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Mg^{2+}-ATP-dependent sodium transport in inside-out basolateral plasma membrane vesicles from guinea-pig small intestinal epithelial cells

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

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

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The transport of sodium into inside-out basolateral plasma membrane vesicles from small intestinal epithelial cells has been examined. It was found, under equilibrium conditions, that binding of ^{22}Na represents approx. 55% of the total uptake during an equilibration period of 30 min; 45% of the total uptake correspond to passive sodium entry in the vesicle space. In addition to binding and to passive Na^+ entry, two distinct mechanisms capable of accumulating sodium in the intravesicular space can be demonstrated when ATP is added to the incubation medium. One transports sodium actively in the absence of potassium, whereas the other requires the presence of potassium in the interior of the vesicles. The two mechanisms can also be differentiated by their affinities for sodium, their optimal pH and by their behaviour towards different inhibitors. Thus, the mechanism that transports sodium in the absence of potassium is refractory to ouabain, but is inhibited by ethacrynic acid and furosemide, whilst the mechanism that accumulates sodium inside the vesicles in the presence of internal potassium is strongly inhibited by ouabain, is weakly inhibited by ethacrynic acid and is insensitive of furosemide. ATP is a specific stimulator of both processes, and the requirement for magnesium is absolute in both cases.

Introduction

In the enterocyte the transcellular movement of sodium is known to be dependent on cellular energy and to involve the participation of carriers. Sodium enters the enterocyte across the mucosal plasma membrane and is extruded from the cell across the basolateral plasma membrane. Sodium extruded against an electrochemical potential gradient and requires coupling to a supply of metabolic energy. Evidence exists that this energy is derived from the hydrolysis of ATP and that there exists at least one enzyme responsible for such hydrolysis, namely the $(Na^+ + K^+)$-ATPase. Experiments performed in vitro are consistent with this notion. For instance, the cardiac glycoside, ouabain, specific inhibitor of the $(Na^+ + K^+)$-ATPAse, only inhibits the active absorption of sodium when added to the serosal face of the tissue [1]. The inhibition to transepithelial sodium transport is accompanied by a loss of potassium and by a gain of sodium by the cells. In addition, autoradiographic [2,3], histochemical [4] and cell fractionation studies [5,6] have located the binding of ouabain and the $(Na^+ + K^+)$-ATPase activity almost exclusively at the basolateral plasma membrane, with little or no activity in the apical pole of the cell.

In the small intestine, removal of potassium

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

† Deceased January 4th, 1983

Abbreviations. p[NH]ppA, adenosine 5'-[β,γ-imido]triphosphate

from the mucosal and serosal media causes a reduction in intracellular potassium and an equivalent gain in intracellular sodium, without change in cell water; the removal of potassium from the mucosal medium has no effect; whereas removal of potassium from serosal medium leads to a reduction in cell potassium without concomitant changes in cell sodium or water content [7]. These observations suggest that the maintenance of high intracellular potassium and low sodium activities depend on the presence of potassium at the serosal face of the cell and that the apical membrane of the cell is little permeable to potassium. The removal of sodium from the mucosal or serosal solutions leads to a fall in intracellular sodium levels, but does not affect the intracellular potassium concentrations, nor the flux of potassium across the basolateral plasma membrane; the bilateral removal of sodium causes a reduction in both intracellular sodium and potassium, a decrease in cell water and a diminution of potassium movement across the serosal membrane. In addition, ouabain reduces cell potassium and increases cell sodium by equivalent amounts without changing the cell water content [7]. These data support the hypothesis that a Na^+-K^+-exchange pump is responsible for the maintenance of the normal intracellular concentrations of sodium and potassium, but appear to indicate that the regulation of the cellular volume is independent of this process. On the other hand, it is known that solutes such as D-glucose and L-alanine strongly enhance transcellular movement of sodium by stimulating the entry of the cation across the apical pole of the cell [8], but they do not influence the rate of exchange of ^{42}K across the basolateral plasma membrane [7]. These observations agree with the findings of Lee and Armstrong [9] who determined the intracellular activities of sodium and potassium in bullfrog small intestine using cation-selective microelectrodes and observed that in the presence of 3-*O*-methylglucose, both ion activities were significantly reduced despite the stimulation of transcellular sodium transport caused by this sugar. If there existed an absolute relationship between the sodium transport and Na^+-K^+ exchange, an increment in cell potassium would be expected. Indeed, these observations have been recently confirmed with isolated enterocytes [10] where it was found that non-metabolizable hexoses elicit no rise in cell potassium despite a clear decrease in intracellular Na^+ concentration.

In addition to the small intestine, there are other epithelia where there appears to be no strict relationship between transcellular sodium transport and sodium-potassium exchange [11–15]. In the proximal tubular cells of the guinea-pig kidney, two different mechanisms for sodium transport across the basolateral plasma membrane of the cell have been described and characterized [16–18]. Once mechanism exchanges intracellular sodium for extracellular potassium, whilst the other actively expells sodium, the cation being followed passively by chloride and water. The first of these mechanisms is inhibited by ouabain, weakly inhibited by ethacrynic acid and insensitive to furosemide and triflocin (α,α,α-trifluoro-*m*-toluidinonicotinic acid), whereas the second mechanism is refractory to ouabain, but inhibited by ethacrynic acid, furosemide and triflocin. Both mechanisms are dependent on cellular energy since both are suppressed by 2,4-dinitrophenol or anoxia, and it is believed that they both derive their energy from the hydrolysis of ATP. In this respect, it is significant that two Na^+-dependent ATPase activities have been found associated with basolateral plasma membranes isolated from guinea-pig renal proximal cells [19–21].

