Biochimica et Biophysica Acta 814 (1985) 363–373
Elsevier

BBA 72523

Active sodium transport in basolateral plasma membrane vesicles from rat kidney proximal tubular cells

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(Received October 3rd, 1984)

Key words: Na^+ transport; Epithelial transport; K^+ dependence; Ouabain inhibition; (Rat kidney)

Inside-out vesicles prepared with basolateral plasma membranes from rat kidney proximal tubular cells can accumulate Na^+ actively in two ways. Mode 1, which is K^+-independent, is ouabain-insensitive and is inhibited by furosemide and mode 2, which is K^+-dependent, is inhibited by ouabain and is insensitive to furosemide. The presence of Mg^{2+} and ATP in the incubation medium is essential for both modes of Na^+ uptake to proceed and in both cases, the nucleotide is hydrolyzed during the process. These results are consistent with the idea of the existence, in these membranes, of two Na^+ pumps: one, which can work in the absence of K^+ (Na^+ pump) and another, which needs K^+ to work ($Na^+ + K^+$ pump).

Introduction

Cells of mammalian renal cortex of several species, when incubated in a cold medium without K^+, gain Na^+, Cl^- and water and lose K^+. If after the cooling period, the tissue is rewarmed in a medium with K^+, the cells extrude the gained Na^+ in two ways: (1) Na^+ extruded accompanied by Cl^- and water and (2) Na^+ extruded in exchange for K^+ [1–4]. Mode 1 does not need K^+ in the external medium, is insensitive to ouabain and is totally inhibited by ethacrynic acid [5,7], furosemide or triflocin [6,7]. Mode 2 needs K^+ in the external medium, is totally inhibited by ouabain, is only partially inhibited by ethacrynic acid and is insensitive to furosemide and triflocin [5–7].

Two Na^+-stimulated ATPase activities have been described to be present in renal tissue [8,9]. One activity (($Na^+ + K^+$)-ATPase)), is stimulated by $Na^+ + K^+$, is totally inhibited by ouabain, is only partially inhibited by ethacrynic acid and is practically insensitive to furosemide and triflocin. The other activity (Na^+-ATPase), is stimulated by Na^+, is insensitive to ouabain and is totally inhibited by ethacrynic acid, furosemide and triflocin. The Na^+- and the ($Na^+ + K^+$)-ATPase activities have different affinities for their ligands and different optimal pH and temperature values. Both ATPase systems were shown to be present in basolateral plasma membranes from guinea-pig and rat kidney proximal tubular cells [10,11].

The parallelism between the characteristics of the two ways of Na^+ extrusion and the two Na^+-stimulated ATPase activities has been considered to be the expression of two different active mechanisms involved in the control of the Na^+ extrusion across the basolateral plasma membrane of proximal tubular cells [8–11]. However, considering the limitations of the slice preparations, the existence of the two Na^+ extrusion cellular mechanisms is still a controversial point.

In this work, another approach was utilized to obtain further evidence. The Na^+ uptake and its energy requirement were studied in inside-out

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vesicles, prepared with basolateral plasma membranes from rat kidney tubular cells.

It was found that the vesicles, similar to the slices, can move Na^+ actively in two ways. One way needs K^+ to work, is inhibited by ouabain (inside the vesicles) and is not inhibited by furosemide. The other way does not need K^+ to work, is insensitive to ouabain (inside or outside the vesicles) and is totally inhibited by furosemide. Paralleling the Na^+ movements, there is a concomitant hydrolysis of ATP, which is necessary for the two ways of Na^+ movement to proceed.

Materials and Methods

Preparation of fractions enriched in basolateral plasma membranes. Healthy male Sprague-Dawley rats of 3 months of age were anesthetized with ether and killed by cervical dislocation. The kidneys were immediately removed, decapsulated and collected in a medium containing 150 mM sucrose/50 mM NaCl/20 mM Tris-HCl (pH 7.2) (sucrose/Na^+/Tris medium), at 4°C. Outermost slices of the kidney cortex (which is rich in proximal tubules) were homogenized and centrifuged at 4°C as previously described [8,10,11]. Briefly: the slices were homogenized at 4°C, with eight strokes at 2500 rpm in an Eberbach homogenizer with a Teflon pestle, in 3 vol./g of tissue of sucrose/Na^+/Tris medium. The homogenate (usually 240 mg protein) was filtered through two sheets of gauze to remove clumps and spun at $1475 \times g$ for 10 min. The supernatant was discarded. The pellet was resuspended in 3 vol. of 1.9 M sucrose/50 mM NaCl and rehomogenized with two strokes in the same homogenizer used before. The resuspension was spun at $13\,300 \times g$ for 10 min. The pellet was discarded and the supernatant was taken back to its original tonicity with 50 mM NaCl cold solution and then spun at $35\,000 \times g$ for 15 min. The supernatant and the lower pellet (brown layer) were discarded and the upper pellet (pink layer) was resuspended in enough quantity of sucrose/Na^+/Tris medium, to give about 2 mg protein/ml of final suspension. 3 vol. of the sucrose/Na^+/Tris solution were added to this final suspension and then centrifuged at $48\,000 \times g$ for 1 min. The resulting supernatant was discarded and the pellet (P_{3c}) was resuspended in the same medium, to give a protein concentration of about 3–4 mg/ml. The preparations were immediately used for the experiments.

