Journal of Bioenergetics and Biomembranes, Vol. 23, No. 1, 1991

Cl^-, Na^+, and H^+ Fluxes during the Acidification of Rabbit Reticulocyte Endocytic Vesicles

Victoria Gaete,[1] Marco Tulio Núñez,[1] and Jonathan Glass[2]

Received March 7, 1990

Abstract

The ionic fluxes associated with the ATP-dependent acidification of endocytic vesicles were studied in a preparation isolated from rabbit reticulocytes enriched for transferrin–transferrin receptor complexes. No vesicle acidification was observed in the absence of intra- and extravesicular ions ($sucrose_{in}$/$sucrose_{out}$), while maximal acidification was observed with $NaCl_{in}/KCl_{out}$. K^+_{in} was a poor substitute for Na^+_{in}, and Cl^-_{out} could be replaced by other anions with the following efficacy of acidification: $Cl^- > Br^- > I^- > PO_4^{3-} >$ gluconate $> SO_4^{2-}$. Flux studies using $^{36}Cl^-$ and $^{22}Na^+$ showed that the vesicles had a permeability for Cl^- and Na^+, and that ATP-dependent H^+ pumping was accompanied by a net influx of Cl^- and a net efflux of Na^+ provided that there was a Na^+ concentration gradient. After 3 mins, the time necessary to maximal acidification, the electrical charge generated by the entrance of H^+ was countered to about 45% by the Cl^- influx and to about 42% by the Na^+ efflux. These studies demonstrated that both Cl^- and Na^+ fluxes are necessary for optimal endocytic vesicle acidification.

Key Words: Acidification; endocytic vesicle; Na^+ fluxes; Cl^- fluxes; H^+ fluxes; H^+-ATPase.

Introduction

Acidification of the endosome is an important event in the routing of internalized ligands and receptors. An NEM[3]-sensitive, H^+-translocating

[1]Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Santiago, Chile.
[2]Department of Medicine, Louisiana State University School of Medicine, Shreveport, Louisiana.
[3]Abbreviations used: NEM, *N*-ethylmaleimide; DIDS, 4,4′-diisothiocyanostilbene-2,2′-sulfonic acid; DCCD, *N,N′*-dicycohexylcarbodiimide; FITC, fluorescein isothiocyanate; Hepes, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid; EGTA, ethylglycol bis(aminoethyl)-*N,N,N′,N′*-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecylsulfate; MES, 2-(*N*-morpholino)ethanesulfonic acid.

0145-479X/91/0200-0147$06.50/0

ATPase is present in endocytic vesicles (Choe *et al.*, 1987; Yamashiro *et al.*, 1983; Forgac *et al.*, 1983; Galloway *et al.*, 1983; Stone *et al.*, 1983a; Van Dyke *et al.*, 1984) as well as cellular organelles such as lysosomes (Schneider, 1981; Harikumar and Reeves, 1983; Dell'Antone, 1984), Golgi vesicles (Glickman *et al.*, 1983), neurosecretory vesicles (Russell, 1984), and chromaffin granules (Njus and Rodda, 1977; Apps and Scaz, 1979; Johnson *et al.*, 1985). The ATPase from bovine brain coated vesicles has been purified and reconstituted in lipid vesicles (Xie *et al.*, 1984, 1986a). Studies on the H^+-ATPase indicates a complex subunit composition (Arai *et al.*, 1987a; Xie *et al.*, 1986b). A 73,000 molecular weight peptide has been identified as the catalytic subunit (Arai *et al.*, 1987a), and a 17,000 molecular weight peptide as the proton channel subunit (Arai *et al.*, 1987b; Sun *et al.*, 1987). Although the effect of anions on acidification has been studied (Yamashiro *et al.*, 1983; Van Dyke *et al.*, 1984; Xie *et al.*, 1983; Xie *et al.*, 1989), demonstration of ionic permeability and quantification of ionic fluxes associated with the influx of protons has not been addressed. Because of the dependence of acidification on Cl^- in the incubation medium (Yamashiro *et al.*, 1983; Van Dyke *et al.*, 1984; Xie *et al.*, 1983, 1989) and of the sensitivity of acidification to DIDS (Yamashiro *et al.*, 1983; Xie *et al.*, 1983), it is thought that the H^+-pump is electrogenic, and that the charges generated by the influx of H^+ are compensated by an influx of Cl^-.

In the particular case of the ligand ferrotransferrin, it has been proposed that acidification of the endocytic vesicle is necessary for the delivery of iron to the cell by (1) promoting the dissociation of iron from transferrin (Lestas, 1976); (2) facilitating the translocation of the dissociated iron through the endosome membrane (Núñez and Glass, 1985); and (3) allowing apotransferrin to remain bound to the transferrin receptor, which in turn allows the recycling of the complex to the plasma membrane (Escarot-Charrier *et al.*, 1980; Klausner *et al.*, 1983). Using endocytic vesicles isolated from rabbit reticulocytes, we have recently reported that vesicle acidification and iron reduction were necessary and sufficient for the mobilization of iron originally present in transferrin (Núñez *et al.*, 1990). During the course of the above experiments we observed that dissociation of iron from transferrin was dependent on acidification which in turn was dependent on the intra- and extravesicular ionic composition. Here we report on the process of acidification and quantitate the related ion fluxes in endocytic vesicles. Using FITC-labeled transferrin, and a novel approach to measure H^+ pumped, we quantitated the acidification in vesicles devoted to iron transport. Using $^{36}Cl^-$ and $^{22}Na^+$, we quantitated the Cl^- and Na^+ fluxes associated with the movement of H^+.