The evidence quoted above in favour of the existence of a sodium transport mechanism independent of Na^+-K^+ exchange in the small intestinal epithelial cells, is indirect. Therefore we decided to evaluate the possibility of the existence, in the enterocyte, of a mechanism analogous to that described for guinea-pig renal proximal cells. It was considered that a preparation of vesicles from basolateral plasma membranes would give a direct answer, since the composition of both the external and the internal milieu of the vesicles could be easily controlled.

In the present work, we demonstrate, using basolateral plasma membrane vesicles of small intestinal epithelial cells, the existence of two ATP-dependent active Na^+-transport mechanisms.

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(1982) and to the 15th annual USSBE (USGEB) Meeting, Fribourg, Switzerland (1983).

Materials and Methods

Materials

All chemicals were of their highest available purity. Dowex 50W-X8 resin (50–100 mesh) was purchased from Fluka (Bushs), ^{22}Na from Amersham International (Amersham), Tris-ATP, and valinomycin from Sigma (St. Louis) and p[NH]ppA, GTP, ITP, UTP, CTP, ADP from Boehringer (Mannheim).

Preparation of basolateral plasma membrane vesicles

Basolateral plasma membrane vesicles were obtained as described by Del Castillo and Robinson [22]. Briefly, guinea-pig small intestinal scrapings were homogenized in a medium containing 250 mM sucrose and 20 mM Tris-HCl (pH 7.2) and basolateral vesicles were isolated by differential centrifugation and application to a Percoll gradient. The final fraction (F-II) containing vesicles was resuspended in the homogenization medium, such that the protein concentration was greater than 4 mg/ml. F-II was immediately used for the transport experiments. It has been previously shown that this fraction contains 60% inside-out basolateral plasma membrane vesicles and is practically free of others membrane contaminants.

Proteins were routinely determined by the Folin method [23], using crystalline bovine albumin as standard. Since high concentrations of Percoll can interfere with this method, blanks were prepared from gradients run without membranes. In addition, a modified Coomassie blue method [24,25] with which Percoll does not interfere [26] was also used to check the results obtained for fractions emerging from Percoll gradients.

Sodium transport assays

All transport experiments were started by the addition of the suspension of basolateral plasma membrane vesicles to the appropriated incubation medium. Aliquots of this mixture were then removed after different incubation times for the determination of radioactivity in the interior of the vesicles. Separation of the vesicles from the suspension medium was performed on Dowex 50W-8X columns. Generally, 50 μl of the incubation medium were applied to the columns and immediately eluted with 1 ml of washing solution of the following composition: 150 mM sucrose and 20 mM Tris-HCl (pH 7.2). The eluate was collected in counting tubes and its ^{22}Na activity was determined in a γ-counter.

In general, the incubation medium contained (final concentrations): 50 mM sucrose, 20 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 20 mM NaCl and 20 mM KCl (or choline chloride) and 5 mM of the appropriate nucleotide. The final volume of the incubation mixture was 100 μl and the quantity of protein added varied between 40 and 200 μg. Sodium uptake was expressed in nmol/mg protein.

In each experiment, two types of blank were run. The first contained no membranes but the same specific activity of ^{22}Na; 50 μl of this solution were applied to the column and eluted in the same manner as the experimental specimens. In this way, the quantity of radioactivity that passes through the column in a non-specific manner can be assessed; in practice, less than 0.01% of the applied radioactivity passed through the column. The second blank determined the binding of ^{22}Na to the membrane preparation. For this purpose, the vesicles were pretreated with a mixture of deoxycholate and EDTA (final concentration 0.06% and 1 mM, respectively) for 30 in at room temperature, in order to open them [22]. The resulting membrane sheets were incubated in the same way as the sealed vesicle preparation and passed through the Dowex columns. It was found that the quantity of ^{22}Na bound to membrane sheets represents approx. 55% of the radioactivity measured in sealed vesicle preparation after an incubation of 30 min in the absence of ATP. This percentage, incidentally, agrees with that calculated when the passive uptake of sodium is expressed as a function of the reciprocal of the medium osmolarity (see later).

All transport experiments were carried out in triplicate at 25°C. The elution time was 30 s. A comparison of the turbidity of the elute from the columns with that of an aliquot of the vesicle suspension added to 1 ml of the washing medium confirms that essentially all the vesicles passed through the column.

The Dowex columns were prepared in the following manner [27,28]: Dowex, purchased in the H^+ form, was transformed to the $Tris^+$ form by the addition of Tris-base (1 M) until a basic pH was attained. A small glass fiber plug was placed at the bottom of a Pasteur pipette (14.5 cm long) and then a height of 4–5 cm of Dowex ($Tris^+$ form) were introduced. The columns were rinsed with 1 ml of the washing solution containing 2.5% albumin and stored at 4°C. Immediately before use, the columns were again rinsed with the same solution at 4°C.

