ELSEVIER

Biochimica et Biophysica Acta 1282 (1996) 182–192

BBA Biochimica et Biophysica Acta

Biochemical characterization of a V-ATPase of tracheal smooth muscle plasma membrane fraction

Gladys Pacheco, Itala Lippo de Bécemberg, Ramona Gonzalez de Alfonzo, Marcelo J. Alfonzo *

Catedras de Bioquímica y Patología General y Fisiopatología, Sección de Biomembranas, Instituto de Medicina Experimental, Universidad Central de Venezuela, Apdo 50587, Sabana Grande, Caracas, Venezuela

Received 8 August 1995; revised 12 February 1996; accepted 14 February 1996

Abstract

A biochemical characterization of a Mg^{2+}-ATPase activity associated with a plasma membrane fraction isolated from airway (tracheal) smooth muscle was performed. This enzyme is an integral part of the membrane remaining tightly bound after 0.6 M KCl extraction. This enzyme activity showed a cold inactivation in the presence of ATP and Mg^{2+}. Also, this Mg^{2+}-ATPase was stimulated by monovalent anions being Cl^-, the best anion for such stimulation, even though Br^- and I^- were good substitutes and F^- was ineffective. This Cl^--stimulated activity showed a powerful nucleosidetriphosphatase activity having the following divalent cation specificity: $Mg^{2+} > Mn^{2+} > Ca^{2+}$, where Zn^{2+} and Fe^{2+} were ineffective. This ATPase activity was not inhibited by ouabain, oligomycin C and vanadate indicating that neither P- or F-ATPases were associated with this enzyme activity. However, the existence of a V-ATPase was shown by the significant inhibition causes by bafilomycin A_1. Additionally, this V-ATPase seems to be coupled to Cl^- conductor because duramycin inhibited this ATPase activity. The presence of a H^+ pump associated to this V-ATPase was shown indirectly, through the stimulatory effect produced by uncouplers such as FCCP and 1799, which were able to produce significant stimulation of this V-ATPase indicating the existence of a H^+-ATPase. Finally, the immunodetection of a 72 kDa polypeptide using a specific antibody against the A subunit (72 kDa) of V-ATPase from chromaffin granule demonstrated the presence of a V-ATPase in this plasma membrane fraction.

Keywords: Proton pump; ATPase, V-; Plasma membrane; Tracheal smooth muscle

1. Introduction

It is well established that mammalian cells have pH_i levels near or slightly above neutrality and the maintenance of the cytoplasmic pH (pH_i) in the physiological range is essential for cell function and survival [1]. Mammalian cells have transmembrane potentials in excess of 50 mV (negative inside), which is a driving force for H^+ to accumulate electrophoretically inside the cell. Moreover, after metabolic cell activation, a dramatic increment in H^+ production occurs in contractile cells like smooth muscle as a consequence of ATP hydrolysis [2]. These findings imply that in order to maintain pH_i within the physiological range, active process have to evolve to extrude excess H^+ (equivalents) from the cytoplasm to the extracellular medium. One of the primary mechanisms involved in the extrusion of H^+ from mammalian cells is a H^+ pump associated with plasma membranes [3,4]. The biochemical expression of this H^+ pump is a membrane bound Mg^{2+}-ATPase activity generally associated to a vacuolar (V-ATPase) [5–7]. The V-ATPases are found in fungi and plant vacuoles [8,9] and also in eukaryotic intracellular membrane compartments such as lysosomes

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; GMP-PNP, 5'-guanylimidodiphosphate; H^+-ATPase, H^+-translocating ATPase (H^+ pump); QNB, quinuclidinyl benzylate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NaN_3, sodium azide; NEM, *N*-ethylmaleimide; p-CMB, *p*-chloromercuribenzoate; PMSF, phenylmethylsulfonyl fluoride; V-ATPase, vacuolar adenosine triphosphatase; 1799, bis(hexafluoroacetonyl)acetone.

* Corresponding author. Fax: +58 2 6627460.

PII S0005-2736(96)00038-7

[10,11], clathrin-coated vesicles [12], Golgi vesicles [13], endocytic vesicles [14,15], and synaptic vesicles [16]. However; a vacuolar H^+-ATPase is found in the plasma membrane of renal intercalated cells, where it is thought to be involved in the process of urinary acidification [4]. However, these proton pumps are thought to originate from intracellular membrane vesicles which, under stimulation, are fused with the plasma membrane. A similar mechanism has been suggested for osteoclast acidification [17]. In this present study, we described a biochemical characterization of an anion-stimulated Mg^{2+}-ATPase (V-ATPase). Moreover, this V-ATPase activity is linked to a H^+ pump and a Cl^- conductor at plasma membrane vesicles isolated from tracheal smooth muscle. Parts of this work have previously been published in abstract form [18].

2. Materials and methods

Reagents were purchased from the following sources: ATP(A-5394) or Tris-ATP, other tri-, di- and monophosphate nucleosides, valinomycin, sodium vanadate, sucrose, PMSF, DTT, p-CMB, ouabain, creatine phosphokinase (rabbit muscle, type I), creatine phosphate, GMP-PNP, 5'-AMP, Trizma, Mes and peroxidase conjugated goat-anti-rabbit immunoglobulin G were from Sigma. The reagents for the P_i determination and EDTA from Merck. The 1799 and FCCP protonophores, duramycin and rutamycin (oligomycin C) were kindly provided by E. Racker (Cornell University, Biotechnology Center, USA). RO-201724 was purchased to RBI (USA). The kits for the quantification of cAMP and [^{3}H]QNB (35.5 Ci/mmol) were purchased from the Radiochemical Centre, Amersham (UK). Other reagents were the highest purity available and the electrophoresis and immunoblotting reagents were purchased from Bio-Rad. An antibody against A subunit of V-ATPase and bafilomycin A_1 were kindly provided by N. Nelson from Roche Institute (Nutley, New Jersey, USA).

2.1. Preparation of subcellular fractions from tracheal smooth muscle

The fractionation of bovine tracheal smooth muscle was performed as described previously [19] and briefly summarized: Bovine trachea and bronchii from 10 cows were transported on ice from the slaughterhouse to the laboratory. The thin layer of smooth muscle holding the cartilage was dissected on ice after the removal of serosa, mucosa and submucosa layers. All subsequent manipulations were performed at 4°C. The smooth muscle (about 250 g) was rinsed with 20 mM Tris-HCl (pH 7.2) containing 0.3 M sucrose, 0.5 mM DTT and 0.25 mM PMSF (Buffer IS), minced and homogenized twice with 3 vol. of Buffer IS/g wet weight tissue, in a Waring blender at full speed for 30 s with 1 min interval on ice. The dispersed material was centrifuged at $850 \times g$ for 10 min and the supernatant (E-I) was saved. The sediment was re-extracted with 2 vol. of buffer IS/g wet tissue and processed as described above. The supernatant (E-II) was saved and the sediment was again extracted with 2 vol. of Buffer IS/g wet tissue and filtered sequentially through 2, 4, 8 layers of cheesecloth. The filtrate was centrifuged at $1000 \times g$ for 10 min to separate the Nuclear fraction (N) and the supernatant (E-III) was pooled with E-I and E-II. The combined extract is referred to as Fraction E. It was centrifuged at $31\,000 \times g$ for 15 min to sediment a mitochondrial fraction (Fraction M) and the postmitochondrial supernatant was centrifuged at $150\,000 \times g$ for 1 h yielding a microsomal fraction (Fraction P) and the soluble fraction (S) or cytosol. All particulate fractions were suspended in small volume of Buffer IS.

