Biochimica et Biophysica Acta 812 (1985) 413–422
Elsevier

BBA 72357

Na^+-stimulated ATPase activities in basolateral plasma membranes from guinea-pig small intestinal epithelial cells

J.R. Del Castillo * and J.W.L. Robinson †

Département de Chirurgie expérimentale, Centre Hospitalier Universitaire Vaudois, 1011 Lausanne (Switzerland)

(Received May 10th, 1984)

Key words Na^+-ATPase, $(Na^+ + K^+)$-ATPase, Na^+ transport, Plasma membrane, Epithelial transport; (Guinea pig small intestine)

Two ATPase activities, a Na^+-ATPase and a $(Na^+ + K^+)$-ATPase, have been found associated with sheets of basolateral plasma membranes from guinea-pig small intestinal epithelial cells. The specific activity of the former is 10–15% of the latter. The two ATPase activities differ in their affinity for Na^+, their optimal pH, their K^+ requirement and particularly in their behaviour in the presence of some inhibitors and of Ca^{2+}. Thus the Na^+-ATPase is refractory to ouabain but it is strongly inhibited by ethacrynic acid and furosemide, whilst the $(Na^+ + K^+)$-ATPase is totally suppressed by ouabain, partially by ethacrynic acid and refractory to furosemide. In addition, the Na^+-ATPase is activated by micromolar concentrations of calcium and by resuspension of the membrane preparation at pH 7.8. The Na^+-ATPase is only stimulated by sodium and to a lesser extent by lithium; however, this stimulation is independent of the anion accompanying Na^+. The latter rules out the participation of an anionic ATPase. The relation between the characteristics of the sodium transport mechanism in basolateral membrane vesicles (Del Castillo, J.R. and Robinson, J.W.L. (1983) Experientia 39, 631) and those of the two ATPase activities present in the same membranes, allow us to postulate the existence of two separate sodium pumps in this membranes. Each pump would derive the necessary energy for active ion transport from the hydrolysis of ATP, catalyzed by different ATPase systems.

Introduction

The epithelial cells of the small intestine are capable of transporting sodium from the lumen into the interstitium against an electrochemical gradient. The interior of the cells is electronegative with respect to surroundings, and the sodium concentration in the cytoplasm is only on tenth of the extracellular concentration. Sodium enters the cell passively across the luminal membrane, following its electrochemical gradient and is extruded actively across the basolateral plasma membrane of the cell. The physiological machinery involved in this latter phenomenon has been called the sodium pump. At the present time, it is known that the $(Na^+ + K^+)$-activated ATPase the Na^+-K^+-exchange pump constitute the same functional entity. There are different types of indirect evidence indicating that this ATPase is related to the movements of sodium and potassium ions across cell membranes, but the most unequivocal demonstration that the $(Na^+ + K^+)$-ATPase participates directly in sodium and potassium transport comes from experiments in which the enzyme is incorpo-

* To whom correspondence should be addressed at (present address) Instituto Venezolano de Investigaciones Cientificas (IVIC) Centro de Biofisica y Bioquímica Apartado 1827, Caracas 1010A Venezuela

† Deceased January 4th, 1983

rated into phospholipid vesicles which are then capable of actively driving the movement of Na^+ and K^+ between outside and inside in the present of ATP [1,2].

It has been shown that basolateral plasma membrane vesicles prepared from intestinal epithelial cells evidence two ways of active sodium transport, which depend on the hydrolysis of ATP [3]. One of these processes exchanges sodium for potassium, whereas the other transport sodium alone, cation presumably being accompanied by chloride, the only anion present in the incubation medium. The first of these mechanisms must be the one related to the functioning of the ($Na^+ + K^+$)-ATPase, located exclusively in the basolateral plasma membrane of these cells. If there exists a direct relationship between the second sodium-pumping mechanism and ATP hydrolysis, it should be possible to demonstrate in these membranes the existence of an ATPase system with properties similar to those of the second transport mechanism found in basolateral plasma membrane vesicles.

It should be pointed out that, previously, Proverbio et al. [4,5] have been able to differentiate two sodium-stimulated ATPase systems in basolateral membranes derived from renal proximal tubular cells. One of them was stimulated by sodium alone (the Na^+-ATPase) and the other by combination of sodium and potassium (the ($Na^+ + K^+$)-ATPase); both required the presence of magnesium. The two systems were distinguished by their sensitivities to different inhibitors. The Na^+-ATPase was insensitive to ouabain, but inhibited by ethacrynic acid, furosemide and triflocin, whereas the ($Na^+ + K^+$)-ATPase was fully inhibited by ouabain, and partially by ethacrynic acid, but was refractory to furosemide and triflocin [6,7]. The characteristics of the two ATPase activities conform perfectly with the properties of the two sodium transport mechanisms described from renal cortex slices rich in proximal tubular cells [8–11]. It was thus proposed that these two ATPase systems constituted the molecular machinery responsible for the movement of sodium in these cells.