Results

Sodium uptake by basolateral plasma membrane vesicles in the absence of ATP

Fig. 1 illustrates the uptake of sodium by basolateral plasma membrane vesicles as a function of the incubation time in the absence of ATP. Equilibrium is attained within 15–30 min of the start of the incubation. It is noteworthy that the uptake after 5 min is about 70% of the equilibrium value. Identical results were obtained if K^+ was removed from the incubation medium and replaced by choline chloride.

It is well known that biological membranes can bind ions to their surfaces, and it is probable that the uptake shown in this figure represents not only movement of sodium into the interior of the vesicles but also binding of the cation to the surface of the membrane vesicles. Fig. 2 shows the uptake of sodium by basolateral plasma membrane vesicles after attainment of equilibrium, as a function of the reciprocal of the osmolarity of the incubation medium. As can be seen, the uptake decreases with the increment of the external osmolarity, indicating that part of the uptake measured responds to an osmotically active space. Extrapolation of the straight line to the ordinate, where the osmolarity is theoretically infinite and the intravesicular space zero, indicates that the binding of sodium is of the order of 63% of the value obtained under conditions in which the transport experiments are generally performed, that is to say that osmolarities in the incubation medium varying between 150 and 200 mosmol/l. This value agrees with that obtained in experiments in which membranes sheets, prepared by treating vesicles with deoxycholate/EDTA [22], were incubated in the same way as membrane vesicles (see above in Methods). The results obtained after an incubation of 30 min showed that the binding in the absence or presence

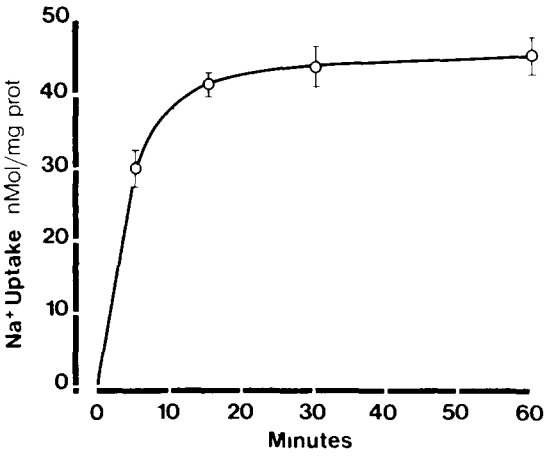

Fig. 1. Uptake of sodium by basolateral plasma membrane vesicles as a function of the incubation time in the absence of ATP. The incubation medium consisted (final volume 0.1 ml) of 100 mM sucrose, 10 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 20 mM NaCl, 20 mM KCl and 1 μCi ^{22}Na Incubation temperature: 25°C. Results are means ± S.E of three experiments

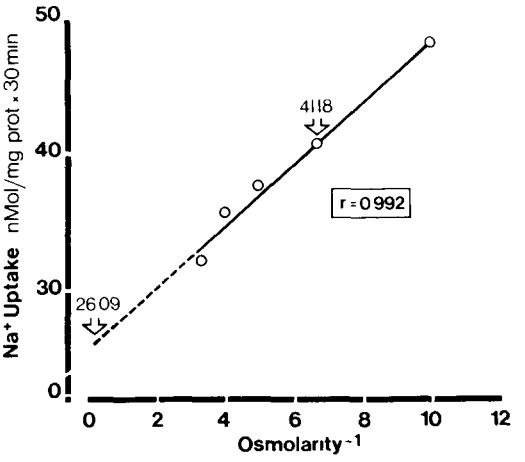

Fig. 2 Relationship between sodium uptake and the reciprocal of the osmolarity of the incubation medium. The basic incubation medium was the same as in the experiment illustrated in Fig 1. Its osmolarity was varied by addition of different sucrose concentrations Results are the means of three experiments, r is the coefficient of correlation of the least-squares regression line The intercept on the ordinate is given by the value 26.09, whereas the value of 41 18 refers to the sodium uptake at 150 mosmol/l

of ATP represents 55% of the uptake observed at equilibrium.

Effect of ATP on sodium uptake by basolateral plasma membrane vesicles

It is well known that the binding sites for potassium and ouabain of the Na^+-K^+ pump are on the external face of the membranes, whereas binding sites for magnesium, sodium and ATP are located at the cytoplasmic face of the membrane. Our preparation has 60% of vesicles oriented inside-out [22], with the result that in this inside-out vesicles the binding sites for magnesium, sodium and ATP are in contact with the external medium. Thus, the effect of ATP on sodium transport in our vesicles can be studied directly. The results of these tests are illustrated in Fig. 3A. At time zero, ATP or its non-hydrolyzable analogue p[NH]ppA were added to different incubation media which contain vesicles that had been equilibrated with the medium for 30 min. The sodium uptake was then measured at different times after addition of the nucleotides. It may be seen an additional increment of sodium uptake when ATP is added to the medium containing magnesium plus sodium or magnesium, plus sodium plus potassium. This additional increment of sodium uptake did not occur with p[NH]ppA. These results suggest that basolateral plasma membrane vesicles are capable of accumulating sodium ions above the equilibrium value when ATP is present in the external medium. Since the results obtained in the presence and absence of potassium are the same, it would appear that there exists one single sodium transport mechanism which was independent of potassium. However, the fact that potassium has no additional effect could simply mean that this cation does not gain access to its binding site which is at the internal face of the inside-out vesicle. The free passage of potassium towards the intravesicular space can be achieved by addition to the incubation medium of the potassium ionophore, valinomycin. In Fig. 3A can be seen that there is an additional component of ATP-dependent sodium uptake, above that observed in the presence of magnesium plus sodium or magnesium plus sodium plus potassium, when valinomycin is added to the incubation medium. The two components of ATP-dependent sodium transport reach a