2.2. Isolation of plasma membrane fractions

Fraction P was dispersed into 75 ml of Buffer IS. 15-ml samples were fractionated on a discontinuous sucrose gradient (0.3 M/0.82 M/1.28 M) in a SW 28 type Beckman rotor at $80\,000 \times g$ for 1 h. Three fractions, P_1(in the interphase between 0.3 M and 0.82 M), P_2 (in the interphase 0.82 M/1.28 M) and P_3 (at the bottom) were thus obtained. P_1 and P_2 (combined from six tubes) were each diluted separately with 80 ml of 20 mM Tris-HCl (pH 7.2), 0.5 mM DTT, 0.25 mM PMSF (Buffer I) and centrifuged at $150\,000 \times g$ for 30 min. Fractions P_1, P_2 and P_3 were suspended in 10 ml of Buffer IS divided into small aliquots, frozen in liquid nitrogen and stored at −80°C.

2.3. Enzyme determination

ATPase activity. In order to measure the membrane bound ATPase, all subcellular fractions were extracted with 0.6 M KCl at 4°C as follows: Approximately, 2 mg of protein was extracted under gentle agitation with 20 ml of a medium containing 0.3 M sucrose, 0.5 mM DTT, 0.6 M KCl, 20 mM Tris-HCl (pH 7.2) by 1 h and later centrifuged at $150\,000 \times g$ for 30 min. Sediments were washed twice with a buffer containing 0.3 M sucrose, 0.5 mM DTT, 20 mM Tris-Mes (pH 7.0) to remove traces of KCl and suspended in small volume of the last buffer. The standard assay of the membrane bound ATPase was 980 μl of buffer consisting of 30 mM Tris-Mes (pH 6.9), 5 mM $MgCl_2$, 5 mM ATP in the presence or absence of 0.15 M KCl, preincubated at 37°C for 5 min and the reaction was started by the addition of 20 μl (20–50 μg protein) and incubated for 1 min. Unless otherwise specified, all assays were performed at 37°C. Reactions were terminated

by addition of 0.1 ml of 50% cold TCA, followed by centrifugation at 3000 × *g* for 10 min. The enzyme activity (ATPase) was quantified by measuring the amount of P_i released using the procedure of Fiske-SubbaRow modified by Grant and us as previously described [20]. Protein determination was performed using bovine serum albumin as standard following a procedure described by Bensadoun and Weinstein [21].

Adenylyl cyclase activity (EC 4.6.1.1). This enzyme was assayed [22] in a total volume of 100 μl containing 50 mM Tris-HCl (pH 7.6); 5 mM $MgCl_2$; 3.2 mM ATP; an ATP regenerating system (5 mM creatine phosphate and 10 IU phosphocreatine kinase per assay in 0.1% defatted bovine serum albumin); 1 mM DTT; 20 μM RO20-1724; 0.1 mM GMP-PNP, and 10–50 μg of protein were added. The sediments of the particulate fractions were resuspended and later diluted with a 50 mM phosphate buffer (pH 7.4) and 4 mM $MgCl_2$. The incubation was carried out at 37°C for 5 min and determined by addition of 10 μl of 167 mM EDTA-Tris (pH 7.5), followed by heating for 3 min in a boiling water bath, and cooling on ice. The cAMP was determined by radioanalysis (Amersham, Kit No. TRK432) in 25 μl supernatant of the reaction mixture obtained after centrifugation at 12000 × *g* for 10 min at 4°C.

5'-Nucleotidase activity (EC 3.1.3.5). This activity was estimated in an incubation media (0.5 ml) containing 50 mM Tris-HCl; pH 7.5 buffer; 10 mM $MgCl_2$; 5 mM 5'-AMP. Incubations were initiated by addition of the enzyme sample (50–500 μg of protein) and the reaction was continued for 5 min at 37°C. Reactions were terminated by addition of 1 ml of 10% cold TCA, followed by centrifugation at 1000 × g for 10 min. P_i was determined in the supernatant by the method of Bartlett [23].

[^{3}H]QNB-binding activity. Muscarinic receptor was determined by using the [^{3}H]QNB-binding activity was performed according the procedure described by Lippo de Bécemberg et al. [24] with 2–5 μg protein per assay at 1200 pM of [^{3}H]QNB.

Gel electrophoresis and immunoblotting procedure for detection of A-subunit of V-ATPase. Plasma membrane fraction (P_1) having the V-ATPase activity was dissolved and reduced with 2% SDS and 2% of 2-mercaptoethanol, electrophoresed through 10% polyacrylamide gels according to the procedure described by Laemmli [25]. Protein bands were transferred onto nitrocellulose membranes. Potential non-specific binding sites on the transferred membranes were blocked for 1 h with 5% (wt/vol) dry milk and 0.05% (vol/vol) Tween 20 dissolved in PBS solution (PBS-T). The latter solution was removed and membranes were washed with PBS-T buffer and incubated for 1 h with a polyclonal antibody against A subunit of chromaffin granule V-ATPase previously diluted in PBS-T with 5% dry milk. After being washed, the membranes were incubated for 1 h with peroxidase conjugated goat-anti-rabbit immunoglobulin G, previously diluted in PBS-T with 5% dry milk. Bound antibody was visualized by an enhanced chemiluminescense detection system (E.C.L. Western blotting detection Kit from Amersham) and exposed to Kodak-X-Omat film. The polypeptide molecular weight standards (Sigma) were detected by staining with Coomassie brilliant blue.

Table 1
Subcellular distribution of V-ATPase, 5'-nucleotidase, GMP-PNP-stimulated adenylyl cyclase and L-[^{3}H]QNB binding as muscarinic receptor activity from bovine tracheal smooth muscle

Fractions	V-ATPase		5'-Nucleotidase		GMP-PNP-adenylyl cyclase		L-[^{3}H]QNB binding	
	S.A.	(*)	S.A.	(*)	S.A.	(*)	S.A.	(*)
E	0.26 ± 0.02	1.0	65 ± 6	1.0	70 ± 5	1.0	200 ± 17	1.0
N	0.15 ± 0.06	0.6	35 ± 5	0.5	155 ± 12	2.2	181 ± 34	0.9
M	1.13 ± 0.15	4.3	98 ± 7	1.5	295 ± 35	4.2	596 ± 33	2.9
P	0.14 ± 0.03	0.5	128 ± 9	1.9	215 ± 20	3.1	886 ± 47	4.4
S	0.07 ± 0.02	0.2	44 ± 3	0.7	n.d.		28 ± 7	
P_1	4.00 ± 0.20	15.3	360 ± 8	5.5	400 ± 40	5.7	1468 ± 138	7.3
P_2	1.14 ± 0.05	4.4	134 ± 9	2.0	338 ± 40	4.8	1378 ± 169	6.9
P_3	0.18 ± 0.03	0.7	99 ± 7	1.4	41 ± 10	0.6	217 ± 49	1.1