Experimental

Labeling of Transferrin

Purified rabbit ferrotransferrin was labeled with FITC as described by Musgrove *et al.* (1984), stored at 4°C, and used within one week of preparation. The FITC: transferrin molar ratio ranged between 4 and 5. In control experiments, FITC-labeled ^{59}Fe-transferrin underwent a normal endocytic and ^{59}Fe delivery cycle when incubated with reticulocytes.

Vesicle Preparation

Endocytic vesicles were prepared as reported (Núñez *et al.*, 1990). When acidification was determined, the cells were incubated with FITC-labeled transferrin for 5 min at 37°C prior to vesicle preparation. The preparation buffer contained 10 mM HEPES-Tris (pH 7.0), 1 mM EGTA-Tris, 1 mM $MgSO_4$, 3 mM NaN_3, and 100 mM either of NaCl, KCl, a mixture of both, or 200 mM sucrose. The salt composition of the preparation buffer depen-dended on the desired internal ionic composition for the particular experiment. The vesicles were used the same day of isolation.

Measurement of Vesicle Acidification

The internal pH of endocytic vesicles was determined as the decrease in the fluorescence of FITC-transferrin measured upon the addition of ATP (Van Renswoude *et al.*, 1982). A normal incubation mixture contained, in a final volume of 1.5 ml, 30–50 μg (5–15 μl) of vesicle protein and lysis buffer (pH 7.0) in which the content of NaCl, KCl, or sucrose was adjusted for the experimental conditions. The reaction was started by the addition of 1 mM ATP-Tris and 1 mM $MgSO_4$ (ATP-Mg) (pH 7.0), and fluorescence was followed in a Perkin-Elmer MPF-2A spectrofluorimeter with the detection chamber thermostated at 25°C. Calibration of fluorescence as a function of pH was determined for every vesicle preparation, with the vesicles solubilized with 0.2% Nonidet P-40 and suspended in isolation buffer at various pHs in the pH range 7.0–5.0. The last precaution was necessary because (1) the fluorescence at all pHs in the presence of sucrose-containing buffers was slightly quenched compared to fluorescence in KCl- or NaCl-containing buffers; and (2) FITC-transferrin fluorescence was quenched when bound to the transferrin receptor in vesicles compared to FITC-transferrin in solution.

ATPase Activity

The ATPase activity of intact vesicles was detected spectrophotometrically at 25°C using the pyruvate kinase/lactate dehydrogenase enzyme system

as reported (Yamashiro *et al.*, 1983). Prior to use the enzyme mixture was filtered through a small Sephadex G-25 column to eliminate the $(NH_4)_2SO_4$ present in the enzyme mixture.

$^{36}Cl^-$ Fluxes

$^{36}Cl^-$ influx was determined by incubation of 120–150 μg of vesicles in 100 μl final volume of a buffer containing: 10 mM (pH 7.0) Hepes-Tris, 1 mM EGTA-Tris, 1 mM $MgSO_4$, 3 mM NaN_3, 80 mM KCl, and 20 mM (5 μC) $Na^{36}Cl$, with or without 1 mM ATP-Mg. The interior of the vesicles contained: 10 mM Hepes-Tris (pH 7.0), 1 mM EGTA-Tris, 1 mM $MgSO_4$, 3 mM NaN_3, and 100 mM NaCl. Aliquots of the incubation mixture were taken at different times of incubation and filtered through 0.22-μm Millipore GSWP filters pretreated with the incubation buffer. Control experiments using ^{125}I-transferrin containing vesicles indicated a greater than 98% retention of vesicles by the filters. In experiments of $^{36}Cl^-$ efflux, the vesicles were preloaded with 100 mM $Na^{36}Cl$ for 60 min at 37°C and then diluted 100-fold in 80 mM KCl and 20 mM NaCl buffer at 25°C. Aliquots were taken at different times of incubation at 25°C and filtered as above.

$^{22}Na^+$ Fluxes

Unless otherwise stated, the ionic composition of the intravesicular medium established for influx and efflux experiments was 10 mM Hepes-Tris (pH 7.0), 1 mM EGTA-Tris, 1 mM $MgSO_4$, 3 mM NaN_3, and 100 mM NaCl; and the ionic composition of the external medium was 10 mM Hepes-Tris (pH 7.0), 1 mM EGTA-Tris, 1 mM $MgSO_4$, 3 mM NaN_3, 80 mM KCl, and 20 mM NaCl, with or without 1 mM ATP-Mg. In experiments of $^{22}Na^+$ influx, the external medium contained 5 μCi of ^{22}NaCl in a total volume of 100 μl. In experiments of efflux the vesicles were preloaded for 60 min at 37°C in a 100 mM NaCl medium containing 5 μCi $^{22}Na^+$, and then diluted 250-fold in a medium so as to give the final external ionic composition stated above. Aliquots of the incubation medium were taken at different times of incubation at 25°C and filtered as described above.