Enzymatic assays and [^{3}H]ouabain binding. Before the enzymatic assays, the fractions were treated with 0.06% deoxycholate/2 mM EDTA, for 30 min at room temperature, according to the method of Jørgensen and Skou [12], in order to disrupt the vesicles formed during the membrane extraction procedure. In previous esxperiments, it was found that for this tissue, the deoxycholate/EDTA treatment is able to disrupt totally any vesicle present in the preparation.

The following methods were used for the enzymatic assays: acid phosphatase (EC 3.1.3.2) was determined using β-glycerophosphate at pH 5.4 as substrate, according to Hübscher and West [13]; 5′-nucleotidase (EC 3.1.3.5) was determined with 5′-AMP as substrate at pH 8.5 according to the method of Heppel and Hilmoe [14]. Glucose-6-phosphatase (EC 3.1.3.9) was determined in the presence of 4 mM EDTA and 2 mM potassium fluoride to inhibit the nonspecific phosphatase [14]. Succinate dehydrogenase (EC 1.3.99.1) was determined following the succinate-dependent reduction of potassium ferricyanide according to the method of King [15]. The ATPase activities (EC 3.6.1.3) were determined with Na^+-free Tris-ATP (2 mM final concentration) as substrate, at pH 7.2, according to the method already described [10,11]. The Mg^{2+}-ATPase is referred as the ATPase activity measured in the presence of Mg^{2+} alone in the incubation medium. The Na^+-ATPase and the ($Na^+ + K^+$)-ATPase are referred as the difference in activity of ($Mg^{2+} + Na^+$)-ATPase minus Mg^{2+}-ATPase and the difference in activity of ($Mg^{2+} + Na^+ + K^+$)-ATPase minus ($Mg^{2+} + Na^+$)-ATPase, respectively. The liberated phosphate was determined in the deproteinized solution [16]. The protein content of the original suspensions was measured by means of the Folin reagent [17]. All samples were run in triplicate or quadruplicate. In some experiments, the ($Na^+ + K^+$)-ATPase activity was determined in membranes treated with valinomycin. The treatment consisted of preincubation of the membranes with 6.25 μg valinomycin/mg protein and 20 mM KCl, for 10 min, at room temperature. The incorporation of [^{3}H]ouabain was carried out following the

method developed by Tobin and Sen [18].

Na^+ uptake by the vesicles. The vesicles (about 40 μg total protein) were preincubated for 2 h at 0°C in the different media in which they were going to be incubated, to allow the ^{24}Na to reach equilibrium. The media were complete, except for Mg^{2+}, which was added later, to start the incubation. Control tubes were run in the absence of Mg^{2+} and with 0.5 mM EDTA. The experimental medium contained (mM): $MgCl_2$, 5; NaCl, 50, with 50 μCi/ml ^{24}Na; Tris-HCl (pH 7.2), 10; sucrose, in enough quantity to obtain an osmolarity of 300 mosM; ATP, 5 and, according to the experimental design: KCl, 20; ouabain, 7; furosemide, 2; valinomycin, 6.25 μg/mg protein. The incubations were carried out at 37°C over different periods of time. The final volume of the reaction was 120 μl. Each point was run in triplicate. At the end of the experimental time, 100 μl of the incubation mixture were passed through a Dowex 50W-X8 column, in order to separate the vesicles from the incubation medium. Immediately, the column was washed with 3 ml of cold stop solution containing (mM): sucrose, 250; $MgCl_2$, 5; Tris-HCl (pH 7.2), 20. The eluates were collected in counting vials and the radioactivity was determined. The Na^+ uptake by the vesicles is expressed in nmol/mg protein. The columns were prepared as follows [19,20]: the ion exchange resin, Dowex 50W-X8 in the H^+ form, was transformed to $Tris^+$ form by the addition of 1 M Tris-base until a basic pH was attained and then it was washed with distilled water until reaching a pH value of 7.2. The columns were prepared introducing 7–8 cm of Dowex resin ($Tris^+$ form) into Pasteur pipettes (5 and 3/4 inch long), at the bottoms of which small glass-fiber plugs were placed. Before the assays, 100 μl of 10% bovine serum albumin were added to the columns, which were then rinsed with 3 ml of cold stop solution. Control experiments showed that, under these conditions, practically 100% of the Na^+ is retained by the resin, while about 98% of the proteins pass through the columns.