All determinations were performed in the sediments of the fractions with the exception of fractions S. V-ATPase was calculated as the difference of the Mg^{2+}-ATPase activities in the presence of 0.15 M KCl minus the activity in absence of KCl. These ATPases were measured as described in Section 2. V-ATPase is expressed as μmol P_i/min per mg protein. 5'-Nucleotidase is expressed as μmol P_i/min per mg protein. GMP-PNP-stimulated adenylyl cyclase as pmol cAMP/min per mg protein resulting from subtracting the basal specific activity from the specific activity in the presence of 0.1 mM GMP-PNP. L-[^{3}H]QNB binding (muscarinic receptor) activity as fmol L-[^{3}H]QNB/mg protein; was measured in the presence of 1200 pM L-[^{3}H]QNB. Data are means ± S.E. of duplicate determinations from 5–7 separate subcelullar fractionations. n.d., not detected; E, total extract; N, nuclear fraction; M, mitochondrial fraction; P, microsomal fraction; P_1, light plasma membrane fraction; P_2, heavy plasma membrane fraction and P_3, sarco(endo)plasmic reticulum fraction. S.A., specific activity and (*), purification was calculated as the ratio of specific activity of each fraction/specific activity of fraction E.

3. Results

3.1. Location of V-ATPase on subcellular fractionation of tracheal smooth muscle

Initial experiments were performed to estimate the activity of Mg^{2+}-ATPase in the different subcellular fractions isolated from airway smooth muscle according with a procedure described in Section 2. These results are shown in Table 1. It can be observed that fraction P_1, which is an enriched in plasma membrane vesicles fraction showed the highest specific activity for the chloride-stimulated ATPase (V-ATPase) and other plasma membrane markers such as 5'-nucleotidase, GMP-PNP-adenylyl cyclase and muscarinic receptor were highly enriched in this P_1 fraction, which supports the origin of this P_1 plasma membrane fraction.

3.2. Characterization of the V-ATPase activity

The high ATPase activity remains tightly bound to the plasma membrane fraction (P_1), after a high-salt extraction procedure (0.6 M KCl). This ATPase activity was found to be linear with respect to time for at least 2 min. Moreover, this enzyme activity was linear between 5 to 50 μg of membrane protein (data not shown).

Cold inactivation in the presence of ATP and Mg^{2+} is an important property exhibited by V-ATPases. In order to evaluate this specific property, a similar classic cold inactivation experiment was performed and the results are shown in Fig. 1. Under these experimental conditions, 50% of the ATPase activity was lost in 5 min at 4°C while the ATPase at 25°C remained for longer times.

3.3. Effects of specific inhibitors for P-, F- or V-ATPases

The effects of specific inhibitors for P-, F- and V-ATPases on this chloride-stimulated Mg^{2+}-ATPase are summarized in Table 2. Ouabain, a classical inhibitor of the Na^+,K^+-ATPases produces a 30% inhibition of the basal activity at 1 mM whereas the Cl^--stimulated activity was not significantly affected for this inhibitor. Sodium vanadate, an specific inhibitor of P-ATPases produced a similar percentage inhibition of the basal activity found for ouabain; suggesting that at least 30% of the basal activity may be P-ATPases associated to this plasma membranes. In these experiments, cross contamination with inner mitochondrial membranes was ruled out because these latter membranes also contain a Mg^{2+}-ATPase (F_0-F_1-ATPase), which is well known to be sensitive to oligomycins or NaN_3. Both the basal activity and the Cl^--stimulated activity are not affected in the presence of 0.5–5 μg/ml of oligomycin C (rutamycin) and 1–5 mM NaN_3 (data not shown). This evidence indicates the absence of mitochondrial contaminants in this plasma membrane fraction. However, bafilomycin A1 at low concentration (nM range) was able to inhibit in more than 80% of the Cl^--stimulated ATPase demonstrating the presence of a V-ATPase in this plasma membrane fraction as shown in Table 2.

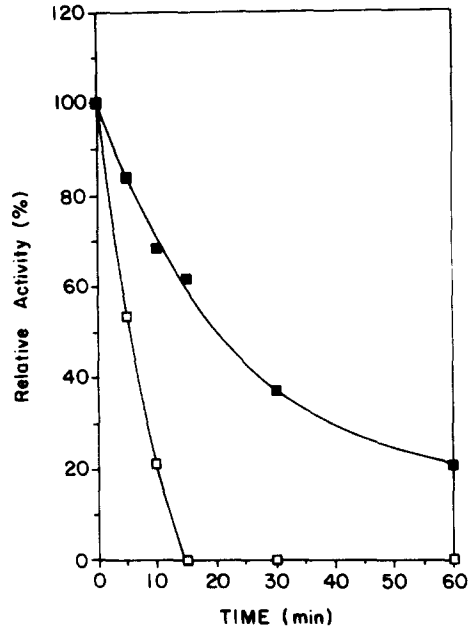

Fig. 1. Cold inactivation of chloride-activated Mg^{2+}-ATPase. Plasma membranes (0.4–0.6 mg/ml) were incubated in the presence of 5 mM ATP, 0.2 M NaCl, 5 mM $MgCl_2$, 0.5 mM DTT, 20 mM Tris-Mes (pH 6.8) containing 5 mM creatine phosphate and 20 μg creatine kinase at room temperature (■) or at 0°C (□). Aliquots (20 μl) were taken at indicated time and the ATPases activities were measured as described in Section 2. The 100% activity was 7.89±0.7 μmol P_i/min per mg protein.

Table 2
Effect of several inhibitors on Mg^{2+}-ATPase

Inhibitor		μmol P_i/min per mg protein		
		+KCl	−KCl	Δ
None		7.80±0.16	4.86±0.15	2.94
Ouabain	1 mM	6.84±0.16	4.44±0.18	2.40
	10 mM	6.84±0.20	4.45±0.13	2.40
Vanadate	50 μM	7.73±0.13	3.81±0.10	3.92
	500 μM	7.71±0.11	2.76±0.10	4.74
Oligomycin C	0.5 μg/ml	7.80±0.15	4.76±0.12	3.04
	5 μg/ml	6.59±0.14	3.08±0.23	3.51
Bafilomycin A1	100 nM	5.43±0.12	4.85±0.12	0.58

The Mg^{2+}-ATPase activity was measured in P_i fraction (20–50 μg protein) as described in Section 2. Oligomycin C was dissolved in ethanol and control experiments using 2 μl of ethanol for 1 ml of assay mixture were done. Bafilomycin A_1 was dissolved in DMSO and the final concentration of DMSO was always less than 1%. Δ, V-ATPase activity calculated by substracting from the Mg^{2+}-ATPase in the presence of 0.15 M KCl the basal activity. Data represent the means±S.E of three separate experiments performed in triplicate.