Determination of the H^+/ATP Ratio

To quantitate the movement of charges associated with ATP-induced acidification, it was necessary to determine the amount of protons pumped into vesicles under experimental conditions similar to those used for determination of $^{36}Cl^-$ and $^{22}Na^+$ fluxes. This was achieved by determining the ratio of protons pumped into the vesicles to the ATP hydrolyzed by the NEM-sensitive ATPase. To calculate the ratio of H^+ pumped to ATP

hydrolyzed, the following approximation of the equation derived by Thayer and Hinkle (1973) was used:

$$\ln(H^+/ATP) = \ln h - k_1 t$$

in which t is the time of incubation, h is the stoichiometry of protons pumped to ATP hydrolyzed, H^+ is the measured moles of protons accumulated in the vesicle at time t, ATP is the moles of ATP hydrolyzed at time t, and k_1 is the rate constant for proton leak from the vesicles. A semilogarithmic plot of H^+/ATP *vs.* time gives a line with a slope of $-k_1$ and an intercept at $t = 0$ equal to h, the H^+/ATP stoichiometry. The experimental protocols of Thayer and Hinkle (1973) were modified to allow measurement of the stoichiometry of proton pumping at pH 7.0, the same external pH at which the ionic fluxes were measured. At pH 7.0 the release of protons from the hydrolysis of ATP (Alberty, 1968; Nishimura *et al.*, 1961) prevented the direct measurement of pH change to determine the kinetics of H^+ pumping, and induced the design of an alternative method to determine pH changes. Vesicles suspended at 1 mg of vesicle protein/ml in a medium that contain 0.5 mM MES-Na (pH 7.0), 1 mM EGTA-Na, 1 mM $MgSO_4$, 3 mM NaN_3, and 100 mM NaCl were equilibrated in a buffer containing 0.5 mM MES-Na (pH 7.0), 1 mM EGTA-Na, 1 mM $MgSO_4$, 3 mM NaN_3, 80 mM KCl, and 20 mM NaCl. After about 5 min necessary for pH equilibration, the reaction was started by the addition of 1 mM ATP-Mg. The reaction was allowed to proceed for 3 min at 25°C, at which point it was stopped with 0.2% Nonindet P-40. The addition of detergent produced an acidification of the medium corresponding to the protons accumulated within the vesicles during the 3 min interval. Further additions of detergent did not produce further pH changes. No pH change occurred if the vesicles were incubated in the presence of 2 mM NEM. The buffer capacity of the medium was determined by the addition of aliquots of a 1 mM HCl standard solution with a total pH change of 0.045 pH units after three additions. The above procedure allowed determination of the total protons pumped into the vesicles during a 3-min interval. In parallel experiments, the kinetics of acidification were determined in vesicles containing FITC-transferrin as described above. Both procedures taken together allowed estimation of the amount of protons pumped as a function of time. The pH changes were followed with a Beckman 71 pH meter attached to an operational amplifier and recorder to give 10 mV full scale (Thayer and Hinkle, 1973). The quantification of ATP hydrolyzed by 1 mM NEM-sensitive ATPase was as described above (Forgac *et al.*, 1983).

Reagents

$H^{36}Cl$ (15.07 μCi/g), $^{22}NaCl$ (1071.56 mCi/mg), and $^{59}FeCl_3$ (20.17 mCi/mg) were from Dupont (Wilmington, Delaware). Carrier-free Na ^{125}I was

from Comisión Chilena de Energía Nuclear (Santiago, Chile). FITC, isomer I, was from Calbiochem (San Diego, California). Percoll, DIDS, DCCD, ATP, and other biochemical reagents used were of analytical grade.

Results

ATPase Activity and Acidification

The ATPase activities of reticulocyte endocytic vesicles have been partially characterized (Choe *et al.*, 1987). The overall initial ATPase activity of these vesicles was $3.10 \pm 0.78 \times 10^{-8}$ moles/min/mg of protein (mean $\pm$ s.d., $n = 11$). In order to determine which of the ATPases present in reticulocyte endocytic vesicle was responsible for the acidification process, the effect of different inhibitors was tested in the overall ATPase activity and in the acidification process. The results are shown in Table I. The ATPase activity was unaffected by oligomycin and strophanthidin. As with the ATPase of rat liver and bovine brain coated vesicles (Dell'Antone, 1984; Sun *et al.*, 1987), the ATPase activity of reticulocyte vesicles was sensitive to NEM, with 1 and 2 mM NEM inhibiting the ATPase activity 25.5 and 46%, respectively. As 1 mM NEM almost completely inhibited acidification, it is clear that the vesicles contain additional ATPase activities not sensitive to NEM. The ATPase responsible for acidification was also inhibited by DIDS, since this compound affected the acidification. As in coated vesicles from calf brain (Sun *et al.*, 1987), DCCD affected mildly the total ATPase activity, but