ATP hydrolyzed by the vesicles during Na^+ uptake. Vesicle suspensions (about 200 μg total protein) were added to the different incubation media utilized to study Na^+ uptake, which were already at 37°C. The osmolarity of the media was adjusted, in each case, with sucrose. The final volume of the reaction was 600 μl and, at the end of the incubation periods, 1 ml of ice-cold $HClO_4$ was added to the incubation tubes. The liberated phosphate was determined in the deproteinized solution according to the method of King [16]. Each point was run in triplicate. The ATP hydrolyzed is expressed as nmol of phosphorus liberated during the incubation time per mg of protein. In order to determine the hydrolysis of ATP that is not associated with the Na^+ uptake by the vesicles (Mg^{2+}-ATPase), parallel experiments were run without adding Na^+ to the incubation medium and in the presence of 2 mM furosemide. This agent inhibits totally the Na^+-ATPase without affecting the Mg^{2+}-ATPase nor the $(Na^+ + K^+)$-ATPase [10,11]. Its presence in these experiments was necessary to inhibit any expression of the Na^+-ATPase produced by the Na^+ present in the vesicle preparations.

Chemicals. ATP, ouabain (strophanthin-G), β-glycerophosphate, glucose 6-phosphate, AMP, potassium ferricyanide, potassium fluoride, deoxycholate, EDTA, Dowex 50W-X8 resin and valinomycin, were purchased from the Sigma Chemical Company, St. Louis, MO, U.S.A.; furosemide was generously provided by Medicamentos York S.A. (Caracas); [^{3}H]ouabain and Instagel, were purchased from New England Nuclear, Boston, MA, U.S.A.; $^{24}Na^+$ (as $NaNO_3$) was provided by Reactor Venezolano I, IVIC, Caracas.

Results

Basolateral plasma-membrane-enriched fractions

Fig. 1 shows the enzymatic content of P_{3c} fractions as related to the initial homogenate. Notice that the ouabain-sensitive, $(Na^+ + K^+)$-ATPase, a recognized marker for basolateral plasma membranes [21,22] and the ouabain-insensitive, Na^+-ATPase, shown to be also associated with these membranes [10,11], are enriched in this fraction by a factor of 4.7, with a recovery of 16%. All the other tested enzymatic markers have a specific activity similar or lower than the specific activity of the homogenate, and their recoveries are very low in this fraction. Consequently, P_{3c} is a fraction enriched in basolateral plasma membranes with a low contamination of brush-border membranes

(5′-nucleotidase [23]); endoplasmic reticulum membranes (glucose-6-phosphatase [24,25]); mitochondrial membranes (succinate dehydrogenase [26]) and lysosomal membranes (acid phosphatase [24]). All these enzymes were enriched in some other fractions and the recovery for all the tested enzymes was near 100%. The degree of enrichment of the Mg^{2+}-ATPase activity is probably due to the presence of contaminants from mitochondrial and luminal membranes.

Determination of vesicles and their sideness

All the preparations were tested for the presence of vesicles by determining their $(Na^+ + K^+)$-ATPase activity before and after treating the membranes with 0.06% deoxycholate/2 mM EDTA at 23°C, according to the method of Jørgensen and Skou [12]. This approach is based on the fact that the $(Na^+ + K^+)$-ATPase is assymetric. In order to work, it needs to see Mg^{2+}, Na^+ and ATP at the internal side of the cell membrane and K^+ at the external side. In the presence of vesicles, the access to the sites of one or more of the ligands becomes restricted or impossible. Consequently, there will be a low $(Na^+ + K^+)$-ATPase activity in the case of inside-out vesicles, since K^+ must diffuse into them in order to reach its site unless the vesicles are treated with valinomycin or another K^+ ionophore. On the other hand, the right-side-out vesicles will not show any $(Na^+ + K^+)$-ATPase activity, since ATP is not able to diffuse through the cell membranes. The deoxycholate/EDTA treatment, by rupturing the vesicles, provides free access of all of the ligands to their sites, permitting the $(Na^+ + K^+)$-ATPase activity, to express at its maximal rate. The effect of the treatment of the preparations with deoxycholate/EDTA or with valinomycin, on the $(Na^+ + K^+)$-ATPase activity, is shown in Table I. It can be seen that the activity increases upon treatment with deoxycholate/EDTA from 41 ± 11 to 448 ± 39 nmol P_i/mg protein per min. The increase of 407 nmol P_i/mg protein per min represents 91% of 448 nmol P_i/mg protein per min, which is considered as the maximal activity. This 91% represents approximately the total percentage of vesicles. The percentage of inside-out vesicles was estimated from the results with valinomycin, shown in Table I. The $(Na^+ + K^+)$-ATPase activity increases with the treatment in 314 nmol P_i/mg protein per min. This number

TABLE I

EFFECT OF THE TREATMENT WITH DEOXYCHOLATE/EDTA OR VALINOMYCIN ON THE $(Na^+ + K^+)$-ATPase ACTIVITY OF P_{3c} FRACTIONS

The deoxycholate/EDTA and valinomycin treatments were carried out as indicated under Materials and Methods. Values expressed as mean ± S.E. ($n = 6$).