3.4. pH dependency

The pH dependency of this V-ATPase was evaluated. Thus, this enzyme activity was assayed over a wide range

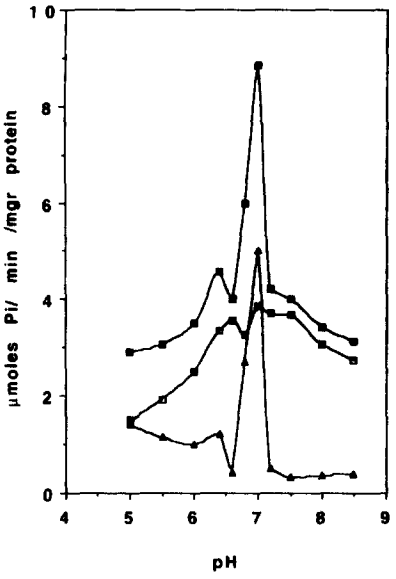

Fig. 2. The pH profile of the chloride-stimulated Mg^{2+}-ATPase. The different pH solutions were prepared using Tris-Mes buffers. The ATPase activity was measured as described in Section 2. □, −KCl; ■, +KCl 0.15 M. △, chloride-stimulated ATPase was calculated by subtracting from the KCl-stimulated activity the basal activity. Each value is the mean of three experiments done in triplicate.

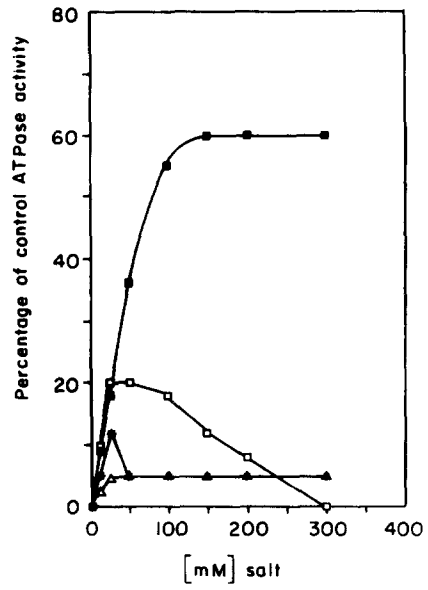

Fig. 3. The effect of different K^+ salts on the Mg^{2+}-ATPase activity. The ATPase was measured following the procedure described in Section 2. The enzyme activity is expressed as the percentage of activation of basal activity of each group of experiments. Basal activity (100%) = 4.0 ±0.8 µmol P_i/min per mg protein. ■, KCl; □, KNO_3; △, K gluconate; ★, K_2SO_4. Each value is the mean of three experiments done in triplicate.

of pH from 5.0 to 9.0 using Tris-Mes mixtures as free chloride buffers. The chloride stimulation was observed to remain the same from pH 5.0 to 6.5, it showed an optimum at pH 6.9–7.0, it decreased sharply and it was virtually absent at pH values higher than 7.2 (Fig. 2). Nevertheless, the basal activity did not show a sharp pH dependency.

3.5. Anion specificity

The addition of KCl seems to be a requirement for the stimulation (twice) of the basal ATPase activity. Thus, the anion specificity was explored using different chloride salts as KCl, KNO_3, K^+ gluconate and K_2SO_4 as shown in Fig. 3. From these data, KCl was the best K^+ salt for activating this ATPase followed by KNO_3, which stimulated the activity by 20% at low concentration (10–50 mM) and this stimulation disappeared at concentrations higher than 100 mM. On the other hand, K_2SO_4 produced only 10% stimulation (10–25 mM) and was ineffective at concentrations higher than 50 mM. However, K^+ gluconate (10–300 mM) was totally ineffective.

3.6. Effect of monovalent cations

The cation specificity of this enzyme was done using KCl, NaCl, RbCl and Tris-HCl at 0.15 M and all these salts produced stimulation of the Mg^{2+}-ATPase as shown in Table 3A. Thus, the following sequence of activation was found for monovalent cations: $K^+ \geq Na^+ > Tris \geq Rb^+$ suggesting that K^+ is more effective than Na^+ and Rb^+. It is important to point out that there was not additional

Table 3
Dependence on different chloride salts (A) and halogen salts (B) of the activity of Mg^{2+}-ATPase

	µmol P_i/min per mg protein	
	activity	Δ
(A) Chloride salt		
None (basal)	4.78±0.20	0
KCl	8.94±0.19	4.16
NaCl	7.92±0.21	3.14
Tris-HCl	7.20±0.13	2.42
RbCl	6.40±0.16	1.62
(B) Halogen salt		
None (basal)	4.78±0.16	0
KCl	10.20±0.15	5.42
KI	10.16±0.08	5.38
KBr	10.09±0.11	5.31
KF	5.46±0.14	0.68
NaCl	9.92±0.12	5.14
NaI	9.11±0.14	4.33
NaBr	9.92±0.09	5.14
NaF	5.46±0.15	0.68

(A) This Mg^{2+}-ATPase activity was measured as described in Section 2. The final concentration of chloride salts was 0.15 M, using 5 mM ATP and 5 mM Mg^{2+} acetate. In the case of Tris-HCl, a solution of Trizma-HCl was adjusted with 2 M Tris to pH 6.9.
(B) The experimental conditions were similar to those described in (A). All different K^+ or Na^+ salts were prepared to obtain a final concentration of 0.15 M. Δ, the difference in activity after subtracting the activity under basal conditions from the activity under each experimental condition. Data represent the means ± S.E. of four separate experiments done in triplicate.

Table 4
The effect of different nucleotides (A) and divalent cations (B) on Mg^{2+}-ATPase

	Activity (μmol P_i /min per mg protein)		
	+KCl	−KCl	Δ
(A) Nucleotides			
None	0	0	0
ATP	9.65±0.20	5.70±0.26	3.95
CTP	10.20±0.29	7.81±0.25	2.39
UTP	8.98±0.28	4.39±0.17	4.59
GTP	7.50±0.29	4.56±0.10	2.94
ADP, CDP, UDP, GDP	0	0	0
AMP, CMP, UMP, GMP	0	0	0
(B) Ion condition			
Without addition	1.04±0.07	0.74±0.04	0.30
$MgCl_2$	8.86±0.13	4.47±0.18	4.39
$MnCl_2$	7.88±0.09	4.41±0.14	3.47
$CaCl_2$	6.03±0.04	5.02±0.09	1.01
$ZnCl_2$	1.39±0.10	1.22±0.03	0.17
$FeCl_2$	1.10±0.05	0.72±0.05	0.38

(A) The Mg^{2+}-ATPase activity was measured as described in Section 2. All nucleotide solutions were neutralized to pH 6.9 with Tris. Blank experiments without membranes were run to evaluate the hydrolysis of these nucleotides, which was negligible. The final concentration of the nucleotide was 5 mM and 5 mM $MgCl_2$ was employed in all assays.
(B) The enzyme activity was measured as described in Section 2. The amount of ATP was 5 mM and the final concentration of the divalent cation was 5 mM. Δ, the enzyme activity obtained by subtracting the ATPase activity in the absence of KCl from the activity in the presence of KCl. Data represent the means ± S.E. of three separate experiments done in triplicate.

activation by chloride salts as 300 mM NaCl added on top of 150 mM KCl did not provide additional stimulation (data not shown).