Table I. Effect of Various Compounds on ATPase Activity and Acidification

Inhibitor	ATPase activity	Acidification
	(% of control)	
1–5 μM oligomycin	96.2 $\pm$ 7.2 (4)	104.4 $\pm$ 13.6 (3)
50 μM strophantidin	94.2 $\pm$ 6.8 (4)	N.D.
10 μM DCCD	N.D.	72.7 $\pm$ 3.3 (2)
100 μM DCCD	82.9 $\pm$ 8 (4)	N.D.
1 mM NEM	74.5 $\pm$ 7.3 (4)	12.7 $\pm$ 5.4 (3)
2 mM NEM	54.2 $\pm$ 17.6 (7)	N.D.
10 μM DIDS	29.9 $\pm$ 0.9 (2)	55.3 $\pm$ 15.3 (2)
100 μM DIDS	N.D.	7.8 $\pm$ 0.42 (2)
0.5–1 mM amiloride	N.D.	85.1 $\pm$ 10.2 (5)

[a] The intravesicular composition of vesicles was 10 mM Hepes-Tris (pH 7.0), 1 mM EGTA-Tris, 1 mM $MgSO_4$, 3 mM NaN_3, and 100 mM NaCl. In the extravesicular buffer, NaCl was substituted by KCl. Prior to detection of ATPase activity or acidification, the vesicles were incubated with the various inhibitors for 30 min at 4°C. The temperature of the experiments was 25°C. The means $\pm$ standard deviation are presented; in parentheses is the number of determinations. N.D., not determined.

acidification was strongly inhibited. Acidification was slightly inhibited by 1 mM amiloride, while 1 μM tetrodotoxin and 10 nM caribdotoxin did not produce inhibition (data not shown).

Effect of the Internal and External Ionic Composition on the Generation of the H^+ Gradient

In an attempt to determine the ions involved both in cotransport and countertransport processes of charge compensation, the extent of acidification was determined in vesicles with various internal and external ionic compositions. The effects of various ionic compositions are seen in Table II. No acidification was observed in the absence of ions ($sucrose_{in}/sucrose_{out}$), and a cation was needed in the intravesicular medium, with Na^+ ($NaCl_{in}/sucrose_{out}$) better than K^+ ($KCl_{in}/sucrose_{out}$). The intermediate degrees of acidification obtained with the other conditions listed in Table II allow conclusions to be drawn regarding the relative influence of ions in the acidification of endocytic vesicles. The presence of Cl^- in the external medium was necessary, but not enough, to support maximal acidification: it allowed for 59% of maximal acidification for the condition $sucrose_{in}/KCl_{out}$ and 65% for $sucrose_{in}/NaCl_{out}$. Similarly, the contribution of a Na^+ gradient to acidification was about 44% ($NaCl_{in}/sucrose_{out}$). In contrast, little acidification was observed with ($KCl_{in}/sucrose_{out}$). From the above analysis it can be concluded that maximal acidification of the vesicles required Cl^- in the extravesicular medium and Na^+ inside the vesicles.

The role of Cl^- in supporting acidification was further explored in experiments in which the extravesicular anion was changed. Cl^- was the anion that best supported acidification. The relative efficiency of various anions was: chloride = 1, bromide = 0.71, iodide = 0.30, phosphate = 0.28,

Table II. The Effect of Ionic Composition on Acidification[a]

External	Internal	pH change
Sucrose	Sucrose	0.04 ± 0.03
Sucrose	KCl	0.09 ± 0.01
Sucrose	NaCl	0.34 ± 0.03
KCl	Sucrose	0.46 ± 0.05
KCl	KCl	0.46 ± 0.11
KCl	NaCl	0.78 ± 0.10
NaCl	Sucrose	0.51 ± 0.05
NaCl	KCl	0.42 ± 0.16
NaCl	NaCl	0.63 ± 0.12

[a]The internal and external ionic composition of the vesicles was 10 mM Hepes-Tris (pH 7.0), 1 mM $MgSO_4$, 1 mM EGTA-Tris, 3 mM NaN_3, and 100 mM KCl or NaCl, or 200 mM sucrose. The change in pH upon the addition of 1 mM Mg-ATP was determined for 3 min; the mean pH ± s.d. of three experiments is presented.

gluconate = 0.19, and sulfate = 0.14. These results are in agreement with data obtained in endocytic vesicles isolated from rat liver (Van Dyke *et al.*, 1984) and bovine brain (Stone *et al.* 1983b).

$^{22}Na^+$ *Fluxes in Endocytic Vesicles*

As indicated by the experiments of Table II, a Na^+ efflux may also be involved in the acidification process. In order to determine net Na^+ fluxes, parallel studies of $^{22}Na^+$ influx and efflux in the presence and absence of ATP were done (Fig. 1). Under conditions of a Na^+ gradient, there was a considerable net $^{22}Na^+$ efflux, considered as the difference between efflux and influx, both in the presence and in the absence of ATP. An increased Na^+ influx was