Treatment	$(Na^+ + K^+)$-ATPase activity (nmol P_i/mg protein per min)	Increment	% of the maximal activity
Control	41 ± 11	–	–
Deoxycholate/EDTA	448 ± 39	407	91
Valinomycin	355 ± 27	314	70

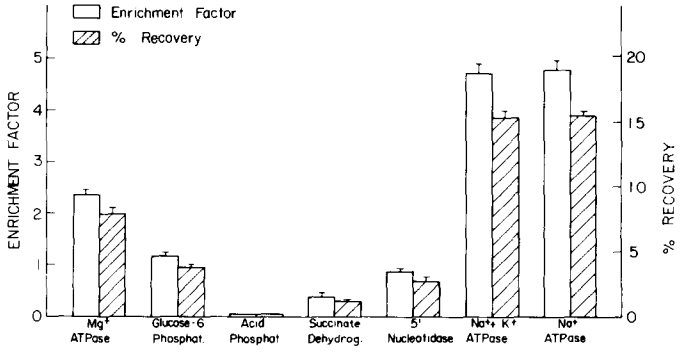

Fig. 1. Enrichment factor (specific activity of each enzyme in fraction P_{3c}/specific activity in homogenate) (white columns), and percentage of recovery in fraction P_{3c} of the different enzyme markers referred to the homogenate (hatched columns). Values expressed as the mean ± S.E. ($n = 6$).

represents 70% of the maximal activity obtained with the treatment with deoxycholate/EDTA. In other words, in our preparations there is about 70% of inside-out vesicles. The difference between the percentage of total vesicles (91%) minus the percentage of inside-out vesicles (70%) gives an estimate of the percentage of right-side-out vesicles, which is 21%.

The percentage of total vesicles was confirmed by [^{3}H]ouabain-binding experiments. Ouabain does not bind at the internal side of the membranes, and under our experimental conditions, ouabain binds at the external side of the membranes if ATP is present at their internal side [27]. Accordingly, ouabain should not bind the sealed vesicles regardless of their orientation. If the vesicles are ruptured with the deoxycholate/EDTA treatment, the [^{3}H]ouabain binding would be maximal. In fact, treatment with deoxycholate/EDTA elicited the membranes to increase their specific [^{3}H]ouabain binding. This increment represents approx. 72–80% of the maximal binding. This number gives an estimate of the percentage of total vesicles. This percentage is somehow lower than the percentage calculated from the ATPase experiments (91%). This difference could be due to some ouabain binding to right-side-out vesicles.

The size of the vesicles determined by electron microscopy, is between 0.1 and 0.2 μm. The intravesicular volume, estimated from passive uptake values at equilibrium is approx. 1.5 μl/mg protein, a value similar to that already reported of 2 μl/mg protein [28].

Binding of Na^+ to the vesicles

Vesicle preparations were incubated for 30 min at 37°C in a medium containing 50 mM NaCl with 50 μCi/ml ^{24}Na, 10 mM Tris-HCl (pH 7.2) and sucrose in enough quantity to achieve the different tested osmolarities. As shown in Fig. 2, the Na^+ content of the vesicles was inversely related to the osmolarity of the medium. The binding of Na^+ to the vesicles was estimated by extrapolating the line to infinite osmolarity. It was found to be 8.7 ± 0.5 nmol Na^+/mg protein. This value was corroborated by measuring the binding of Na^+ to vesicle preparations treated with deoxycholate/EDTA. The quantity of Na^+ bound to the membrane sheets was 9.5 ± 0.9 nmol/mg protein. The uptake values were all corrected for this number.

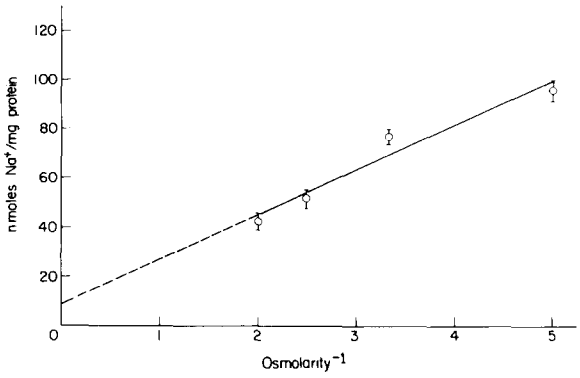

Fig. 2. Na^+ incorporation into basolateral plasma membrane vesicles as a function of the osmolarity of the incubation medium. The vesicles were incubated for 30 min at 37°C in the following medium (mM): NaCl, 50, with 50 μCi/ml ^{24}Na; Tris-HCl (pH 7.2), 10; sucrose, in enough quantity to obtain the different tested osmolarities. Values expressed as mean ± S.E. ($n = 10$).

