J.M. 1977. Anion selectivity in biological systems. *Physiol. Rev.* **57:**109–156
40. Xie, X.-S., Crider, B.P., Stone, D.K. 1989. Isolation and reconstitution of the chloride transporter of clathrin-coated vesicles. *J. Biol. Chem.* **264:**18870–18873
41. Yeh, H.-I., van Rossum, G.D.V. 1991. Proton accumulation and ATPase activity in Golgi apparatus-enriched vesicles from rat liver. *Hepatology* **13:**523–533
42. Young, G.P.-H., Qiao, J.-Z., Al-Awqati, Q. 1988. Purification and reconstitution of the proton translocating ATPase of Golgi-enriched membranes. *Proc. Natl. Acad. Sci. USA* **85:**9590–9594