3.7. Halogen selectivity

It is well known that V-ATPases can be activated by anions (specifically by chloride) but this property is also exhibited by other members of the halogen family. In this sense, several experiments were performed using different Na^+ or K^+ salts of different halogens (Cl^-, Br^-, I^-, F^-). Table 3B shows the effect of these different salts on the Mg^{2+}-ATPase. It can be seen that Br^- and I^-, like chloride were good activators, but F^- was ineffective. From these data, a sequence order of effectiveness for halogen activation can be drawn as follows: $Cl^- \geq Br^- > I^- \gg F^-$ regarding the metal ion employed.

3.8. Nucleotide substrate specificity

A Mg^{2+}ATP complex seems to be the substrate for ATPases and following this assumption, we explored the nucleotide specificity and the divalent requirements of this membrane bound ATPase. Table 4A shows the substrate specificity using different types of tri-, di- and monophoshonucleotides. Neither, the mononucleotides or dinucleotides were hydrolyzed. However, it was found that all nucleotide triphosphates were good substrates for these enzyme activities. Thus, the basal activity was higher in the presence of CTP and ATP and a lower activity with UTP and GTP. One unusual finding was the high UTPase activity and additional experiments in the presence of bafilomycin were performed. It was found that only 20% of the chloride-activated UTPase was sensitive to this inhibitor (data not shown), in contrast with the 80% inhibition for the ATPase activity showed in Table 2. Therefore, these results strongly suggest that this enzyme behaves as a nucleosidetriphosphatase having more specificity for ATP.

3.9. Divalent cation specificity

Table 4B shows the effect of divalent cations on the Cl^--stimulated ATPase and the following sequence of activation was found $Mg^{2+} > Mn^{2+} > Ca^{2+}$. However, Zn^{2+} and Fe^{2+} were ineffective.

3.10. Effects of alkylating reagents

In order to study the regulation exerted by SH groups on this enzyme activity, several alkylating compounds were employed. Two different compounds were used: NEM and p-CMB. It was found that pretreatment of these vesicles with NEM yielded a 35% inhibition of the Cl^--stimulated ATPase and a 55% inhibition of the basal activity (data not shown). Using p-CMB, a dramatic inhibi-

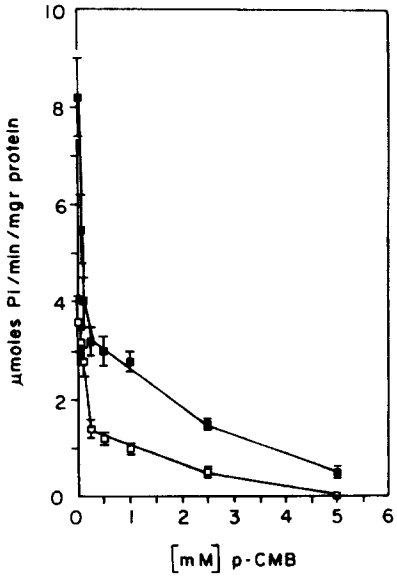

Fig. 4. The p-CMB effect on the Mg^{2+}-ATPase. Plasma membrane fractions were preincubated with p-CMB during 30 min at 4°C. After preincubation for 5 min at 37°C, the enzyme reaction was started upon the addition of ATP. The rest of the ATPase procedure was followed as described in Section 2. □, −KCl; ■, +KCl 0.15 M. Each value is the mean ± S.E. of three experiments done in triplicate.

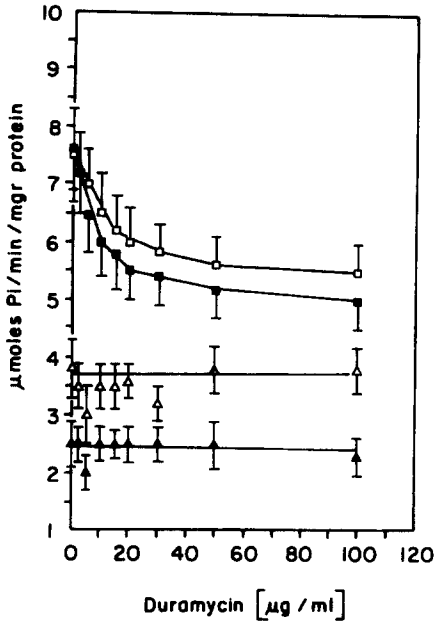

Fig. 5. Effect of duramycin on the Mg^{2+}-ATPase activity. Duramycin at different concentrations was preincubated with plasma membranes (P_1) fraction (20–25 µg) during 5 min at 37°C in the presence or absence of 1 µM valinomycin (Val). The ATPase reaction was started by the addition of ATP and the ezyme was measured as described in Section 2. Controls with 5 µl ethanol were done. □, +Val +KCl; ■, −Val +KCl; △, +Val −KCl; ▲, −Val −KCl. Each value is the mean ± S.E. of three different experiments performed in triplicate.

tion of the Cl^--activated ATPase was observed, which is shown in Fig. 4. The p-CMB produced at 0.05 mM 31% inhibition, from 0.1 mM through 2.5 mM 68% inhibition, and at 5 mM complete inhibition. The basal activity was less sensitive at lower concentrations but at higher concentrations of p-CMB both activities were completely inhibited. These data suggest that SH groups should be involved in the regulation of this ATPase. Additional experiments with other covalently modifying reagents were done, such as DCCD (500 µM), which produced a complete inhibition of Cl^--stimulated and basal Mg^{2+}-ATPase (data not shown).

3.11. Relationship between a Cl^- conductor and the V-ATPase

A H^+-ATPase is associated with clathrin-coated vesicles, which is sensitive to duramycin. In addition; in these systems, a duramycin-sensitive chloride conductor has been postulated. It can be seen that duramycin in a dose dependent manner was able to inhibit more than 50% of the Cl^--stimulated ATPase. These results are shown in Fig. 5. This inhibition was smaller in the presence of 1 µM valinomycin. It is important to emphasize that the basal activity in the presence or absence of valinomycin was completely insensitive to duramycin. These data support the hypothesis that a chloride transporter (conductor) is linked to this H^+-ATPase, which may be responsible for the Cl^- activation. All this evidence together supports the fact that there is a chloride conductor coupled to a V-ATPase in the plasma membrane vesicles isolated from airway smooth muscle.