In the present paper, two sodium-stimulated ATPase activities in basolateral plasma membrane of epithelial cells from small intestine are described. Their different affinities for sodium and different behaviour in the present of various inhibitors have permitted us to related them directly with the sodium-transporting mechanisms that we have characterised in vesicles of the same membranes.

Part of this work was presented to the 5th Meeting of the European Intestinal Transport Group, Egmond aan Zee, Netherlands (1983).

Materials and Methods

Materials

All compounds used were of the highest available purity. Tris-ATP and ouabain (strophanthin-G) were purchased from Sigma (St. Louis) and ethylene glycol bis(2-aminoethyl ether)-*N*,*N'*-tetraacetic acid (EGTA) from Fluka (Bushs). Furosemide was a gift of Hoechst A.G. (Frankfurt am Main) and ethacrynic acid of Merck, Sharp & Dohme (Haarlem).

Preparation of basolateral plasma membranes

The basolateral plasma membranes were obtained as described by Del Castillo and Robinson [12]. Briefly, a membrane fraction (P_3) was isolated from scraping of guinea-pig intestinal mucosa by differential centrifugation. As previously described, the P_3 fraction is a vesicle preparation rich in basolateral plasma membranes although it contains a small contamination of brush-border membranes. Membrane fraction P_3 was used in most experiments. Occasionally, the basolateral plasma membranes were separated from contaminating brush-border membranes present in P_3 fraction by applying the suspension to a Percoll gradient (11.67% v/v). This yields a basolateral plasma membrane vesicle fraction (F-II), which is essentially free of the other membrane contaminants [12]. The P_3 and F-II fractions were resuspended in homogenization medium (sucrose 50 mM + Tris-HCl (pH 7.2 or 7.8)). It should be emphasized that it is most important, for the demonstration of the Na^+-ATPase, to use fresh homogenization media, free of bacterial contamination, preferably prepared immediately before the initial homogenization, and to keep all solutions on ice throughout the preparative procedure.

ATPase assays

Fractions P_3 and FII are formed mainly by vesicles. To assay the ATPase activities, membrane fractions P_3 and F-II were treated with deoxycholate/EDTA [12]. This treatment transforms the vesicles into sheets. The ATPase activities were determined as described previously [5]. The incubation medium, with a final volume of 1 ml, contained 50–100 mM Tris-HCl (pH 7.0 at 37°C), 5 mM $MgCl_2$, and as appropriate, 100 mM NaCl, 20 mM KCl and 1 mM ouabain. The concentration of Tris-HCl was varied to adjust the osmolarity of the medium. The final concentration of proteins was between 25 and 50 μg/ml, range in which ATP hydrolysis was linear function of the protein concentration. The incubation medium was preincubated for 5 min at 37°C and the reaction was initiated by addition of Tris-ATP (2.5 mM final concentration) to the mixture. After an incubation of 15 min the reaction was stopped by the addition of 1 ml of 6% perchloric acid. The samples were well mixed and centrifuged, and the orthophosphate liberated was determined in the deproteinized solution [13]. Preliminary experiments showed that under these conditions, there was a linear relationship between the orthophosphate liberated and the incubation time. All determinations were run in triplicate.

The ATPase activities are expressed in nmol of phosphate produced per mg of protein and per minute, after subtraction of a blank obtained on adding the membrane suspension only after the perchloric acid. The ATPase activity obtained in the presence of magnesium alone is known as the Mg^{2+}-ATPase. The difference in activity between this Mg^{2+}-ATPase and the activity in the presence of both magnesium and sodium is denoted as the Na^+-ATPase, and the difference in activity obtained in the presence of magnesium, sodium and ouabain or magnesium, sodium and potassium is referred to as the $(Na^+ + K^+)$-ATPase.

Proteins were routinely determined by the Folin method [14], using crystalline bovine albumin as standard.

Results

Two Na^+-stimulated ATPase activities in small intestinal tissue

It may be seen in Table I that basolateral plasma membrane sheets prepared from small intestinal epithelium and resuspended in a medium at pH 7.8 possess two Na^+-stimulated ATPase activities, besides the ubiquitous Mg^{2+}-ATPase. One of these activities is dependent on Mg^{2+}, Na^+ and K^+ and is inhibited by ouabain; this corresponds to the well-known $(Na^+ + K^+)$-ATPase. The other, the Na^+-ATPase, is stimulated by Mg^{2+} and Na^+ and is refractory to ouabain.

TABLE I

ATPase ACTIVITIES IN BASOLATERAL PLASMA MEMBRANES OF FRACTION F-II

Membrane sheets were obtained by treating basolateral membrane vesicles with deoxycholate/EDTA (Ref 12) The membrane fractions were resuspended at pH 7 8. Incubation medium: Tris-HCl, pH 7.0 (50–150 mM), $MgCl_2$, 5 mM; NaCl, 100 mM; KCl, 20 mM, Ouabain, 1 mM, ATP, 2 5 mM. The values are expressed as the means ± S E ($n = 4$)

Incubation medium	ATPase activity (nmol P_i/mg protein per min)	Δ
Mg^{2+}	274 ± 2 5	-
$Mg^{2+} + Na^+$	324 ± 2 9	50 ***
$Mg^{2+} + Na^+ + K^+$ + ouabain	328 ± 2 6	54 ***
$Mg^{2+} + Na^+ + K^+$	982 ± 4.5	654 ***

*** $P < 0.001$.

It was found that intestinal Na^+-ATPase is activated by calcium (Table II). In the absence of calcium, sodium is able to enhance the basal ATPase activity determined in the presence of Mg^{2+} and ouabain, but up on addition of 50 μM Ca^{2+}, there is a significant stimulation of the Na^+-ATPase activity. On the other hand, $(Na^+ + K^+)$-ATPase activity is partially inhibited by the same concentration of calcium, and the Mg^{2+}-ATPase activity is unaffected.