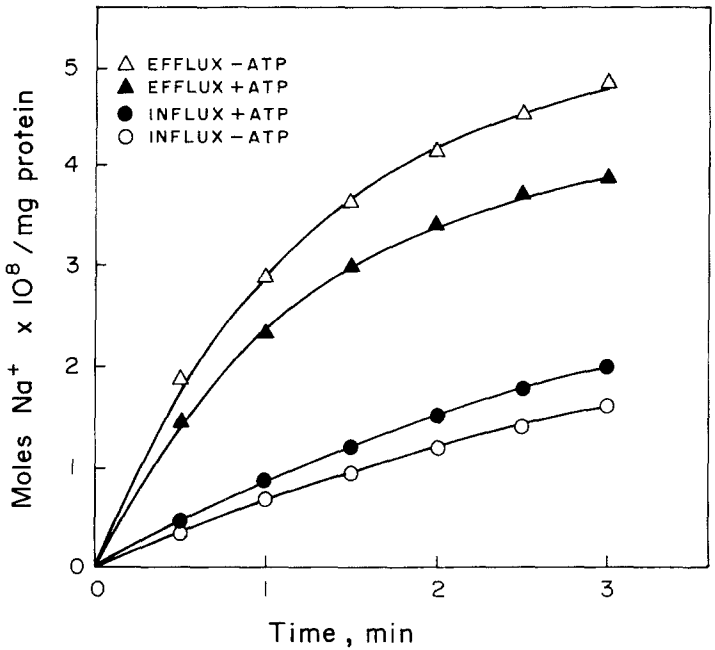

Fig. 1. Sodium fluxes. The ionic composition of the intravesicular medium was 10 mM Hepes-Tris (pH 7.0), 1 mM EGTA/Tris, 1 mM $MgSO_4$, 3 mM NaN_3 and 100 mM NaCl. The ionic composition of the external medium was 10 mM Hepes-Tris (pH 7.0), 1 mM EGTA/Tris, 1 mM $MgSO_4$, 3 mM NaN_3, 20 mM NaCl, and 80 mM KCl. *Influxes*: Vesicles were incubated at 25°C, with 5 μCi of ^{22}NaCl in a total volume of 100 μl in the presence (filled circles) and absence (empty circles) of 1 mM Mg-ATP. Aliquots were taken at different times between 0.5 and 3 min and filtered through Millipore filter. *Effluxes*: Vesicles were preloaded for 60 min at 37°C in 100 mM NaCl buffer, containing 5 μCi ^{22}NaCl, and then diluted in 100 mM KCl buffer so as to give 20 mM NaCl, 80 mM KCl buffer. Incubation times were between 0.5 and 3 min at 25°C, in the presence (filled triangles) and absence (empty triangles) of 1 mM Mg-ATP. The results are from a representative experiment.

observed in the presence of ATP. Therefore, these vesicles contain a Na^+ pump, capable of pumping Na^+ into the vesicular lumen.

Electrogenicity of the H^+-Pump and the Intravesicular pH at the Equilibrium

To corroborate the electrogenic character of the H^+ pump, vesicles containing 100 mM KCl_{in} were suspended in a sucrose buffer and its acidification determined after the addition of ATP (Fig. 2A). The acidification was minimal, but when a K^+ conductance was established by the addition of valinomycin, the acidification was similar to that obtained with vesicles containing $NaCl_{in}$ and KCl_{out} (Fig. 2B). This experiment clearly indicated that the pump was electrogenic, and demonstrated the requirements of

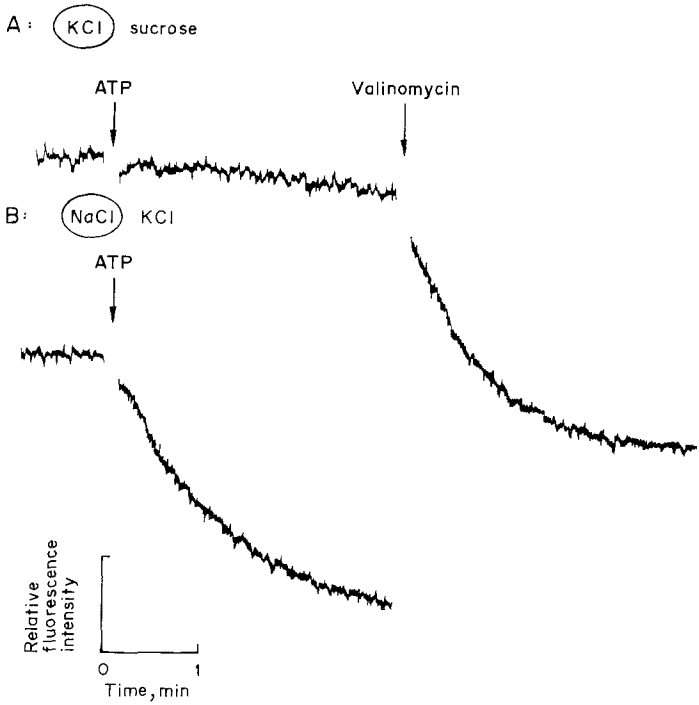

Fig. 2. Acidification dependence on ionic conductance. (A) FITC-transferrin containing vesicles prepared with 100 mM KCl_{in}, as described in Experimental were suspended in an external medium of 10 mM Hepes-Tris (pH 7.0), 1 mM EGTA-Tris, 1 mM $MgSO_4$, 3 mM NaN_3, and 200 mM sucrose. Upon the addition of 1 mM Mg-ATP, little fluorescence quenching was detected; however, with the addition of 1 μM valinomycin significant quenching was observed. (B) As a control for the extent of acidification that could be expected in (A), vesicles prepared in parallel containing 100 mM NaCl were suspended in the same medium with 100 mM KCl and acidification initiated with 1 mM Mg-ATP.

charge dissipation to obtain acidification. The fact that the same pH was achieved with K^+_{in}-valinomycin as with $NaCl_{in}/KCl_{out}$ indicated that these ions were sufficient to fulfill the charge dissipation needed to allow for maximal acidification.