Uptake of Na^+ by the vesicles

The vesicles were preincubated (to achieve equilibrium) for 2 h at 0°C in the different media, which were complete except for Mg^{2+}, which was added later to start the incubations. It was found previously that, in the absence of ATP, the addition of Mg^{2+} and/or K^+ plus valinomycin to the medium, did not have any effect on the Na^+ content of the vesicles. On the other hand, it was also found that the presence of Mg^{2+} was essential for ATP to produce the effects shown below. Accordingly, the experiments were run in the presence of: Na^+ + ATP; Na^+ + ATP + Mg^{2+} or Na^+ + K^+ + ATP + Mg^{2+} + valinomycin. The results of these experiments are shown in Fig. 3. At the indicated times, aliquots were taken from the incubation tubes and passed through the columns. The eluates were assayed for radioactivity. In all the cases, the radioactivity inside the vesicles was higher for the vesicles incubated with Na^+ + ATP + Mg^{2+} than for the vesicles incubated with Na^+ + ATP, which maintained, for all the experimental time, the same quantity of radioactivity. The difference between these two conditions is indicated as ΔNa^+. A further increment of radioactivity was found when the vesicles were incubated with Na^+

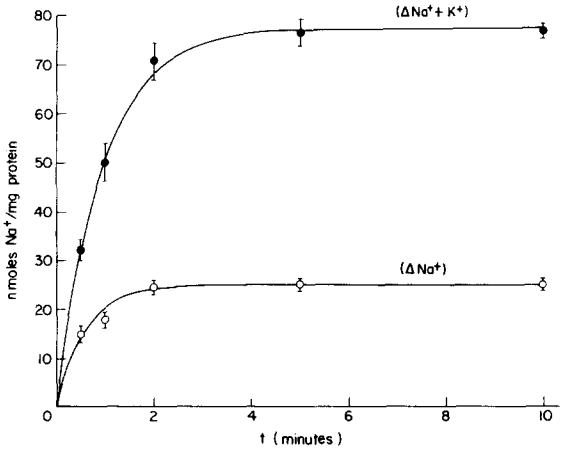

Fig. 3. Na^+ uptake by basolateral plasma membrane vesicles as a function of time. The vesicles were preincubated for 2 h at 0°C in the different media, without Mg^{2+}, which was added to start the incubation. The incubations were carried out at 37°C. ΔNa^+ indicates the difference in radioactivity inside the vesicles when incubated with $Na^+ + ATP + Mg^{2+}$ and $Na^+ + ATP$. $\Delta Na^+ + K^+$ indicates the difference between the vesicles incubated with $Na^+ + ATP + Mg^{2+} + K^+ +$ valinomycin and $Na^+ + ATP + Mg^{2+}$. The concentration of the different substances was (mM): Tris-HCl (pH 7.2), 10; NaCl, 50, with 50 μCi/ml ^{24}Na; $MgCl_2$, 5; KCl, 20; ATP, 5; valinomycin, 6.25 μg/mg protein; sucrose in enough quantity to obtain an osmolarity of 300 mosM. The osmolarity inside the vesicles was 300 mosM. Values expressed as mean ± S.E. ($n = 10$).

$+ ATP + Mg^{2+} + K^+ +$ valinomycin. The difference in radioactivity between the vesicles incubated under these conditions minus the radioactivity present in the vesicles incubated with $Na^+ + ATP + Mg^{2+}$, is referred as $\Delta Na^+ + K^+$. From the data shown in Fig. 3, the initial $Mg^{2+} + ATP$-dependent uptake of Na^+ by the vesicles was calculated. The ΔNa^+ (uptake of Na^+ in the absence of K^+) is 52 ± 4 nmol Na^+/mg protein per min. The $\Delta Na^+ + K^+$ (uptake of Na^+ dependent on K^+) is 99 ± 4.

ΔNa^+ and $\Delta Na^+ + K^+$ reach their plateau after 2 min of incubation, but ΔNa^+ comes only up to about 1/3 of $\Delta Na^+ + K^+$. It seems evident that after a given time and with an identical leak for Na^+ over the membrane, the amount of incorporated Na^+ should be the same although reached with a different time course. This is not the case for our preparation. A possible explanation of this fact could be that the driving forces for the Na^+ leak are different. The $Na^+ + K^+$ pump ($\Delta Na^+ + K^+$) moves Na^+ in exchange for K^+, concentrating Na^+ inside the vesicles. The Na^+ pump (ΔNa^+), as described for the kidney slices, would move Na^+ inside the vesicles with Cl^- and water, increasing the hydrostatic pressure inside the vesicles. The driving forces for the leak would then be different: Na^+ concentration in the first case and hydrostatic pressure in the second. These results can also be explained considering the presence of different membrane vesicle populations in the preparation. However, this explanation is difficult to sustain. We can show Na^+ movement in the absence of K^+, only in preparations where we can demonstrate Na^+-ATPase activity. On the other hand, the distribution of the Na^+-ATPase, always parallels the distribution of the ($Na^+ + K^+$)-ATPase in all the fractions obtained during the preparation procedure. This is an indication that both ATPases are associated to the same membrane: basolateral plasma membrane. Basolateral plasma membranes from different cell populations could also explain these results. However, the kidney cortex slices are very rich in proximal tubules [5]. Accordingly, the contribution of membrane vesicles from other populations must be very small and, consequently, not enough to explain the shown results.