The measurable Na^+-ATPase activity may also be enhanced by preparing the basolateral plasma membranes at pH 7.2 and resuspending the final fraction at pH 7.8. This phenomenon is illustrated in Table III. Under this conditions, the Na^+-ATPase activity is 20 nmol/mg protein per min. Neither the addition of calcium nor the addition of EGTA (which reduces the external Ca^{2+} concentration to less than 10^{-9} M) has any effect on this activity. This results shows that although Ca^{2+}

TABLE II

EFFECT OF 50 μM Ca^{2+} ADDED TO INCUBATION MEDIUM ON THE Na^+-STIMULATED ATPases OF BASOLATERAL PLASMA MEMBRANES OF FRACTION P_3

Mg^{2+}, 5 mM; Na^+, 100 mM, K^+, 20 mM, ouabain, 1 mM, ATP, 2 5 mM· The values are expressed as the means ± S E ($n = 6$)

Incubation medium	ATPase activity (nmol P_i/mg protein per min)	
	$-Ca^{2+}$	$+Ca^{2+}$
Mg^{2+} + ouabain	224 ± 2.2	225 ± 2 9
Mg^{2+} + Na^+ + ouabain	237 ± 2.3	259 ± 3.0
Mg^{2+} + Na^+ + K^+	527 ± 3 7	515 ± 7 0
Δ Na^+	(a) 13 ± 3 1 **	(b) 34 ± 4 1 ***
Δ Na^+ + K^+	(c) 290 ± 4 6	(d) 256 ± 7 6

** $0.005 > P > 0.001$
*** $P > 0.001$
(b − a) $0.005 > P > 0.001$
(c–d) $0.005 > P > 0.001$ according to t-test throughout

TABLE IV

EFFECT OF DIFFERENT Ca^{2+} CONCENTRATIONS ON THE Na^+-ATPase IN THE BASOLATERAL PLASMA MEMBRANE FRACTION P_3

P_3 fractions were resuspended at pH 7 2 Ca^{2+} concentrations refer to added and not to free calcium concentrations Mg^{2+}, 5 mM, Na^+, 100 mM; ouabain, 1 mM The values are expressed as the means ± S E ($n = 6$)

Added Ca^{2+} (μM)	ATPase activity (nmol P_i/mg protein per min)		
	Mg^{2+}	Mg^{2+} + Na^+	Δ
0	270 ± 1 05	284 ± 1 13	14 ***
5	251 ± 2.27	270 ± 2 86	19 ***
10	252 ± 4.83	277 ± 3 00	25 **
20	259 ± 4 55	286 ± 1.37	27 ***
50	258 ± 4 23	285 ± 4 43	28 ***
100	264 ± 1 65	286 ± 1 37	22 ***

*** $P < 0.001$
** $P < 0.005$

stimulates the Na^+-ATPase, it is not an obligatory activator of the enzyme. The Na^+-ATPase activity is observed in full in the absence of Ca^{2+}, provided its activity is unmasked by the change in pH of the medium of resuspension of the membranes.

Table IV illustrates the effect of increasing the added calcium to the incubation medium on the Na^+-ATPase of basolateral plasma membranes suspended at pH 7.2. As the added calcium concentration is increased (in the micromolar range), the Na^+-ATPase activity rises, reaching a maximum when 20 μM of added Ca^{2+} is employed. These data can be transformed on a reciprocal plot to give an apparent K_a for added calcium activation of 5 μM.

TABLE III

EFFECT OF Ca^{2+} AND EGTA ADDED TO INCUBATION MEDIUM ON THE Na^+-ATPase OF BASOLATERAL PLASMA MEMBRANES OF FRACTION P_3 RESUSPENDED AT pH 7.8

Assays performed in the presence of 1 mM ouabain Mg^{2+}, 5 mM, Na^+, 100 mM, Ca^{2+}, 25 μM; EGTA, 0 5 mM, at pH 7 0 The values are expressed as the means ± S E ($n = 6$)

Addition	ATPase activity (nmol P_i/mg protein per min)		
	Mg^{2+}	Mg^{2+} + Na^+	Δ
None	246 ± 4 2	267 ± 4 7	20 **
Ca^{2+}	242 ± 5.0	263 ± 4 9	21 *
EGTA	220 ± 2 9	243 ± 3 4	23 ***

* $0.02 > P > 0.01$
** $P < 0.01$.
*** $P < 0.001$

Cellular localization of the Na^+-ATPase

Having demonstrated the presence of a Na^+-ATPase in fractions enriched in basolateral plasma membranes, we proceeded to explore the distribution of this ATPase activity in the different fractions of the preparation, to compare it with that of the ($Na^+ + K^+$)-ATPase which is known to be present only in basolateral plasma membranes and is therefore used as a marker for this fraction [15].