Quantification of Protons Pumped per ATP Hydrolyzed

Figure 3 shows the parallel determinations of proton pumping and NEM-sensitive ATP hydrolysis as a function of time. As shown in the inset, the plot of ln(H^+ pumped/ATP hydrolyzed) vs. time allows the calculation of both h, the H^+/ATP ratio, from the intercept and k_1, the H^+ leak rate constant, from the slope (Thayer and Hinkle, 1973). The mean values of three independent experiments ($\pm$ standard deviation) were $h = 0.93 \pm 0.07$ and $k_1 = 3.29 \pm 1.13 \times 10^{-3}\ sec^{-1}$. The value obtained for h could then be

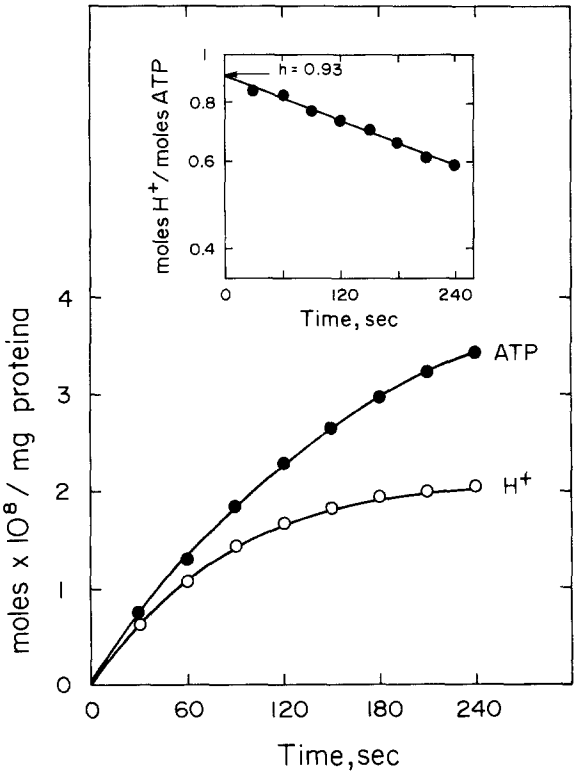

Fig. 3. Determination of H^+/ATP ratio. The kinetics of proton pumping and of NEM-sensitive ATPase activity were determined as described in Experimental. The inset is the plot of ln(H^+/ATP) vs. time with the intercept representing h and the slope k_1. Shown are the means of three independent experiments.

Table III. Stoichiometry of Charges during Proton Pumping[a]

Time (sec)	H^+ pumping	Cl^- influx	Na^+ efflux
	(moles × 10^a × mg protein^{-1})		
30	1.05 ± 0.22	0.67 ± 0.30	0.41 ± 0.08
60	2.09 ± 0.45	1.20 ± 0.55	0.79 ± 0.21
90	2.56 ± 0.37	1.29 ± 0.59	1.07 ± 0.31
120	2.84 ± 0.39	1.35 ± 0.61	1.23 ± 0.42
150	3.05 ± 0.44	1.40 ± 0.63	1.28 ± 0.55
180	3.15 ± 0.46	1.42 ± 0.62	1.32 ± 0.59

[a]The stoichiometry of proton pumping was derived from the NEM-sensitive ATPase activity utilizing a H^+/ATP ratio of 0.93 as derived from the data in Fig. 3. Net Cl^- and Na^+ fluxes were calculated from the differences in influx and efflux of the respective ions in the presence of ATP using the following ionic conditions: *internal*: 10 mM Hepes-Tris (pH 7.0), 1 mM $MgSO_4$, 3 mM NaN_3, 100 mM NaCl; *external*: 10 mM Hepes-Tris (pH 7.0), 1 mM $MgSO_4$, 3 mM NaN_3, 20 mM NaCl, 80 mM KCl, and 1 mM ATP-Mg. The experimental conditions used were as described in Experimental. Data shown are means of three independent experiments.

used to calculate the number of protons pumped from the data of NEM-sensitive ATP hydrolysis.

Balance of Charges in the Process of Acidification

As both Cl^- influx and Na^+ efflux are involved in obtaining maximal acidification, it was of interest to quantify both fluxes in relation to the amount of H^+ pumped into the vesicles after the addition of ATP. To quantitate these processes, all the fluxes under study were measured in the same preparation using the same ionic conditions, i.e., equal Cl^- concentrations inside and outside the vesicle, a higher Na^+ concentration inside the vesicle, and a higher K^+ concentration outside the vesicle. Table III lists the values for H^+, Cl^-, and Na^+ fluxes as a function of incubation time. After 3 min of incubation, by which time more than 95% of the acidification has occurred, the charges from H^+ influx had been balanced to about 45% by the Cl^- influx and 42% by the Na^+ efflux.