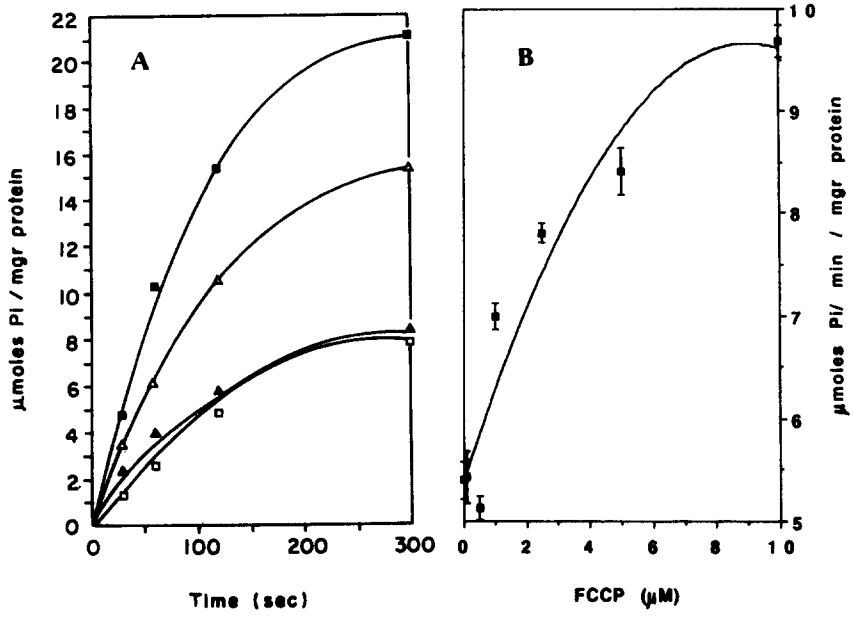

Fig. 6. (A) The activation effect of FCCP on Mg^{2+}-ATPase. Plasma membrane (P_1) (25–30 µg protein) was incubated during different times in the presence of 10 µM FCCP at 37°C. Controls with ethanol (5 µl) were run simultaneously. ■, +FCCP +KCL; □, +FCCP −KCl; △, −FCCP +KCl; ▲, −FCCP −KCl. (B) Dose-dependent activation of FCCP on chloride-stimulated enzyme. Samples were assayed at different FCCP concentration during 1 min of incubation at 37°C. The ATPase activity was measured as described in Section 2. Controls with 5 µl of ethanol were run simultaneously showing activity similar to control experiments. Each value is the mean of three different experiments done in triplicate.

3.12. Effects of uncouplers on the V-ATPase

Proton pumps can build up a pH gradient and a membrane potential (positive inside the vesicle) due to the active proton pumping activity. Protonophores (uncouplers) are able to dissipate the pH gradient and in this way increase the ATPase activity of these H^+ pumps. The latter effect has been classically described for the F_0-F_1-ATPases. V-ATPases have been associated with H^+ pumps in several membranes systems. In this sense, the effects of uncouplers on these enzyme activities were studied. Thus, FCCP was able to produce an activation of the Cl^--activated ATPase as a function of time, which is shown in Fig. 6A. Similarly, FCCP was able to activate this ATPase activity in a dose-dependent manner, which is shown in Fig. 6B. It can be seen that FCCP increased the initial velocity of this ATPase, which suggests that this ATPase activity behaves like an uncoupled ATPase. These results were confirmed using another uncoupler as 1799, which showed a similar behavior as that found for FCCP (data not shown). All these data together indicate that there is a H^+ pump associated with a V-ATPase at plasma membrane vesicles isolated from airway smooth muscle.

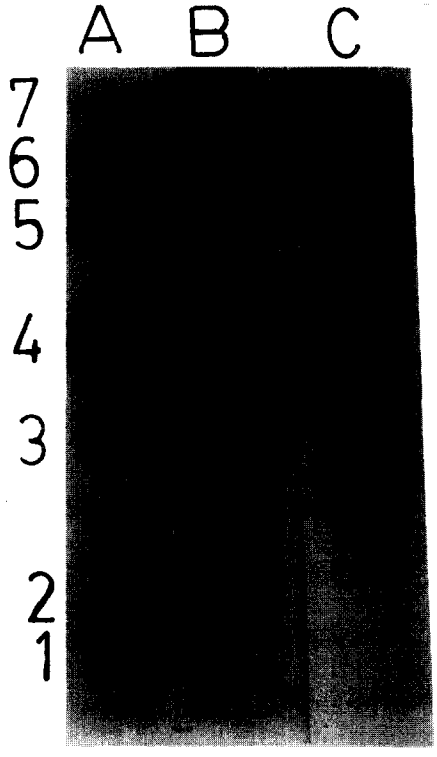

Fig. 7. Inmunoactivities of plasma membrane fraction P_1 with polyclonal antibody against A subunit of V-ATPase. The fraction P_1 was run in SDS-10% polyacrylamide gel electrophoresis as described in Section 2. (Lane A) Protein molecular weight standards containing: lysozyme (1) (14300); β-lactoglobulin (2) (18400); carbonic anhydrase (3) (29000); ovalbumin (4) (43000); fumarase (5) (48500); bovine serum albumin (6) (68000); phosphorylase B (7) (97400). (Lane B) Polypeptide constant of P_1 fraction stained with Coomasie brillant blue. (Lane C) Western blotting of plasma membrane fraction P_1. This fraction (35 μg protein) was transferred to nitrocellulose membranes and reacted with polyclonal antibody against A-subunit of V-ATPase at a dilution (1:100). Bound antibody was visualized by the immunoperoxidase coupled reaction enhanced chemiluminescence detection system and exposed to Kodak-X-Omat film.

3.13. Western blotting for detection of A-subunit of V-ATPase

In order to have solid evidence on the existence of a V-ATPase associated with this plasma membrane fraction, a Western blotting protocol was performed in these plasma membrane samples using a polyclonal antibody against the subunit A (72 kDa) of the purified chromaffin granule V-ATPase. After, the immunoblotting procedure was performed, an immunoreactive band around 72 kDa was found using the experimental conditions. Several experimental conditions as membrane protein, antibodies dilution and exposition times were assayed to guarantee a clear band on the X-rays film, which is shown in Fig. 7 (Lane C). Additionally, molecular weight protein standards were run and the relative mobility of these markers is shown in Fig. 7 (Lane A). The polypeptide content was visualized using Coomassie brilliant blue stain as shown in Fig. 7 (Lane B).

4. Discussion

The existence of highly active Mg^{2+}-ATPases in plasma membrane fractions isolated from smooth muscles seems to be an original and important experimental finding [18,26]. Trying to understand the nature of this Mg^{2+}-ATPase, we decided to characterize this enzyme activity and to correlate it with the ion transport across airway smooth muscle plasma membranes. Transmembrane movements of ions may have an important impact on airway smooth muscle tone and contractibility by influencing a variety of cellular processes such as intracellular ion concentrations, membrane voltage and intracellular pH [27]. The presence of a V-ATPase associated to plasma membrane fractions isolated from the airway (tracheal) smooth muscle should be clearly demonstrated. In this sense, we decided to evaluate the presence of several classic markers of plasma membranes, which were determined. A good correlation was found between the subcellular distribution and the enrichment of plasma membrane markers such as 5′-nucleotidase, GMP-PNP-stimulated adenylyl cyclase, muscarinic receptor activity with the V-ATPase here studied. This evidence strongly supports the origin of these vesicles (P_1 fraction), which derivated from plasma membrane structures. So far, we know this is the first report on the presence of a V-ATPase in plasma membrane structures derived from non H^+-secreting tissues such as smooth muscle cells.