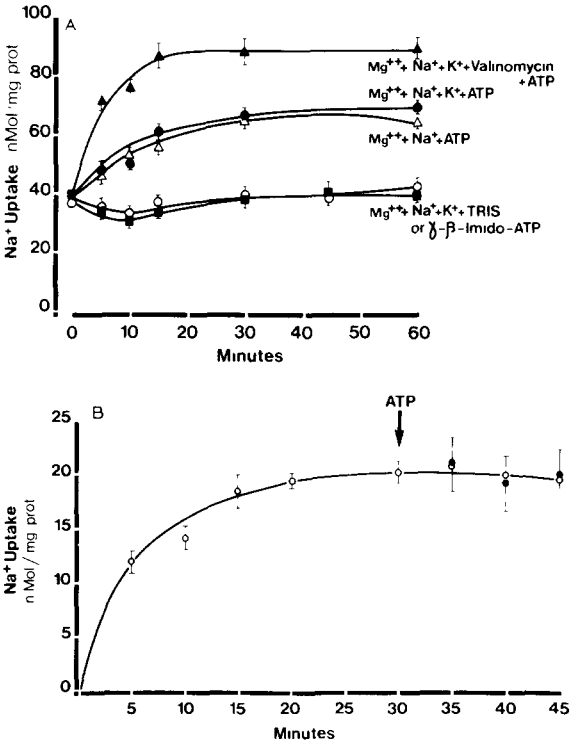

Fig. 3. (A) The effect of ATP on Na^+ uptake by basolateral membrane vesicles Vesicles were preincubated at 25°C for 30 min in the usual medium (see legend Fig. 1), and then the nucleotides were added as shown (time 0 in this graph) Concentrations used valinomycin 10 μM, ATP or p[NH]ppA 5 mM Results are the means ± S.E. of three experiments. (B) The lack of effect of ATP on sodium uptake by brush-border membrane vesicles The vesicles were preincubated in the usual medium for 30 min, and ATP was added (final concentration 5 mM) Results are means ± S.E ($n = 3$)

maximum 20–30 min after addition of the nucleotide. Fig. 3B illustrates the sodium uptake in brush-border membrane vesicles, prepared by the calcium precipitation method described previously [22], incubated in the usual medium in the presence or not of 5 mM ATP. Equilibrium is also attained in this case 20–30 min after the start of the incubation, but addition of ATP has no effect on sodium uptake. This observation emphasizes that the phenomenon observed in the basolateral plasma membrane vesicles does not represent a nonspecific effect of ATP on the membrane.

Dependence of sodium transport on magnesium and on the nature of the accompanying anion

Table I shows that both the Na^+-dependent

TABLE I

EFFECT OF THE PRESENCE OF Mg^{2+} IN THE INCUBATION MEDIUM ON ATP-INDUCED SODIUM UPTAKE BY BASOLATERAL MEMBRANE VESICLES

Experiments performed as in Fig 3, with or without 5 mM $MgCl_2$ Δ^1, Na^+ uptake in the presence of ATP minus Na^+ uptake in the presence of p[NH]ppA. Δ^2, Na^+ uptake in the presence of $Na^+ + K^+ +$ valinomycin + ATP minus Na^+ uptake in the presence of $Na^+ +$ ATP Results are means ± S.E of three experiments

Incubation medium	Na^+ uptake (nmol/mg protein per 15 min)				
	p[NH]ppA, Na^+	ATP, Na^+	Δ^1	ATP, Na^+, K^+, valinomycin	Δ^2
$+MgCl_2$	47 4 ± 1.34	78.8 ± 0.44	31.4	109 0 ± 3.94	30.2
$-MgCl_2$	42.6 ± 2.44	45.2 ± 1.76	2.6	46.2 ± 1 46	1.0

and the $(Na^+ + K^+)$-dependent components of ATP-induced sodium transport in basolateral membrane vesicles depend on the presence of magnesium in the incubation medium. This is not the case for the passive Na^+ uptake.

The uptake of sodium by the Na^+-dependent ATP-induced mechanism was studied when Cl^- was substituted by Br^- in the incubation medium. The Na^+ binding (23 nmol/mg protein per 5 min), the passive Na^+ uptake in the presence or absence of p[NH]ppA (45 nmol/mg protein per 5 min) and the active Na^+ uptake (61.3 nmo/mg protein per 5 min), did not change when Cl^- was substituted by Br^-.