Discussion

The experiments presented here show that endocytic vesicles from rabbit reticulocytes have a proton pump that is similar to that described for bovine brain and rat liver coated vesicles (Choe *et al.*, 1987; Yamashiro *et al.*, 1983; Forgac *et al.*, 1983; Galloway *et al.*, 1983; Stone *et al.*, 1983a; Van Dyke *et al.*, 1984). The studies are unique in several regards, however. First, the use of a preparation of endocytic vesicles dedicated mainly to the transport of a

particular ligand, in this case transferrin, allowed us to determine the ionic fluxes required for acidification of that particular compartment. Second, our studies indicate that in order to optimally neutralize the electric potential generated by proton pumping, both a net Cl^- influx and a net Na^+ efflux are required, with each of these fluxes by itself supporting submaximal acidifications. As far as we know, this is the first demonstration that Na^+ efflux is required for maximal vesicle acidification and that a net Na^+ efflux and a net Cl^- influx can occur in the presence of ATP.

The Na^+ pump activity observed in the present report can be attributed to the Na^+, K^+-ATPase activity, described in endocytic vesicles from other systems (Fuchs *et al.*, 1989; Cain *et al.*, 1989). Under conditions of a Na^+ concentration gradient (inside > outside), the Na^+ flow was outward, even in the presence of ATP. The Na^+, K^+-ATPase could have the inhibitory role described by others (Fuchs *et al.*, 1989; Cain *et al.*, 1989) in the absence of a Na^+ concentration gradient. In this case, inhibition of the acidification mediated by the Na^+ pump should be through the generation of a positive membrane potential (Fuchs *et al.*, 1989).

The experiment shown in Fig. 2 illustrated the essential requirement of ionic conductances for acidification to occur. In the absence of ions little or no acidification occurred. Both Cl^- and Na^+ fluxes seem essential for the full expression of H^+ pump activity. As ^{86}Rb fluxes were two orders of magnitude less than Cl^- and Na^+ fluxes (data not shown), potassium fluxes were considered to be too small to account for charge balance, a conclusion corroborated by the negligible effect of K^+_{in} in acidification (Table II). These experiments also indicated that the intravesicular pH at equilibrium was a function of the intrinsic properties of the vesicle, and not of a positive potential generated within the vesicles: since with acidification of the vesicles the proton pump ATPase activity decreased and the proton leak increased (Fig. 3), the intravesicular pH stabilized when the rates of proton pumping and proton leak from the vesicle were balanced.

In studying the dependence of acidification on ionic composition, maximal acidification was observed with KCl in the extravesicular and NaCl in the intravesicular medium, suggesting a possible role for both Cl^- and Na^+ fluxes in the process of acidification. Cl^- has been proposed as the ion that could dissipate the electrical gradient generated by H^+ pumping (Stone *et al.*, 1983a; Xie *et al.*, 1983, 1989; Ecarot-Charrier *et al.*, 1980). The data presented here indicate that the addition of ATP generates a transient Cl^- influx that can dissipate part of the electrical gradient produced by the entrance of protons. The question arises about the nature of the transport system involved. It is unlikely that the influx of Cl^- occurs through the Cl^-/HCO_3^- antiport since the latter process is electroneutral (Sachs *et al.*, 1982). Experiments of fusion of endocytic vesicles with planar lipid bilayers performed in

our laboratory using techniques previously described (Latorre *et al.*, 1985) have detected an anion selective channel with a conductance of 130 pS, as determined by *i/v* curves in the presence of 500 mM KCl on the *cis* side and 100 mM KCl on the *trans* side (X. Cecchi, personal communication). The above results, and the anion selectivity in supporting acidification, strongly suggest an anion channel with selectivity for Cl^- as the entity responsible for the Cl^- fluxes.

It was possible to demonstrate that endocytic vesicles had a Na^+ permeability and that Na^+ inside the vesicles increased the amount of acidification. Since no net Na^+ efflux was found in the absence of a Na^+ gradient, the capacity of Na^+ to support acidification will depend on a concentration gradient favoring Na^+ efflux. Although no evidence is provided here that such a gradient exists *in situ*, the gradient would be expected if extracellular fluid is trapped during the process of vesicle formation.

Acidification was not inhibited by tetrodotoxin, a potent inhibitor of voltage-dependent Na^+ channels, and was just slightly inhibited by 1 mM amiloride, an inhibitor of the Na^+/H^+ antiport, *per se* an electroneutral process (Sachs *et al.*, 1982). It is therefore unlikely that Na^+ fluxes occur through the above pathways. Na^+ fluxes could be mediated either by a Na^+ channel not regulated by voltage or by the electrogenic $Ca^{2+}/3Na^+$ antiport (Reeves and Hale, 1984; Slaughter *et al.*, 1983).

The net sum of the Cl^- and Na^+ fluxes accounts for a considerable portion of the proton flux. The proton flux was determined using an adaptation of the methods of Thayer and Hinkle (1973). This method assumes a constant buffering capacity both of the extravesicular medium and the intravesicular space, an assumption that is actively being investigated. It is possible that the H^+-ATPase activity was overestimated as we lack a specific inhibitor for the enzyme and as 1 mM NEM sensitivity may include other ATPases. However, the calculated proton fluxes and the sum of the net Cl^- and Na^+ fluxes were similar (see Table III) and therefore we assume that the contribution of nonproton pumping ATPase activities to our calculations was relatively small, and that the flux of H^+ is approximately balanced by the Cl^- and the Na^+ fluxes.

Acknowledgments

This work was sponsored by grants from Universidad de Chile (DTI B 2200), FONDECYT (1003-88), the National Institutes of Health (DK-37866), and the National Science Foundation (INT-8715381).