Effect of ouabain and furosemide on the Na^+ uptake by the vesicles

Similar Na^+ uptake experiments were carried out in the presence of 2 mM furosemide or 7 mM ouabain. The vesicles were incubated for 2 min and then passed through the columns. Vesicles containing ouabain were obtained by carrying out all the membrane purification procedures in the presence of 7 mM ouabain. The results of these experiments are shown in Table II. Furosemide, added to the incubation medium, inhibits practically all the ΔNa^+, but does not affect the $\Delta Na^+ + K^+$. On the other hand, ouabain inside the vesicles, inhibits totally the $\Delta Na^+ + K^+$, leaving unaffected the ΔNa^+. Ouabain in the incubation medium, does not affect either the ΔNa^+ or the $\Delta Na^+ + K^+$. When the vesicles were prepared with ouabain and incubated in the presence of furosemide, the ΔNa^+ and the $\Delta Na^+ + K^+$ were completely inhibited (data not shown).

TABLE II

EFFECT OF OUABAIN OR FUROSEMIDE ON THE Na^+ UPTAKE BY THE VESICLES

The vesicles were incubated for 2 min under the same conditions indicated in Fig. 2, in the presence or absence of 7 mM ouabain (inside the vesicles) or 2 mM furosemide (outside the vesicles). Values expressed as mean ± S.E. ($n = 10$).

Na^+ uptake	Na^+ uptake (nmol Na^+/mg protein per 2 min)		
Incubation conditions:	no inhibitors or ouabain outside the vesicles	furosemide in the incubation medium	ouabain inside the vesicles
In the absence of K^+ (ΔNa^+)	28 ± 2	4 ± 4	25 ± 3
In the presence of K^+ + valinomycin	94 ± 5	67 ± 7	31 ± 1
($\Delta Na^+ + K^+$)	66 ± 5	63 ± 8	6 ± 3

Hydrolysis of ATP during the Na^+ uptake by the vesicles

If the ΔNa^+ and the $\Delta Na^+ + K^+$ are the expressions of active transport processes, they need energy to proceed. Since the presence of ATP in the incubation medium is essential for both ways of Na^+ uptake to occur, the hydrolysis of this nucleotide was studied under the same conditions utilized to determine the Na^+ uptake. The difference in ATP hydrolyzed by the vesicles when incubated with $Mg^{2+} + ATP + Na^+$ minus the value obtained when incubated with $Mg^{2+} + ATP$ + 2 mM furosemide will be referred to as Na^+-ATPase. On the other hand, the difference obtained when the vesicles are incubated with Mg^{2+} + ATP + Na^+ + ouabain + valinomycin, in the presence or absence of K^+, will be referred to as ($Na^+ + K^+$)-ATPase. Fig. 4 shows the time course of the two ATPase activities. In both cases, there is a linear relationship between the ATP hydrolyzed and the incubation time, with slopes of 29 ± 1 nmol P_i/mg protein per min for the Na^+-ATPase and of 59 ± 1 for the ($Na^+ + K^+$)-ATPase. It must be considered that the ATP concentration of the media could be drastically lowered during the Na + uptake experiments, masking the results. However, the total ATP hydrolyzed during the Na^+ uptake experiments, after 5 min of incubation, is about 0.09 μmol. The quantity of ATP present in the medium is 0.6 μmol.

The ($Na^+ + K^+$)-ATPase activity measured during the Na^+ uptake experiments, is about 8-times lower than the value obtained with membrane sheets. This difference may be due to several factors: (a) The Na^+ uptake experiments were done in the presence of ouabain in the external medium, which inhibits the ($Na^+ + K^+$)-ATPase activity of membrane sheets and of vesicles that may be disrupted during the incubation. (b) The activity of the ($Na^+ + K^+$)-ATPase may be limited

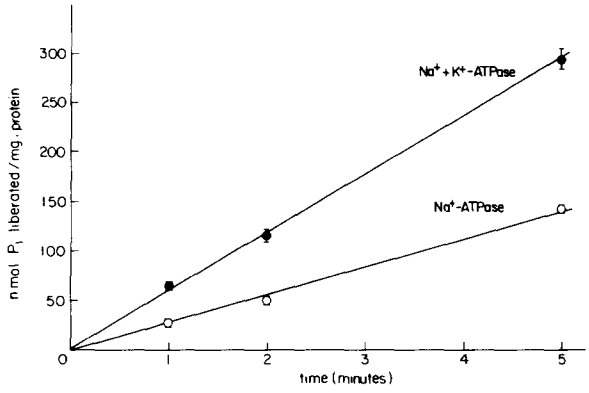

Fig. 4. Hydrolysis of ATP by the vesicles during Na^+ uptake. The vesicles were incubated at 37°C, for the indicated times, in the same media utilized to study Na^+ uptake. Parallel experiments were run without adding Na^+ to the incubation medium and in the presence of 2 mM furosemide, to determine the Mg^{2+}-ATPase. All the experiments were run in the presence of 7 mM ouabain. 'Na^+-ATPase' indicates the difference in ATP hydrolyzed in the presence of $Mg^{2+} + ATP + Na^+$ and Mg^{2+} + ATP + furosemide. '($Na^+ + K^+$)-ATPase' indicates the difference in ATP hydrolyzed in the presence of $Mg^{2+} + ATP + Na^+$ + valinomycin, in the presence or absence of K^+ in the incubation medium. Values expressed as the mean ± S.E. (n = 10).