TABLE II

EFFECT OF DIFFERENT NUCLEOTIDES ON Na^+ UPTAKE IN BASOLATERAL MEMBRANE VESICLES

The vesicles were pre-incubated for 30 min in the usual medium, then the nucleotide (5 mM) was added and the incubation continued for a further 15 min Results are means ± S E. of three experiments

Nucleotide	Na^+ uptake (nmol/mg protein per 15 min)	
	Na^+	$Na^+ + K^+ +$ valinomycin
p[NH]ppA	41.1 ± 6.86	41.2 ± 6.08
ADP	43.2 ± 3.11	38.8 ± 7.39
ATP	86 9 ± 3.16	130.4 ± 10.84
GTP	40.6 ± 1 62	41.8 ± 5.12
CTP	40.8 ± 2.66	46.6 ± 4.26
ITP	40.0 ± 3.19	39.6 ± 5.14
UTP	38.5 ± 3.30	33 8 ± 6.84

Effect of different nucleotides on the two components of sodium transport

The stimulatory effect of ATP is specific for that nucleotide in the case of both components of transport (Table II). None of the other nucleotides tested was able to provoke a significant accumulation of sodium within the basolateral plasma membrane vesicles above the position of equilibrium.

Sodium uptake as a function of the external sodium concentration

A study of each component of sodium uptake into basolateral plasma membrane vesicles as a function of the external sodium concentration is illustrated in Figs. 4 and 5. Sodium uptake was

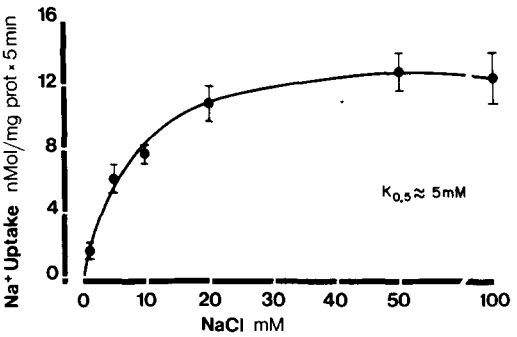

Fig 4. Relationship between the ATP-stimulated uptake of Na^+ through the Na^+-dependent mechanism and the sodium concentration of the incubation medium. The vesicles were preincubated for 30 min, then the nucleotide (5 mM) was added and the incubation continued for a further 5 min. The sodium concentration was varied by replacement with equivalent quantities of Tris-HCl. Results are means ± S.E of four experiments

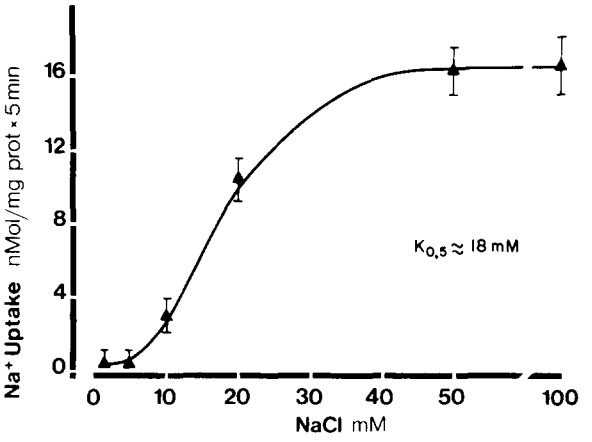

Fig 5 Relationship between the ATP-stimulated uptake of Na^+ through the $(Na^+ + K^+)$-dependent mechanism and the sodium concentration of the incubation medium The vesicles were preincubated for 30 min in the presence of valinomycin (10 μM), then the nucleotide (5 mM) was added and the incubation continued for a further 5 min The sodium concentration was varied by replacement with equivalent quantities of Tris-HCl Results are means ± S E. of four experiments

measured 5 min after addition of ATP or p[NH]ppA to an incubation medium in which the vesicles had been equilibrating during 30 min of preincubation. At each sodium concentration, passive uptake and binding was measured as described and subtracted from the ATP-dependent uptake values. Fig. 4 reveals that the Na^+-dependent component exhibits kinetics of the Michaelis-Menten type and reaches its maximum value at an external sodium concentration of 40–50 mM. By plotting the data according to Woolf transformation of the Michaelis-Menten equation an apparent $K_{0\,5}$ for Na^+ of 5 mM can be obtained. On the other hand, the $(Na^+ + K^+)$-dependent component gives a sigmoid curve as a function of external sodium concentration (Fig. 5), which also reaches its maximum at 50 mM Na^+. These data were evaluated by a Probit transformation, using the experimentally determined V_{max} (16.99 nmol/mg protein per 5 min) as the asymptote of the curve. Then a straight line with a correlation coefficient of 0.971 is obtained. The apparent $K_{0\,5}$ value for Na^+ was 18 mM.

Effect of pH on Mg^{2+}-ATP-dependent sodium uptake in basolateral plasma membrane vesicles

The pH dependency of the two components of ATP-stimulated sodium uptake into basolateral plasma membrane vesicles is illustrated in Fig. 6. The Na^+-dependent component exhibits a pH optimum close to 7.0 and is more sensitive to changes in pH than the $(Na^+ + K^+)$-dependent component whose pH optimum is at 7.2.