Table V gives details of the specific activity, the relative specific activity with respect to the homogenate and percentage of recovery of the two ATPase activities in the different fractions of the preparation. Like the ($Na^+ + K^+$)-ATPase, the Na^+-ATPase is preferentially enriched in the P_3 and F-II fractions which are mainly formed by basolateral plasma membranes [12]. A better way of demonstrating the localization of the Na^+-stimulated ATPase involves the comparison of the distribution of this ATPase activity with that of the ($Na^+ + K^+$)-ATPase. When using the data of

TABLE V

DISTRIBUTION OF Na^+-ATPase AND ($Na^+ + K^+$)-ATPase [a] IN THE DIFFERENT FRACTIONS OF THE PREPARATION

S A , specific activity (nmol P_i/mg protein per min); R.S A , relative specific activity; R%, percentage of total recovery. The values are expressed as means ± S E

Fraction	Na^+-ATPase ($n = 6$)			($Na^+ + K^+$)-ATPase ($n = 7$)		
	S.A	R S A [b]	R%	S.A.	R S A. [b]	R% [b]
Homogenate	5.33 ± 1 21	1		43 ± 3 0		
S	3 16 ± 0.87	0 59	31.36 ± 3.05	26 ± 4.0	0.62	32.5 ± 1.56
P_1	7.33 ± 1.36	1 38	37.68 ± 2.95	61 ± 6.1	1 43	39.1 ± 2.4
S_1	3 00 ± 0.91	0 56	6.02 ± 1.08	15 ± 2.4	0 36	3 8 ± 0 71
P_2	8 67 ± 1.81	1.63	7 48 ± 0 86	47 ± 8 8	1 11	5 0 ± 0 55
P_3	32 56 ± 3 04	6.11	18.33 ± 1.73	256 ± 3 0	6 02	18 1 ± 1 25
% total recovery	–	–	100 87	–	–	98 5
Fraction	Na^+-ATPase ($n = 6$)			($Na^+ + K^+$)-ATPase ($n = 7$)		
	S A	R.S.A [c]	R% [c]	S.A.	R S.A. [c]	R% [c]
P_3	32 6 ± 3 04	1		256 ± 3.0	1	
F-I	not detectable	0	0	not detectable	0	0
F-II	57 8 ± 2 12	1.78	57 87 ± 3.44	538 ± 6.8	2.10	68 5 ± 6.33
F-III	23 7 ± 3 80	0 73	11 06 ± 4.00	247 ± 34.2	0.96	14 6 ± 1 26
F-IV	10.2 ± 1.80	0.31	12.94 ± 3.19	85 ± 8 5	0 33	13 7 ± 1 66
% total recovery	–	–	81 78	–	–	96.8

[a] Data taken from Del Castillo and Robinson (1982) Ref 12. A full description of the different fractions is given in Ref 12.
[b] R.S.A. and R% refer to homogenate
[c] R.S A. and R% refer to P_3 fraction

Table V, we compare the relative specific activity and percentage of recovery of the two ATPase in the different fractions of the preparation, it becomes obvious that the Na^+-ATPase and the ($Na^+ + K^+$)-ATPase are similarly distributed throughout, as witnessed by the high correlation coefficient (0.99 in both cases). We can conclude that the Na^+-ATPase is also localized in basolateral membranes of intestinal epithelial cells.

TABLE VI

EFFECT OF DIFFERENT NUCLEOTIDES ON THE ATPase ACTIVITIES IN BASOLATERAL PLASMA MEMBRANES OF FRACTION P_3

Mg^{2+}, 5 mM; Na^+ 100 mM, K^+ 20 mM, ouabain, 1 mM, nucleotides, 2 5 mM. The values are expressed as the means ± S.E ($n = 6$)

Nucleotide	ATPase activity (nmol P_i/mg protein per min)				
	Mg^{2+}	$Mg^{2+} + Na^+$	$Mg^{2+} + Na^+ + K^+$	ΔNa^+	ΔNa^+-K^+
ATP	211 ± 2 6	236 ± 3.2	481 ± 5 3	+25 **	245 ***
AMP	38 ± 4 2	35 ± 5.1	42 ± 4.8	−3	7
ADP	67 ± 8 6	69 ± 10.3	87 ± 5 4	+2	18
GTP	221 ± 9 8	224 ± 8.7	299 ± 15 4	+3	75 *
UTP	146 ± 16.4	143 ± 14 8	166 ± 4.7	−3	23
CTP	123 ± 15.2	115 ± 11 2	140 ± 15.9	−8	25
ITP	190 ± 7.8	189 ± 9.4	250 ± 9.1	−1	61 *

*** $P < 0.001$
** $P < 0.005$
* $P < 0.01$.

Partial characterization of the intestinal Na^+-ATPase

The Na^+-ATPase possesses certain characteristics which differentiate it from the other tested ATPase activities. To examine these properties, fractions resuspended at pH 7.8 or resuspended at pH 7.2 were used indifferently. The assays were run in the presence of 20 μM Ca^{2+}. Similar results were obtained with both types of preparation.