Several P- or F-ATPases specific inhibitors were used to confirm that such plasma membrane-bound ATPase are

indeed a V-ATPase. Thus; ouabain (a Na^+,K^+,Mg^{2+}-ATPase inhibitor) [28]; rutamycin (oligomycin C, a F_0-F_1-ATPase inhibitor) [29] and vanadate (a general inhibitor for P-ATPases) [30] were unable to inhibit this Cl^--stimulated ATPase. These results have been described for most V-ATPases systems [31,32].

An important piece of evidence that allow us to identify this ATPase as V-ATPase was the dramatic inhibitory effect showed by low concentrations of bafilomycin A_1 on the chloride-stimulated ATPase. The V-ATPase proton pumps are inhibited (non-specifically) by dicyclohexylcarbodiimide and sulfhydryl agents but recently a group of macrolide antibiotics, the bafilomycins, has been identified as specific, potent inhibitors of these V-ATPases [33,34]. The prototype bafilomycin A_1 is a specific inhibitor of the V-ATPases, in the nanomolar range, and a 10000-fold increase in concentration is required before inhibition of other ion translocating ATPase is observed. Moreover, the bafilomycin-binding site seems to be located in the V_0 sector of the V-ATPase [35].

These V-ATPases have been described in several organisms and mammalian tissues, specifically associated with the secretory vesicles [14–16], lysosomes [10], their association with plasma membranes has been shown for several H^+-secreting cells as kidney cells [4,36,37]. This enzyme activity must be an integral part of the membrane structure [7,38] and the oligomeric catalytic sector of V-ATPase is composed of several subunits called A, B, C, D and E [7,38–40], that seem to be held together through hydrophobic interactions, which may be weaken through time at low temperature. A classic cold inactivation in the presence of ATP was performed for this ATPase and it was found that a significant inactivation of the V-ATPase occurred at 4°C in comparison to the enzyme activity incubated with ATP and Mg^{2+} at 25°C. This last property has been described for some of these V-ATPases, which is the case for V-ATPase present in rat liver lysosomes, where the soluble sector of this V-ATPase became unstable at low temperature due to the release of five subunits of the enzyme complex [41,42]. Another possible explanation for the cold inactivation may be due to the proteolytic activity, which can destroy some essential components [10]. A relevant aspect of the regulation of this V-ATPase is associated with the pH activity profile. Thus, the pH profile showed a very sharp optimal pH around 7.0, which has been described for most V-ATPases [4,40,43].

The function of SH groups at the catalytic activity of this V-ATPase was investigated by using NEM and p-CMB as alkylating agents. It was found that p-CMB was more potent than NEM inhibiting the chloride-stimulated and the basal activity suggesting that SH groups may be involved in the catalytic site of these ATPases. Our data are in good agreement with previous studies showing the inhibitory effect of alkylating reagents in several V-ATPases systems [44].

The anion stimulation of V-ATPase has been studied in many biological systems. The anion sensitivity of a V-ATPase has been demonstrated for the first time by Uchida et al. [45] in plant V-ATPase and by Moriyama and Nelson [46] in animal V-ATPase. Thus, several V-ATPases showed great specificity to chloride ions [6,40]. In our case the following sequence of activation was found ($Cl^- > NO_3^-$ > gluconate > SO_4^{2-}), which has been described for other V-ATPases [47,48]. This chloride activation belongs to the halogen family being $Cl^- > Br^- > I^-$ the best activators and F^- was ineffective, which also has been described in some V-ATPases systems [32,49,50].

This V-ATPase is a powerful nucleosidetriphosphatase exhibiting a poor specificity for nucleosidetriphosphates being unable to hydrolyze di- or mononucleotides. Similar results have been shown by V-ATPases from other biological systems [4,13,43,51,52]. However, a high UTPase activity was found being only 20% sensitive to bafilomycin (unpublished results). These data suggested that the real substrate of these V-ATPases is a Mg^{2+}ATP complex. In our system, it was found that Mg^{2+} was the best divalent cation, which can be replace by Mn^{2+} [17,51,53]. However; Zn^{2+} and Fe^{2+} were poor divalent cations for this V- ATPase. The lack of effect of Fe^{2+} seems to be different of the clathrin-coated vesicles [50] and lysosomal [54] V-ATPases.

This chloride requirement can be explained through two mechanisms: (1) chloride may act at the catalytic site inducing activation of the enzyme or (2) chloride ions may dissipate the membrane potential created by the H^+-pump activity of this V-ATPase. This latter hypothesis has been postulated in some systems, where, a V-ATPase seems to be coupled to a chloride conductor [31,32]. In addition, our results are similar on the chloride activation described for the clathrin-coated vesicles and endocytic vesicles [50]. In this sense; duramycin, an antibiotic polypeptide isolated from *Streptomyces cinnamoneus*, is a specific inhibitor of a chloride conductor coupled to the V-ATPase of clathrin-coated vesicles from bovine brain [55]. This antibiotic was able to inhibit this chloride-stimulated ATPase present in plasma membrane vesicles from airway smooth muscle. Thus, duramycin inhibition can be explained by a direct inhibition of the H^+ pump or the chloride conductor or both. Some evidences has been reported that duramycin may accomplish its effects through specific lipid–duramycin interactions instead of protein–duramycin lipid–duramycin [56,57]. Thus, the presence of phosphatidylethanolamine (PE) in the membranes allows the formation of duramycin-PE complex, which in some manner can affect the function of the V-ATPase or the chloride transporter.

V-ATPases have been demonstrated to be associated to H^+ pumps activities. In this sense, it is known that AT-Pases associated to H^+ pumps can be stimulated in the presence of conductive protonophores, which dissipate the proton gradients [7,58–60]. Thus, the effect of some uncouplers such as FCCP and 1799 was studied on this

V-ATPase. In the sense, ATPase activity should increase when protonophores are used to dissipate pH gradients created by the active H^+ pumps. Our results support the idea that proton gradients and membrane potential created by the active accumulation of H^+ inside these vesicles can inhibit the hydrolytic activity of this V-ATPase. Both uncouplers induced a significant stimulation of this V-ATPase here described in a function of time and a dose-dependent manner. These data confirm the existence of a H^+ pump energized by a V-ATPase, which is associated to these plasma membrane vesicles. It is important to emphasize that the basal activity was not stimulated by uncouplers indicating that the chloride-stimulated activity was the only one sensitive to protonophores.

Altogether, the findings here described provide strong evidence about an original description a vacuolar type proton pump (V-ATPase) associated to the airway smooth muscle plasma membrane fractions.

Acknowledgements

We would like to recognize the late Efraim Racker (he was a very enthusiastic investigator), for providing criticisms and reagents for this project. We thank Nathan Nelson for providing the bafilomycin and the antibody against subunit A (V-ATPase) and the suggestion of the cold inactivation experiment. Also to M. F. Correa de Adjounian for the immunoblotting experiments. Additionally, this project was supported in part by grants No. CDCH 0.33.2942/95 (M. Alfonzo); CDCH 09.10.34.69.95 (R. Gonzalez de Alfonzo). We thank to V. Herrera for her assistance in the preparation of subcellular and plasma membrane fractions.