TABLE III

EFFECT OF OUABAIN OR FUROSEMIDE ON THE ATP HYDROLYZED BY THE VESICLES DURING Na^+ UPTAKE

The vesicles were incubated for 2 min under the same conditions indicated in Fig. 3, in the presence or absence of 7 mM ouabain (inside the vesicles) or 2 mM furosemide (outside the vesicles). 7 mM ouabain was always present in the incubation media. Values expressed as mean ± S.E. ($n = 8$).

ATPase	ATPase activity (nmol P_i liberated/mg protein per min)		
Incubation conditions:	no inhibitors or ouabain outside the vesicles	furosemide in the incubation medium	ouabain inside the vesicles
In the absence of K^+ (ΔNa^+)	26 ± 1	2 ± 3	28 ± 1
In the presence of K^+ + valinomycin	83 ± 3	62 ± 3	34 ± 3
($\Delta Na^+ + K^+$)	57 ± 3	60 ± 4	6 ± 3

by the availability of K^+ inside the vesicles, which is not the case for membrane sheets. (c) The protein concentration used for the vesicle experiments is not optimal for the ATPase activity. In this respect, we found that membrane sheets, at the same concentration of proteins used in the vesicle experiments (333 μg/ml medium), show a ($Na^+ + K^+$)-ATPase activity which is only 40% of the activity that they show at optimal protein concentrations in the incubation medium (25–100 μg/ml medium).

In another set of experiments, the vesicle preparations were used to determine the hydrolysis of ATP associated to the two modes of Na^+ uptake, but in the presence of furosemide in the incubation medium or ouabain inside the vesicles. The results are shown in Table III. As for the Na^+ uptake, the hydrolysis of ATP associated to it showed a differential sensitivity to furosemide and to ouabain. The Na^+-ATPase is totally inhibited by furosemide and is insensitive to ouabain, no matter whether it is inside or outside the vesicles. The ($Na^+ + K^+$)-ATPase is insensitive to furosemide and is totally inhibited by internal ouabain.

Discussion

In this work, we studied the characteristics of the uptake of Na^+ under steady-state conditions, by inside-out vesicles of basolateral plasma membranes, prepared from rat kidney cortex slices. The vesicles accumulate Na^+ upon addition of Mg^{2+} + ATP to the incubation medium. A higher Na^+ accumulation is achieved, if K^+ + valinomycin are added to the incubation medium, together with Mg^{2+} and ATP (Fig. 3). Possible changes of vesicle volume produced by valinomycin plus K^+ could be responsible for the higher Na^+ uptake that takes place under this condition. However, this is not the case: (1) The passive Na^+ uptake is unaffected by the addition of K^+ + valinomycin, (2) the ΔNa^+ uptake and its associated ATP hydrolysis are the same in the presence or absence of K^+ + valinomycin, in vesicles prepared with 7 mM ouabain inside (Tables II and III).

The incorporation of Na^+ into the vesicles against its concentration gradient (50 mM NaCl inside and outside the vesicles), the necessity of Mg^{2+} + ATP in the incubation medium and the concomitant hydrolysis of ATP produced during the Na^+ incorporation (Fig. 4), clearly indicate that we are observing active transport processes. Working with a similar preparation, Boumedil-Podevin and Podevin [29] have recently demonstrated the existence of a ouabain-sensitive, K^+-dependent Na^+ uptake by the vesicles, that takes place in the presence of ATP. However, they did not find any uptake of Na^+ in the absence of K^+. This difference with our results can be due to the presence of EDTA in their homogenization medium. A 3-h preincubation of the membranes with 2 mM EDTA, abolishes either the incorporation of Na^+ independent of K^+ (unpublished data),

as well as the expression of the ouabain-insensitive, Na^+-stimulated ATPase activity [30]. On the other hand, the low concentration of Na^+ used in their experiments (4 mM) could also be responsible for the different results. In this respect it must be considered that the K_a for Na^+ for the Na^+-ATPase is 8 mM [10,11].