Table VI illustrates the hydrolysis of different nucleotides by the various enzymes. The Na^+-ATPase is only able to act on ATP, whereas the ($Na^+ + K^+$)-ATPase is essentially specific for ATP though it is able to hydrolyse GTP and ITP to a limited extent. The Mg^{2+}-ATPase, on the other hand, hydrolyse efficiently all the nucleotides studied with the exception of ADP and AMP.

Table VII shows that litium is able to replace sodium to a certain extent as activator of the Na^+-ATPase, though other monovalent cations are ineffective. Choline appears to be inhibitory of the Mg^{2+}-ATPase. Different sodium salts activate the enzyme in an analogous manner (Table VIII) showing that the stimulatory effect of sodium is independent of the accompanying anion. Since bicarbonate stimulates the activity of the Mg^{2+}-ATPase it was necessary to measure the difference in activity obtained with a medium containing $KHCO_3$ and Mg^{2+} and that obtained with $NaHCO_3$ and Mg^{2+}, since as already shown, potassium is inert in the system (Table VII).

Fig. 1 illustrates the effect of the pH of the incubation medium on the three ATPase activities tested in basolateral plasma membranes. the pH

TABLE VII

EFFECT OF DIFFERENT CATIONS ON THE Mg^{2+}-ATPase (P_3 FRACTION)

The cations are added as chloride salts The assays are performed in the presence of 1 mM ouabain. Mg^{2+}, 5 mM, cations, 100 mM The values are expressed as the means ± S.E ($n = 6$)

Incubation medium	ATPase activity (nmol P_i/mg protein per min)	Δ
Mg^{2+}	248 ± 2.2	–
$Mg^{2+} + Na^+$	278 ± 3 8	30 **
$Mg^{2+} + Li^+$	262 ± 3 9	14 *
$Mg^{2+} + NH_4^+$	243 ± 2 9	−5
$Mg^{2+} + Rb^+$	246 ± 3 3	−2
$Mg^{2+} + Cs^+$	244 ± 3 7	−4
$Mg^{2+} + K^+$	246 ± 2 8	−2
Mg^{2+} + choline	206 ± 13 3	−42 *

** $P < 0.005$
* $P < 0.05$

TABLE VIII

EFFECT OF DIFFERENT Na^+ SALTS ON THE Mg^{2+}-ATPase OF BASOLATERAL PLASMA MEMBRANES (P_3 FRACTION)

The assays were performed in the presence of 1 mM ouabain Mg^{2+}, 5 mM, salts, 100 mM except Na_2SO_4 (50 mM) The values are expressed as the means ± S E ($n = 4$)

Incubation medium	ATPase activity (nmol P_i/mg protein per min)	Δ
Mg^{2+}	247 ± 2 8	–
Mg^{2+} + NaBr	272 ± 2 7	25 **
$Mg^{2+} + Na_2SO_4$	271 ± 2.4	24 **
Mg^{2+} + NaCl	281 ± 2.5	34 ***
$Mg^{2+} + CH_3COONa$	282 ± 2.3	35 ***
$Mg^{2+} + KHCO_3$	364 ± 3 2	–
$Mg^{2+} + NaHCO_3$	389 ± 3 5	25 *

* $0.02 > P > 0.01$
** $P < 0.01$.
*** $P < 0.005$

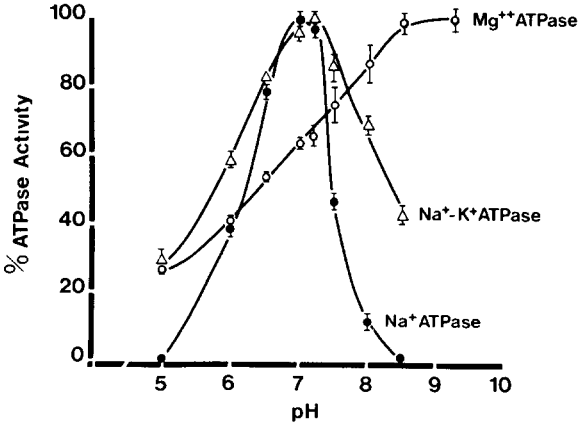

Fig. 1 Effect of pH on the ATPase activities in basolateral plasma membranes of fraction P_3 The buffers used for obtaining the different pH values. Acetate, pH 5, Tris-HCl, pH 6–9.25 Incubation medium contained $MgCl_2$, 5 mM, NaCl, 100 mM, KCl, 20 mM, ouabain, 1 mM The values represent the means ± S E of six determinations