Effect of different inhibitors on the two components of sodium uptake in basolateral plasma membrane vesicles

The fact that our preparation contains 60% inside-out vesicles poses a problem for the exploration of the effects of ouabain, since the binding site of this agent is located within the vesicles. In order to ensure contact of the inhibitor with its membrane receptor, it is necessary to carry out the preparation of the vesicles with 1 mM ouabain in the homogenization medium throughout the preparative procedure. In this way, one can be certain that ouabain is present inside the vesicles at the time of the performance of the transport experiment. Results of two series of experiments, with ouabain either present only in the external medium or present on both sides of the membranes are shown in Table III; the other inhibitors, furosemide and ethacrynic acid, are only added to the

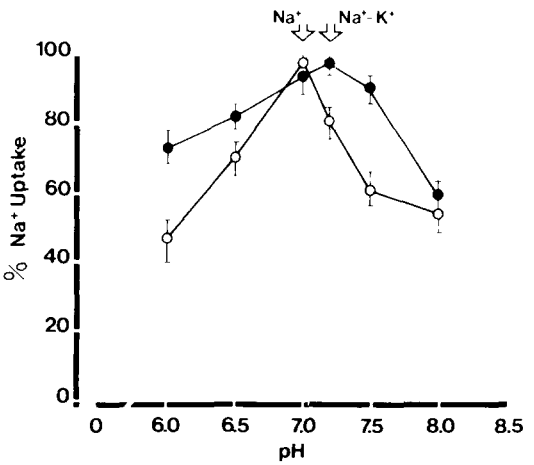

Fig. 6. Relationship between the two components of ATP-stimulated sodium uptake and the pH of the incubation medium The separate components were evaluated as shown in Figs. 4 and 5. The $(Na^+ + K^+)$-dependent component was obtained in the presence of valinomycin The various incubation media were obtained by using buffers of different Tris-HCl compositions. The results are means ± S.E. of three different experiments

TABLE III

EFFECTS OF VARIOUS INHIBITORS ON COMPONENTS OF Na^+ UPTAKE

Experiments were performed as in Fig 3, with or without inhibitors * In these conditions vesicles were prepared in the presence of 1 mM ouabain to insure that the inhibitor was present at the internal face of the vesicles The effects of furosemide (1.5 mM) and ethacrynic acid (1.5 mM) were tested. The values are means ± S E. of three experiments in each case Units in nmol/mg protein per 15 min

	Ouabain only outside	Δ	Ouabain * outside and inside	Δ	Ouabain * inside + furosemide outside	Δ	Ouabain * inside + ethacrynic acid outside	Δ
Na^+ + p[NH]ppA	35 3 ± 3.08	–	39 7 ± 1 91	–	39 5 ± 2 00	–	25 1 ± 2.40	–
Na^+ + ATP	74 5 ± 2 71	39 2	73.9 ± 2 88	34.2	58.9 ± 0 81	19 4	25 2 ± 1.51	0.1
Na^+ + K^+ + ATP + valinomycin	111 8 ± 9.29	37.3	70 3 ± 2.33	−3.6	57 4 ± 2.59	−1.5	24.5 ± 2 38	−0.7
	Control	Δ	Furosemide outside	Δ	Ethacrynic acid outside	Δ		
Na^+ + p[NH]ppA	36.8 ± 1 12	–	37 8 ± 2.88	–	26 5 ± 3.56	–		
Na^+ + ATP	72 7 ± 0 86	35 9	47 4 ± 2 08	9.6	27 5 ± 1 71	1 1		
Na^+ + K^+ + ATP + valinomycin	99 3 ± 4 97	26 6	75 5 ± 6.80	28 1	37.5 ± 1 13	10 0		

incubation medium, since their binding sites are presumed to be at external face of the vesicles, i.e. the cytoplasmatic side of the native membranes. As expected, ouabain has no effect on either component of sodium uptake when added only to the external medium; when it is present inside the vesicles, it inhibits the ($Na^+ + K^+$)-dependent component of the Mg^{2+}-ATP-dependent sodium uptake but has no influence on the Na^+-dependent component. On the other hand, addition of furosemide to the external medium reduces the Na^+-dependent component by 45–60% but does not affect the ($Na^+ + K^+$)-dependent one at all. Ethacrynic acid completely inhibits the Na^+-dependent component when its final concentration in the external medium is 1.5 mM, whilst it is only able to reduce the ($Na^+ + K^+$)-dependent component by 60% under the same conditions; it also partially inhibits the passive uptake. These data indicate that the two components of Mg^{2+}-ATP-dependent sodium uptake in basolateral plasma membrane vesicles can be separated functionally by their behaviour towards different inhibitors.

Discussion

Basolateral plasma membrane vesicles derived from epithelial cells of the guinea-pig small intestine have been shown to be capable no only of binding but also of accumulating sodium ions. The extent of binding has been evaluated in two ways, (a) by plotting sodium uptake against the inverse of the osmolarity of the incubation medium and by extrapolating the resulting straight line to infinite osmolarity, and (b) by incubating membrane sheets (opened vesicles) under identical conditions to those used with the vesicles. The two techniques give rather similar values. It is concluded that binding accounts for some 55% of the uptake observed at an osmolarity of 200 mosm/l. The rest (45%) corresponds to passive sodium entry. The values for passive uptake at equilibrium, following correction for the binding component, permit an estimation of the volume of the vesicles, for every mg of vesicular protein, the intravesicular volume is approx. 1 μl. This value agrees with that obtained earlier on the basis of equilibrium glucose uptake in the same vesicles, which was 1.2 μl/mg protein [22].

The transport experiments described in this paper were performed under equilibrium conditions, that is to say, after the vesicles had been equilibrated with the incubation medium contained ^{22}Na.