References

[1] Roos, A. and Boron, W.F. (1981) Physiol. Rev. 61, 296–434.
[2] Paul, R.J. (1989) Annu. Rev. Physiol. 51, 331–349.
[3] Nandi, J. and Ray, T.K. (1987) J. Biochem. 241, 175–181.
[4] Gluck, S.L. (1992) J. Bioenerg. Biomembr. 24, 351–359.
[5] Pedersen, P.L. and Carafoli, E. (1987) Trends Biochem. Sci. 12, 146–150.
[6] Nelson, N. (1991) Trends Pharmac. Sci. 12, 71–75.
[7] Nelson, N. (1992) J. Exp. Biol. 172, 19–27.
[8] Bowman, B.J., Vásquez-Laslop, N. and Bowman, E.J. (1992) J. Bioenerg. Biomembr. 24, 361–370.
[9] Taiz, L. (1992) J. Exp. Biol. 172, 113–122.
[10] Moriyama, Y. and Nelson, N. (1989) J. Biol. Chem. 264, 3577–3582.
[11] Arai, K., Shimaya, A., Hiratani, N. and Ohkuma, S. (1993) J. Biol. Chem. 268, 5649–5660.
[12] Xie, X.-S., Stone, D.K. and Racker, E. (1988) Methods Enzymol. 157, 634–646.
[13] Moriyama, Y. and Nelson, N. (1989) J. Biol. Chem. 264, 18445–18450.
[14] Mellman, I., Fuchs, R. and Helenius, A. (1986) Annu. Rev. Biochem. 55, 663–700.
[15] Nelson, N. (1992) J. Exp. Biol. 172, 149–153.
[16] Moriyama, Y., Maeda, M. and Futai, M. (1992) J. Exp. Biol. 172, 171–178.
[17] Mattsson, J.P., Lorentzon, P., Wallmark, B. and Keeling, D. (1993) Biochim. Biophys. Acta 1146, 106–112.
[18] Pacheco, G., Bécemberg, I.L. and Alfonzo, M. (1990) Acta Cient. Venez. (Suppl. 1) 41, 37.
[19] Lippo de Bécemberg, I., Peña de Aguilar, A.E., Camarillo, I., Gonzalez de Alfonzo, R. and Alfonzo, M. (1989) FEBS Lett. 253, 16–22.
[20] Alfonzo, M.J., Kandrach, M.A. and Racker, E. (1981) J. Bioenerg. Biomembr. 13, 375–391.
[21] Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241–250.
[22] Doval, M.T., Bécemberg, I.L., Alfonzo, M. and De Venanzi, F. (1979) Acta Cient. Venez. 30, 494–501.
[23] Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468.
[24] Lippo de Bécemberg, I., Ponte-Sucre, A. and Alfonzo, M. (1986) Arch. Venez. Farm. Terap. 5, 244–256.
[25] Laemmli, U.K. (1970) Nature 227, 680-685.
[26] Missiaen, L., Wuytack, F. and Casteels, R. (1988) Biochem. J. 250, 571–577.
[27] Bretag, A.H. (1987) Physiol. Rev. 67, 618–724.
[28] Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1983) in Molecular Biology of the Cell, pp. 291–293, Garland Publishing, New York and London.
[29] Kirley, T.L. (1988) J. Biol. Chem. 263, 12682–12689.
[30] Macara, I.G. (1980) Trends Biochem. Sci. 5, 92–94.
[31] Forgac, M. (1989) Physiol. Rev. 69, 765–796.
[32] Forgac, M. (1992) J. Bioenerg. Biomembr. 24, 341–350.
[33] Bowman, E.J., Siebers, A. and Altendorf, K. (1988) Proc. Natl. Acad. Sci USA 85, 7972– 7976
[34] Hanada, H., Moriyama, Y., Maeda, M. and Futai, M. (1990) Biochem. Biophys. Res. Commun. 179, 873–877.
[35] Crider, B.P., Xiao-Song, X. and Stone, D.K. (1994) J. Biol. Chem. 269, 17379–17381.
[36] Gluck, S. and Nelson, R. (1992) J. Exp. Biol. 172, 205–218.
[37] Gluck, S.L., Nelson, R.D., Lee, B.S., Wang, Z.-Q., Guo, X.-L., Fu, J.-Y. and Zhang, K. (1992) J. Exp. Biol. 172, 219–229.
[38] Forgac, M. (1992) Ann. N.Y. Acad. Sci. 671, 273–283.
[39] Forgac, M. (1992) J. Exp. Biol. 172, 155–169.
[40] Nelson, N. and Taiz, L. (1989) Trends Biochem. Sci. 14, 113–116.
[41] Nelson, N. (1989) J. Bioenerg. Biomembr. 21, 553–571.
[42] Moriyama, Y. and Nelson, N. (1989) Biochim. Biophys. Acta 980, 241–247.
[43] Anraku, Y., Unemoto, N., Hirata, R. and Wada, Y. (1989) J. Bioenerg. Biomembr. 21, 589–603.
[44] Gerencser, G.A. (1990) Biochim. Biophys. Acta 1023, 475–477.
[45] Uchida, E., Ohsumi, Y. and Anraku, Y. (1985) J. Biol. Chem. 260, 1090–1095.
[46] Moriyama, Y. and Nelson, N. (1987) J. Biol. Chem. 262, 9175–9180.
[47] Lubansky, H.J. and Arruda, J.A.L. (1985) J. Biol. Chem. 260, 4035–4040.
[48] Arai, H., Pink, S. and Forgac, M. (1989) Biochemistry 28, 3075–3082.
[49] Xie, X.-S., Crider, B.P. and Stone, D.K. (1989) J. Biol. Chem. 264, 18870–18873.
[50] Xie, X.-S., Stone, D.K. and Racker, E. (1983) J. Biol. Chem. 258, 14834–14838.
[51] Bowman, B.J. and Bowman, E.J. (1986) J. Membr. Biol. 94, 83–97.
[52] Harikumar, P. and Reeves, J.P. (1983) J. Biol. Chem. 258, 10403–10410.
[53] Moriyama, Y. and Nelson, N. (1988) J. Biol. Chem. 263, 8521–8527.
[54] Ohkuma, S., Moriyama, Y. and Takano, T. (1982) Proc. Natl. Acad. Sci. USA 79, 2758–2762.

[55] Stone, D.K., Xie, X.-S. and Racker, E. (1984) J. Biol. Chem. 259, 2701–2703.
[56] Navarro, J., Chabot, J., Sherrill, K., Aneja, R., Zahler, S.A. and Racker, E. (1985) Biochemistry 24, 4645–4650.
[57] Sokolove, P.M., Westphal, P.A., Kester, M.B., Wierwille, R. and Sikora-Van Meter, K. (1989) Biochim. Biophys. Acta 983, 15–22.
[58] Gluck, S., Kelly, S. and Al-Awqati, Q. (1982) J. Biol. Chem. 257, 9230–9233.
[59] Thomas-Reetz, A., Hell, J.W., During, M.J., Walch-Solimena, C., Jahn, R. and De Camilli, P. (1993) Proc. Natl. Acad. Sci. USA 90, 5317–5321.
[60] Forgac, M. and Berne, M. (1986) Biochemistry 25, 4275–4280.