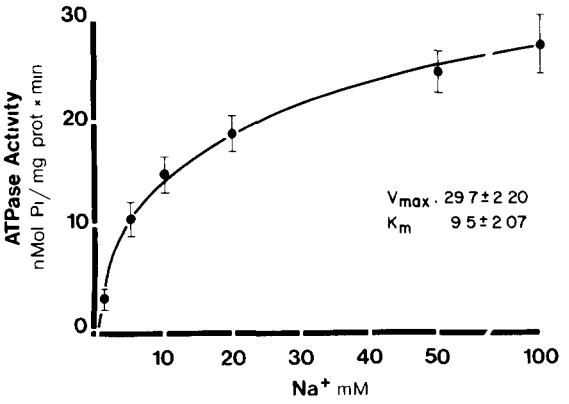

Fig 2. Effect of different sodium concentrations on the Na^+-ATPase activity of basolateral plasma membranes of fraction P_3 The fractions were resuspended at pH 7.2 and 25 μM calcium ions were added to obtain maximal enzymatic activity. $MgCl_2$, 5 mM; ouabain, 1 mM and ATP, 2.5 mM were also present in the incubation medium and the osmolarity was maintained constant with Tris-HCl. The values are the means $\pm$S E. of nine different experiments.

optimum for the Mg^{2+}-ATPase is above 8.5, whilst that of the Na^+-ATPase is 7.0 and that of the $(Na^+ + K^+)$-ATPase is 7.2. The Na^+-ATPase activity is much more sensitive to changes in pH over a narrow range than the other two ATPase activities.

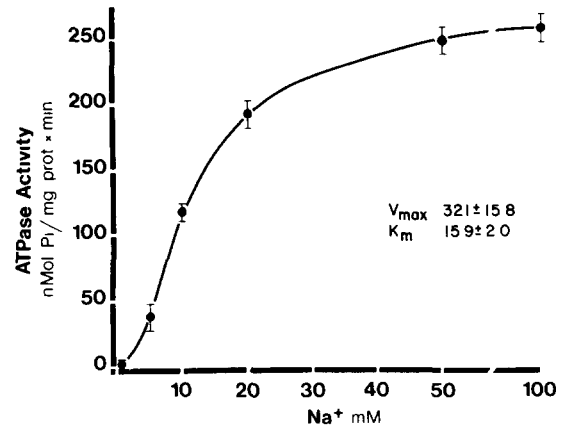

Fig. 3. Effect of different sodium concentration on the $(Na^+ + K^+)$-ATPase activities of basolateral plasma membranes of fraction P_3. Incubation conditions: $MgCl_2$, 5 mM, KCl, 20 mM; ATP, 2.5 mM with or without ouabain, 1 mM Osmolarity was maintained constant with Tris-HCl The values are the means $\pm$ S.E of six different experiments

The effect of increasing the concentration of sodium of the incubation medium on intestinal Na^+-ATPase is shown in Fig. 2. In each case, the ionic strength was maintained constant by addition of Tris-HCl (pH 7.0). The maximal enzymatic activity is attained at a sodium concentration of 50 mM. A linear transformation of these data permitted to calculate a K_m of 9.5 ± 2.1 mM and a V_{max} of 29.7 ± 2.2 nmol/mg protein per min. Somewhat different behaviour is observed for the $(Na^+ + K^+)$-ATPase (Fig. 3). Although the maximal velocity is again reached at a concentration of 50 mM Na^+, the apparent K_m for sodium is 15.9 ± 2.0 mM and the V_{max} is 321 ± 15.8 nmol/mg protein per min.

Effect of different inhibitors on the ATPase activities of intestinal basolateral plasma membranes

Ouabain completely inhibits the $(Na^+ + K^+)$-ATPase without affecting the other two ATPases tested (Fig. 4A). Note that at a concentration of 1 mM ouabain no detectable $(Na^+ + K^+)$-ATPase activity remains; this is the concentration of the inhibitor that is added to the incubation medium in the majority of our experiments to assess the levels of the other two ATPases and constitutes the principal mean used to differentiate the $(Na^+ + K^+)$-ATPase from the other enzymes in the preparation.

Fig. 4B shows the effect of the diuretic, ethacrynic acid, on the different ATPases tested. At a concentration of 1 mM, this drug essentially abolishes all Na^+-ATPase activity, inhibits the $(Na^+ + K^+)$-ATPase by approximately 60% and the Mg^{2+}-ATPase by 25%. Besides the differences in sensitivities to ouabain and ethacrynic acid, the enzymes are also affected differently by furosemide (Fig. 4C); this drug, at a concentration of 2 mM, inhibits about 60% of the Na^+-ATPase activity, whereas the other two ATPase are refractory to this diuretic. The intestinal Na^+-ATPase was less sensitive to furosemide than the renal enzyme which was almost completely inhibited by 2 mM furosemide [7]. Both systems are inhibited by 25 μM vanadate (data not shown).