Several considerations must be discussed in order to try to explain the ATP + Mg^+-dependent accumulation of Na^+ shown by the vesicles in the absence of K^+ (ΔNa^+): (1) Furosemide has been shown to inhibit chloride-dependent cation transport systems in several tissues [31–40], including mammalian thick ascending limb of the Henle's Loop [41–43]. The $Na^+ + Cl^-$ or the $Na^+ + K^+ + 2Cl^-$ cotransport systems are described to be passive or secondary active, depending on the activity of the $Na^+ + K^+$ pump. The ouabain-insensitive, K^+-independent accumulation of Na^+ by the vesicles, is inhibited by furosemide. However, the characteristics of this Na^+ uptake clearly differentiate it from the cotransport systems. Thus, (a) it is seen in the absence of Na^+ or Cl^- gradients, (b) is seen under conditions where the $Na^+ + K^+$ pump is not working, i.e., in the absence of K^+ and in the presence of ouabain inside the vesicles and (c) ATP is hydrolyzed when the system is working. Finally, there is no evidence of the existence of a $Na^+ + Cl^-$ cotransport system in mammalian proximal tubule [44]. (2) The ΔNa^+ may be taken to be an expression of the activity of the $Na^+ + K^+$ pump. Working at a lower rate in one case, because of the absence of K^+, and working at a higher rate in the other case, when K^+ is added to the incubation medium. K^+ enters passively into the vesicles and then is extruded by the $Na^+ + K^+$ pump interchanged by Na^+, which is pumped into the vesicles. If this were the case, it would be necessary to explain why furosemide inhibits the K^+-independent Na^+ accumulation, but does not inhibit the K^+-dependent Na^+ accumulation, while ouabain does exactly the opposite: inhibits this latter expression of Na^+ accumulation without affecting the other one. (3) An ATP-driven electrogenic proton pump, which extrudes H^+ from the interior of inside-out basolateral plasma membrane vesicles of proximal tubular cells, has been suggested [29]. This proton pump could increase the Na^+ uptake of the vesicles by the development of an inside-negative membrane potential. The existence of this mechanism could explain the ΔNa^+ observed in our preparations. However, ΔNa^+ and its concomitant ATP hydrolysis are inhibited by furosemide (Tables II and III) which, to our knowledge, has never been described as an inhibitor of proton pumps. Even more, as shown in Tables II and III, the ΔNa^+ and its concomitant ATP hydrolysis are unaffected when assayed in the presence of valinomycin, K^+ and ouabain (inside the vesicles). Since valinomycin abolishes the alteration of the membrane potential produced by the proton pump [29], the results mentioned above demonstrate that ΔNa^+ is not linked indirectly to an electrogenic proton pump in the vesicles. (4) An alternative explanation may derive from the experiments with kidney cortex slices [1,3,6]. The cells pump Na^+ across the basolateral plasma membrane by the activity of two mechanisms or pumps: pump 1, which moves Na^+ accompanied by Cl^- and water, and pump 2, which moves Na^+ exchanged by K^+. Pump 1 is ouabain-insensitive and is inhibited by furosemide. Pump 2 is inhibited by ouabain and is insensitive to furosemide [1,7]. ΔNa^+ and its associated hydrolysis of ATP show similar characteristics of pump 1. $\Delta Na^+ + K^+$ and its associated hydrolysis of ATP show similar characteristics of pump 2. Accordingly, ΔNa^+ and $\Delta Na^+ + K^+$, may be taken to be the expressions of pump 1 and pump 2, respectively. The ratio of the quantity of Na^+ transported by each system to the quantity of ATP concomitantly hydrolyzed, calculated as the ratio of the slopes of the Na^+ uptake curves (Fig. 3) and the ATP hydrolysis curves (Fig. 4) is 1.78 ± 0.05 for the ouabain-insensitive, K^+-independent system and 1.67 ± 0.04 for the ouabain-sensitive, K^+-dependent system. This latter value is somewhat lower than the value reported for other cells, which is about 3 [45,46]. It may be due to some loss of Na^+ from the vesicles as they pass through the columns. Obviously, the presence of vesicles leaky to Na^+ would not affect the value of the $(Na^+ + K^+)$-ATPase activity, but would affect the amount of intravesicular Na^+. This fact would underestimate the Na^+ transported by the $Na^+ + K^+$ pump. The Na^+/ATP ratio for the ouabain-insensitive, K^+-independent system, has never been reported before, and could also be affected by the

presence of vesicles leaky to Na^+.

In spite of all the experimental evidence presented in this paper, the ouabain-insensitive Na^+ uptake could be still considered the result of differences in the passive 'leak' for $Na^+ + Cl^-$, rather than the result of the activity of a second Na^+ pump. This conclusion can be reached considering that when the vesicles are passed through the ion-exchange columns, they lose Na^+. This loss of Na^+ is reduced by the presence of Mg^{2+} + ATP. This possibility is unlikely because: (1) the ouabain-insensitive Na^+ uptake is associated to a concomitant ATP hydrolysis, (2) both Na^+ uptake and ATP hydrolysis are furosemide-sensitive and finally, (3) both Na^+ uptake and ATP hydrolysis are enhanced by the addition of micromolar quantities of Ca^{2+} to the incubation medium. This effect of Ca^{2+} is furosemide-sensitive and is not due to the activity of the Na^+/Ca^{2+} exchange system (unpublished data).

Further characterization of the two modes of Na^+ uptake by the vesicles must be done in order to confirm and substantiate these new findings, which constitute a very strong support for the existence of a second Na^+ pump in the basolateral plasma membrane of renal proximal tubular cells.

Acknowledgements

The authors wish to acknowledge Drs. Steven R. Gullans and Anibal G. Lopes for the reading and criticism of this paper. Thanks are also due to Mrs. Isabel O. de Zoppi and Mrs. Aída P. de Espidel for their secretarial help and to Mrs. Dhuwya Otero for the drawings. This work was supported in part with grant from project No. D.F. S1-1245 from CONICIT.

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