Following this equilibration procedure, it was possible to examine the effects of ATP and its

non-hydrolyzable analogue, p[NH]ppA, on the transport of sodium. The addition of the analogue never influence the uptake of sodium by the vesicular preparation. On the other hand, addition of ATP provoked an accumulation of sodium inside the vesicle to attain a level that was 2.5-times the equilibrium value (calculated from Fig. 3A after subtraction of a binding component of 55%). An additional component of sodium uptake was observed in the presence of magnesium, sodium, potassium and valinomycin, a potassium ionophore which allows a constant concentration of this cation to be maintained within the vesicle; under this conditions, the intravesicular sodium level reached a value that was 3.8-times that observed at equilibrium. The demonstration of an active uptake of sodium, induced by Mg^{2+}-ATP indicates that Na^+ influx is larger than Na^+ efflux. This indicates that the vesicular membrane remains relatively impermeable to sodium.

The stimulatory effect on sodium transport across the vesicular membrane is specific for ATP, as shown in Table II; no other nucleotide examined was able to elicit a significant enhancement of sodium uptake above the equilibrium level. The fact that both components of ATP-induced sodium uptake absolutely depend on the presence of magnesium ions in the incubations medium (Table I) suggests that the true substrate for the transport mechanisms is a Mg^{2+}-ATP complex, rather than ATP alone.

The two components of Mg^{2+}-ATP-stimulated sodium transport react differently to certain experimental situations, such as changes in external sodium concentration, alterations in pH or the presence of different inhibitors. Thus, both the Na^+-dependent and the $(Na^+ + K^+)$-dependent components, measured, respectively, in the presence of sodium alone or sodium, potassium and valinomycin, are saturable functions of the sodium concentration in the incubation medium; this is not the case for the passive component of sodium uptake. However, the Na^+-dependent component exhibits a Michaelis-Menten type relationship with an apparent $K_{0.5}$ of approximately 5 mM; but the $(Na^+ + K^+)$-dependent component reveals a sigmoid curve, from which an apparent $K_{0.5}$ of about 18 mM can be derived with the aid of a probit transformation. As far as the dependence of the transport mechanisms of pH is concerned, the Na^+-dependent component has its pH optimum at 7.0 and it is rather sensitive to changes in pH, whereas the $(Na^+ + K^+)$-dependent component has its pH optimum at 7 2 and is less sensitive to pH changes.

Finally, the two components differ in their sensitivities to some inhibitors. As shown in Table III, the $(Na^+ + K^+)$-dependent component is completely inhibited when ouabain is introduced into the intravesicular space, though this drug has no effect when added simply to incubation medium; the ouabain binding site is known to occur at the external face of native membranes and therefore at the interior of inside-out vesicles. In addition, ouabain never has any effect on the Na^+-dependent component of sodium transport. Conversely, the latter is inhibited to the extend of about 60% by furosemide which does not influence the $(Na^+ + K^+)$-dependent component. Lastly, ethacrynic acid completely inhibits the Na^+-dependent component but only partially inhibits the $(Na^+ + K^+)$-dependent component

The existence of a furosemide-sensitive component of sodium movement in the basolateral membrane vesicles opens the questions as to whether this process bears any relationship to the furosemide-sensitive Na^+-Cl^- symport which has been described in the basolateral plasma membrane of several epithelia [29]. This mechanism is believed to effect the uphill transport of chloride from the interstitial space into the cell, the movement being coupled to the entry of sodium down their electrochemical gradient; it is though to play a central role in the secretion of ions across the epithelia from the serosal to the mucosal side of the cell. To our knowledge, its existence in the basolateral plasma membrane of the enterocyte has not yet been demonstrated experimentally, though it has often been inferred in discussions on intestinal secretion mechanisms [29]. There are two compelling reasons to negate any connection between this hypothetical symport and our Na^+-dependent component of Mg^{2+}-ATP-induced sodium uptake. First, the symport is believed to be responsible for movement towards the interior of the cell, whereas our mechanism is involved in sodium extrusion from the cell; and secondly, the symport mechanism is supposed to be activated by

the sodium electrochemical gradient across the basolateral plasma membrane, which does not exist in our experiments at the moment in which ATP is added. The symport mechanism is thought also be independent of ATP hydrolysis. Our mechanism is specifically stimulated by ATP. Nevertheless, we performed a series of experiments on sodium uptake in the absence of chloride; since the mechanism proceeded normally when all chloride ions were excluded it is clear that any relationship between our sodium extrusion mechanism and the hypothetical sodium-chloride symport is excluded. If both exist and are sensitive of furosemide, this is purely fortuitous.

Another possibility is whether the two systems are indeed present in the same vesicles or whether they are present in vesicles derived from different cells. On this regard, vesicles prepared from isolated villus cells showed similar results to those reported in this paper. Remains to be determined if there is any difference between jejunum and ileum.

On the basis of our experimental results, we are in position to define clearly two modes of sodium transport across basolateral plasma membrane vesicles derived from small intestinal epithelium. Both components of transport are active, since sodium is accumulated within the vesicles above its equilibrium concentration. The source of energy for these active transport mechanisms is ATP, since is the only nucleotide capable of stimulating sodium uptake, but the substrate for the reaction in both cases is Mg^{2+}-ATP. The similarities between the two components end there: their ionic requirements, their kinetic behaviour with respect to sodium ions, their pH optima and their sensitivities to different inhibitors are different.