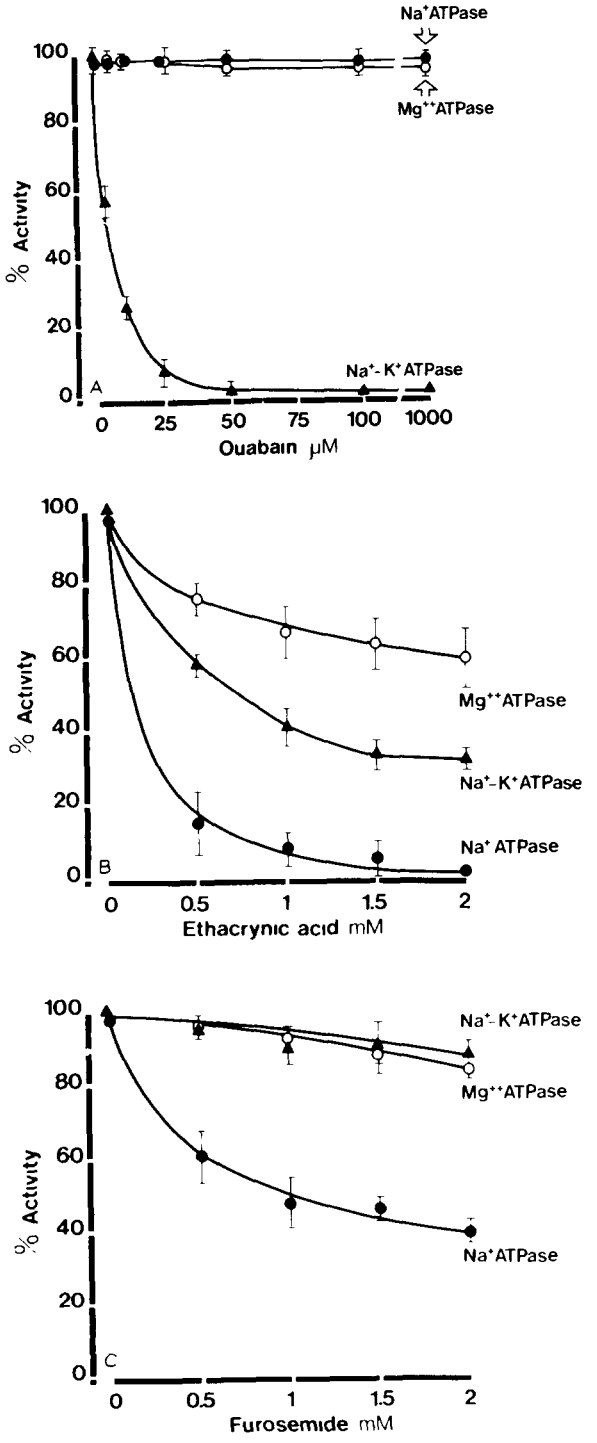

Fig 4. Sensitivity of the different ATPases of basolateral plasma membranes of fraction P_3 to ouabain (A), ethacrynic acid (B) and furosemide (C). Incubation conditions as in Table I. Results are means ± S E of four determinations

Discussion

We have demonstrated previously [3] that basolateral plasma membrane vesicles derived from epithelial cells of the small intestine possess two mechanisms for the active transport of sodium, one of which is independent of potassium and is refractory to ouabain, but is inhibited by ethacrynic acid and furosemide, whilst the other requires the presence of potassium at the extracellular face of the plasma membrane, is completely inhibited by ouabain, partially inhibited by ethacrynic acid and insensitive to furosemide. Both mechanisms derive their energy from the hydrolysis of ATP. We have consequently studied, in this paper, the presence and characteristics of ATPase systems in purified fractions of basolateral plasma membranes from intestinal epithelial cells. The presence of two Mg^{2+}-dependent sodium-stimulated ATPase activities in such fractions was showed; one, denoted the Na^+-ATPase, is stimulated by sodium alone, whereas the second, the $(Na^+ + K^+)$-ATPase, requires both sodium and potassium for activation. Each of these ATPase activities has particular properties which allow it to be characterized, differentiated and related to the appropriate sodium transport mechanism. Thus for instance, the Na^+-ATPase is stimulated by 50 μM calcium added to the incubation medium, while this same concentration inhibits the $(Na^+ + K^+)$-ATPase (Table II). This activation by calcium is not due to the stimulation of a Ca^{2+}-ATPase, since at this calcium concentration, calcium had no effect on the Mg^{2+}-ATPase (Table II) nor on the Na^+-ATPase preactivated by suspension at pH 7.8, and in addition, the Na^+-ATPase resuspended at pH 7.8, remains demonstrable in the presence of EGTA, a calcium chelator (Table III). Thus even if calcium activate the Na^+-ATPase, calcium is not an essential activator. In the kidney, the effect of calcium on the Na^+-ATPase is to increase the V_{max} without affecting the K_m for sodium [16]; possibly it exerts a regulatory role on the activity of this enzyme in the cell, as has been suggested by Proverbio et al. [16].

It is noteworthy that although for preactivation, the enzyme requires resuspension at pH 7.8, incubation should be carried out at pH 7.0, to obtain maximal Na^+-ATPase activity (Fig. 1). This

observation indicates that the activation by raising pH or by the presence of calcium may be of a conformational nature.