References

Alberty, R. A. (1968). *J. Biol. Chem.* **243**, 1337–1343.

Apps, D. K., and Scaz, G. (1979). *Eur. J. Biochem.* **100**, 411–419.

Arai, H., Berne, M., Terres, G., Puopolo, K., and Forgac, M. (1987a). *Biochemistry* **26**, 6632–6638.

Arai, H., Berne, M. J., and Forgac, M. (1987b). *J. Biol. Chem.* **262**, 11006–11011.

Cain, C. C., Sipe, D. M., and Murphy, R. R. (1989). *Proc. Natl. Acad. Sci. USA* **86**, 544–548.

Choe, H-R., Moseley, S. T., Glass, J., and Núñez, M. T. (1987). *Blood* **70**, 1035–1039.

Dell'Antone, P. (1984). *FEBS Lett.* **168**, 15–22.

Ecarot-Charrier, B., Grey, V. L., Wilczynska, A., and Schulman, H. M. (1980). *Can. J. Biochem.* **58**, 418–426.

Forgac, M., Cantley, L., Wiedenman, B., Alstiel, L., and Branton, D. (1983). *Proc. Natl. Acad. Sci. USA* **80**, 1300–1303.

Fuchs, R., Schmid., S., and Mellman, I. (1989). *Proc. Natl. Acad. Sci. USA* **86**, 539–543.

Galloway, C. J., Dean, G. E., Marsh, M., Rednick, G., and Mellman, I. (1983). *Proc. Natl. Acad. Sci. USA* **80**, 3334–3338.

Glickman, J., Croen, K., Kelly, S., and Al-Awqati, Q. (1983). *J. Cell. Biol.* **97**, 1303–1308.

Harikumar, P., and Reeves, J. P. (1983). *J. Biol. Chem.* **258**, 10403–10410.

Johnson, R. G., Carty, S. E., and Scarpa, A. (1985). *Ann. N.Y. Acad. Sci.* **456**, 254–267.

Klausner, R. D., Ashwell, G., Van Renswoude, J., Harford, J., and Bridges, K. R. (1983). *Proc. Natl. Acad. Sci. USA* **80**, 2263–2266.

Latorre, R., Alvarez, O., Cecchi, X., and Vergara, C. (1985). *Annu. Rev. Biophys. Biophys. Chem.* **14**, 79–111.

Lestas, A. N. (1976). *Br. J. Haematol.* **32**, 341–350.

Musgrove, E., Taylor, R. I., and Hedley, D. (1984). *J. Cell. Physiol.* **118**, 6–12.

Nishimura, M., Ito, T., and Chance, B. (1961). *Biochim. Biophys. Acta* **59**, 177–188.

Njus, D., and Rodda, G. K. (1977). *Biochim. Biophys. Acta* **463**, 219–244.

Núñez, M. T., and Glass, J. (1985). *J. Biol. Chem.* **260**, 14707–14711.

Núñez, M. T., Gaete, V., Watkins, J. A., and Glass, J. (1990). *J. Biol. Chem.* **265**, 6688–6692.

Reeves, J. P., and Hale, C. C. (1984). *J. Biol. Chem.* **259**, 7733–7739.

Russell, J. T. (1984). *J. Biol. Chem.* **259**, 9496–9507.

Sachs, G., Faller, L. D., and Rabon, E. (1982). *J. Membr. Biol.* **64**, 123–135.

Schneider, D. R. (1981). *J. Biol. Chem.* **256**, 3858–3864.

Slaughter, R. S., Sutko, J. L., and Reeves, J. P. (1983). *J. Biol. Chem.* **258**, 3183–3190.

Stone, D. K., Xie, X-S., and Racker, E. (1983a). *J. Biol. Chem.* **258**, 4059–4062.

Stone, D. K., Xie, S-S., and Racker, E. (1983b). *J. Biol. Chem.* **258**, 4059–4062.

Sun, S-Z., Xie, X-S., and Stone, D. (1987). *J. Biol. Chem.* **262**, 14790–14794.

Thayer, W. S., and Hinkle, P. C. (1973). *J. Biol. Chem.* **248**, 5395–5402.

Van Dyke, R. W., Steer, C. J., and Scharschmidt, B. F. (1984). *Proc. Natl. Acad. Sci. USA* **81**, 3108–3112.

Van Renswoude, J., Bridges, K. R., Harford, J. B., and Klausner, R. D. (1982). *Proc. Natl. Acad. Sci. USA* **79**, 6186–6190.

Xie, X-S., Stone, D. K., and Racker, E. (1983). *J. Biol. Chem.* **258**, 9676–9680.

Xie, X-S., Stone, D. K., and Racker, E. (1984). *J. Biol. Chem.* **259**, 11676–11678.

Xie, X-S., Tsai, S-J., and Stone, D. (1986a). *Proc. Natl. Acad. Sci. USA* **83**, 8913–8917.

Xie, X-S., and Stone, D. K. (1986b). *J. Biol. Chem.* **261**, 2492–2495.

Xie, X-S., Crider, B. P., and Stone, D. K. (1989). *J. Biol. Chem.* **264**, 18870–18873.

Yamashiro, D. J., Fluss, S. R., and Maxfield, S. R. (1983). *J. Cell Biol.* **97**, 929–934.