The characteristics of the two components of sodium transport in basolateral plasma membranes of enterocytes correspond closely to those described for the sodium-pumping mechanism of renal proximal cell [30]. One is responsible for the transport of sodium accompanied by chloride and water, whereas the other exchanges sodium for potassium. The former is insensitive to ouabain, but inhibited by ethacrynic acid, furosemide and triflocin, whereas the latter is totally inhibited by ouabain but is refractory to furosemide and is only partially inhibited by ethacrynic acid. These marked similarities lead us to propose for the small intestine a similar model for sodium pumping as that developed for the proximal tubules of the kidney, namely two ATP-dependent mechanisms in parallel in the same membrane. The existence of two parallel pumps in the intestine could explain the lack of correlation between the movement of sodium and potassium observed experimentaly when an organic solute is transported [31], and the lack of dependence of cell volume on the sodium/potassium pumping mechanism sensitive to ouabain [7].

Recently, Boumendil-Povedin and Povedin [32] have studied Na^+ transport in inside-out basolateral plasma membrane vesicles isolated from rabbit kidney cortex. They demonstrated an ATP-dependent Na^+ uptake in K^+-loaded vesicles, sensitive to internal ouabain. However, they failed to show Na^+ uptake in absence of potassium. This difference with our results may be due to their utilization of EDTA (1 mM) in the homogenization medium, which inhibits the Na^+-ATPase [33] and probably the mechanism of Na^+ uptake independent of potassium.

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References

1 Schultz, S.G (1977) Yale J. Biol. Med. 50, 99–113
2 Dibona, D.R and Mills, J.W. (1979) Fed. Proc 38, 134–143
3 Stirling, C.E (1972) J. Cell Biol. 53, 704–714
4 Ernst, S.A. and Hootman, S.R. (1981) Histochem. J. 13, 397–418
5 Kinne, R. and Kinne-Saffran, E.M. (1978) in Molecular specialization and symmetry in membrane function (Solomon, A.K. and Karnovsky, M., eds.), pp. 272–293, Harvard University Press, Cambridge
6 Quigley, J.P. and Gotterer, G.S (1972) Biochim. Biophys. Acta 255, 107–113
7 Nellans, H.N. and Schultz, S.G. (1976) J. Gen. Physiol. 68, 441–463
8 Schultz, S.G. and Curran, P.F (1970) Physiol. Rev. 50, 637–718
9 Lee, C.O and Armstrong, W McD (1972) Science 175, 1261–1264

10 Sepúlveda, F V , Burton, K A and Brown, P D (1982) J Cell. Physiol 111, 303–308
11 Candia, O.A. and Zadunaisky, J A (1972) Biochim. Biophys. Acta 255, 517–529
12 Essig, A. (1965) Am. J Physiol 208, 401–406
13 MacKnight, A.D C , Civan, M and Leaf, A (1975) J. Membrane Biol 20, 365–386
14 Robinson, B.A. and MacKnight, A.D.C. (1976) J Membrane Biol. 26, 217–238
15 Giebisch, G , Sullivan, L P and Whittembury, G (1973) J. Physiol (London) 230, 51–74
16 Whittembury, G and Proverbio, F (1970) Pflugers Arch 316, 1–25
17 Proverbio, F , Robinson, J W L. and Whittembury, G (1970) Biochim Biophys. Acta 211, 327–336
18 Proverbio, F. and Whittembury, G (1975) J Physiol (London) 250, 559–578
19 Proverbio, F., Condrescu-Guidi, M and Whittembury, G (1975) Biochim. Biophys Acta 394, 281–292
20 Proverbio, F and Del Castillo, J.R. (1981) Biochim Biophys. Acta 646, 99–108
21 Del Castillo, J R , Marin, R., Proverbio, T and Proverbio, F (1982) Biochim Biophys. Acta 692, 61–68
22 Del Castillo, J R and Robinson, J.W.L. (1982) Biochim Biophys Acta 688, 45–56
23 Lowry, O H., Rosebrough, N J , Farr, A L and Randall, R J (1951) J. Biol Chem 193, 265–275
24 Bradford, M M (1976) Anal. Biochem 72, 248–254
25 Gadd, K G (1981) Med Lab Sci 38, 61–63
26 Terland, O , Flatmark, T and Kryvi, H (1979) Biochim Biophys Acta 553, 460–468
27 Gasko, O D , Knowles, A F , Shertzer, H G , Soulina, E -M and Racker, E (1976) Anal Biochem 72, 57–65
28 Karlish, S J D and Pick, U (1981) J Physiol (London) 312, 505–529
29 Frizzell, R A and Schultz, S G (1979) in International Review of Physiology, Gastrointestinal Physiology (Crane, R K , ed), Vol 19, pp 205–225, University Park Press, Baltimore
30 Proverbio, F , Del Castillo, J R , Marin, 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
31 Schultz, S G (1978) in Membrane transport processes (Hoffman, J F., ed), Vol 1, pp 213–227, Raven Press, New York
32 Boumendil-Povedin, E F and Povedin, R A (1983) Biochim Biophys Acta 728, 39–49
33 Proverbio, F , Proverbio, T and Marin, R (1982) Biochim Biophys Acta 688, 757–763