The Na^+-ATPase specifically hydrolyzes ATP, as does the $(Na^+ + K^+)$-ATPase, though the latter had some action on GTP and ITP (Table VI). This property defines the two enzymes as ATPase. Like the renal Na^+-ATPase [4,7], the intestinal enzyme is only activated by Na^+ and to a lesser degree by Li^+ (Table VII). This fact links the enzyme with the sodium transport system. The fact that the enzyme is stimulated indifferently by different sodium salts (Table VIII) essentially excludes the possibility that we are dealing with an anion-stimulated ATPase. Moreover, the Na^+-ATPase shows characteristics strongly different to the anion ATPase described for the intestine [17].

The Na^+-ATPase and the $(Na^+ + K^+)$-ATPase can also be differentiated by their slightly different pH optima and different sensitivities to pH. They also reveal somewhat different affinities for sodium, the apparent K_m values for sodium being 9.5 mM and 15.9 mM, respectively (Figs. 2 and 3).

The two enzymes can also be distinguished by their different behaviour towards a series of inhibitors. The Na^+-ATPase is insensitive to ouabain but is totally suppressed by 1.5 ethacrynic acid and partially inhibited by furosemide; in contrast, the $(Na^+ + K^+)$-ATPase is fully inhibited by ouabain, partially inhibited by 1.5 mM ethacrynic acid and unaffected by furosemide (Fig. 4). These features are of extreme importance, since they correspond exactly to the sensitivities of the two sodium-transporting mechanisms that have been characterized in basolateral plasma membrane vesicles from intestinal epithelial cells [3]. This correspondence furnishes the strongest evidence that each of the enzymes provides the machinery responsible for each one of the transport systems.

The possibility that the Na^+-ATPase activity represents residual contamination by other subcellular membrane fragments seems remote. In the preparative procedure described in detail in a previous paper [12], we have studied the distribution of different subcellular membrane enzymatic markers. None of tested markers paralleled the distribution of the $(Na^+ + K^+)$-ATPase. In the present paper we have shown that the distribution of the Na^+-ATPase in the different fractions of the preparation and that of the $(Na^+ + K^+)$-ATPase are similar (Table V), indicating that the Na^+-ATPase is associated with basolateral plasma membranes, for which the $(Na^+ + K^+)$-ATPase is considered a marker, and not with membranes from other subcellular organelles.

The showed results have permitted us to characterize two Mg^{2+}-dependent ATPases, activated by sodium, and located in the basolateral plasma membrane of the enterocytes. We are therefore in a position to postulate that, like renal proximal tubular cells, the intestinal epithelial cells possess two distinct sodium pumps in their basolateral membranes, both which derive their energy from the hydrolysis of ATP through the intervention of separate ATPase systems. It is possible that the Na^+-ATPase and its associated Na^+-transport mechanism participate in the volume regulation of the intestinal cell, as suggested for proximal tubule [11]. Its role in transepithelial sodium transport and in the control of the intracellular environment remain to be determined.

Acknowledgements

We are extremely grateful to Drs. F. Proverbio and G. Whittembury for their suggestions and constructive criticism of this manuscript. J.R. Del Castillo received grants from the Fundación Gran Mariscal de Ayacucho, Caracas, and from Zyma S.A., Nyon.

References

1 Anner, B.M (1980) Acta Physiol. Scand. Suppl 481, 15–19
2 Goldin, S M and Sweadner, K.J (1975) J. Biol Chem 250, 4022–4024
3 Del Castillo, J R. and Robinson, J.W.L (1983) Experientia 39, 631
4 Proverbio, F., Condrescu-Guidi, M. and Whittembury, G (1975) Biochim Biophys. Acta 394, 281–292
5 Proverbio, F and Del Castillo, J.R. (1981) Biochim. Biophys. Acta 646, 99–108
6 Pérez-González de la Manna, M., Proverbio, F. and Whittembury, G (1980) Curr. Topics Membranes Transp. 13, 315–335
7 Del Castillo, J R , Marín, R., Proverbio, T and Proverbio, F (1982) Biochim Biophys Acta 692, 61–68
8 Whittembury, G. and Proverbio, F (1970) Pflugers Arch 316, 1–25
9 Proverbio, F., Robinson, J.W.L. and Whittembury, G. (1970) Biochim Biophys. Acta 211, 327–336

10 Proverbio, F and Whittembury, G. (1975) J Physiol 250, 559–578
11 Proverbio, F, Del Castillo, J R., Marín, R. and Whittembury, G. (1981) in Epithelial ion and water transport (Macknight, A.D C. and Leader, J.P., eds), pp 349–356, Raven Press, New York
12 Del Castillo, J.R. and Robinson, J W L (1982) Biochim Biophys Acta 688, 45–56
13 King, E J (1932) Biochem. J 26, 292–297
14 Lowry, O H, Rosebrough, N J, Farr, A L and Randall, R J (1951) J Biol. Chem 193, 265–275
15 Ernst, S.A and Hootman, S R (1981) Histochem J 13, 397–418
16 Proverbio, F, Proverbio, T and Marín, R (1982) Biochim Biophys. Acta 688, 757–763
17 Humphreys, M H and Chou, L Y N (1979) Am J Physiol, 236, E70–E76