Biochimica et Biophysica Acta, 728 (1983) 39–49
Elsevier Biomedical Press

BBA 71530

EFFECTS OF ATP ON Na^+ TRANSPORT AND MEMBRANE POTENTIAL IN INSIDE-OUT RENAL BASOLATERAL VESICLES

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(Received July 16th, 1982)

Key words: ($Na^+ + K^+$)-ATPase; Ouabain inhibition; Membrane potential; Proton transport; Ionophore; Vanadate; (Rabbit kidney)

We have studied Na^+ transport in inside-out basolateral membrane vesicles isolated from rabbit kidney cortex. The addition of ATP in the presence of Mg^{2+} to the outside of K^+-loaded vesicles induced a rapid influx of Na^+ against its chemical gradient. Whereas intravesicular K^+ was required, extravesicular K^+ was inhibitory. ATP-dependent Na^+ uptake was inhibited by intravesicular, but not extravesicular ouabain, while extravesicular vanadate was inhibitory. Evaluation of changes in membrane potential using the lipophilic cation triphenylmethylphosphonium ($TPMP^+$) demonstrated hyperpolarization of the membrane voltage after MgATP addition. Changing membrane potential from zero to −40 mV had no effect on ATP-dependent Na^+ transport. The potential produced by MgATP was inhibited by valinomycin and by protonophores, but not by vanadate or ouabain. By contrast, the hyperpolarization that occurred in mitochondria after MgATP addition was inhibited by 75% by vanadate. We conclude that the properties of the renal basolateral sodium pump are qualitatively similar to those found in red cell and nerve and that these membranes probably also contain an electrogenic proton pump.

Introduction

It is generally agreed that transport of Na^+ out and K^+ into animal cells is coupled to ATP hydrolysis catalyzed by the membrane-bound ($Na^+ + K^+$)-ATPase which is the enzymatic equivalent of the sodium pump. With regard to the kidney, which is one of the richest sources of ($Na^+ + K^+$)-ATPase, much work has been done to relate changes in this enzyme activity to tubular Na^+ reabsorption. In recent experiments, a good correlation was found between these two events in various nephron segments [1,2]. In contrast to the wealth of information on ($Na^+ + K^+$)-ATPase localization along the nephron little is known about the mechanism by which Na^+ is translocated across the renal basolateral membrane in which ($Na^+ + K^+$)-ATPase is located predominantly, if not exclusively [3,4[. With respect to proximal tubules, practically all our understanding on Na^+ transport mediated by the ($Na^+ + K^+$)-ATPase derives from studies using intact cell systems. In such complex systems, however, unequivocal interpretation of the data is not possible since Na^+ transport may be mediated via other carriers located at the luminal membrane. These include Na^+-cotransport with sugars, amino acids, organic and inorganic acids and an Na^+-H^+ antiport system (for reviews, see Refs. 3, 5–8). Moreover, in contrast to studies using simple cell systems, such as erythrocytes or resealed erythrocyte ghosts, it is not feasible to adjust the composition of the in-

Abbreviations: $TPMP^+$, triphenylmethylphosphonium; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

tracellular fluid of renal cells to desired values and thus to assess with certainty the effects and the sidedness of interaction of various ligands on the sodium pump.

We thought, therefore, that a more direct approach is warranted to characterize further Na^+ transport mediated by the $(Na^+ + K^+)$-ATPase. To that end we have isolated from the rabbit kidney cortex a purified preparation of basolateral membranes. This preparation consisted of sealed, inside-out vesicles, an orientation which allows direct ATP access to the catalytic site and thus overcome the problem of impermeability of the renal basolateral membrane to this substrate [1,2].

Materials and Methods

Preparation of basolateral membranes and mitochondria

Basolateral membranes from rabbit kidney cortex were isolated by differential and sucrose density centrifugations according to the method described by Marx et al. [9] for rat kidney with some modifications. White male rabbits, 2–4 kg in weight, were anesthetized with sodium pentobarbital. The kidneys were placed on frozen buffer 1 (STE) (0.3 M sucrose, 0.001 M Na_2EDTA and 0.01 M Tris-Hepes, pH 7.4) and the cortex was minced. All subsequent steps were performed at 0–14°C. The tissue was homogenized using five strokes in a Dounce homogenizer (A pestle) and three strokes at 750 rev./min in a glass-teflon homogenizer. The homogenate was then filtered through a double layer of cheese cloth. The homogenate was adjusted to 50 ml with buffer 1. This homogenate was centrifuged at $800 \times g$ for 10 min in the 59 100 swing out rotor of a MSE-25 centrifuge with the brake off to remove cell debris and nuclei. The $800 \times g$ supernatant was then centrifuged at $6000 \times g$ for 10 min (brake off). The supernatant was saved. The resulting pellet consisted of three distinct colored layers. The top, loosely packed layer, which is almost white, was collected by slowly swirling the tube with about three ml of 0.3 M sucrose + 10 mM Tris-Hepes, pH 7.4 (buffer 2 (ST buffer)). The $6000 \times g$ supernatant was centrifuged at $16\,000 \times g$ for 10 min with the brake off. The supernatant fluid was discarded and the top, loosely packed layer was recovered in buffer 2 by repeating the procedure used above. These two partially purified membrane fractions were combined and the volume was adjusted to 10 ml with buffer 2. After homogenization in a Dounce homogenizer (A pestle) with 5 strokes this suspension was layered onto 32–45% linear sucrose gradient (w/w) in 10 mM Tris-Hepes, pH 7.40. The gradients were linear as indicated by the measure of the refractive index using a Bausch and Lomb refractometer. After centrifugation for 200 min at $286\,000 \times g$ in the SW 41 rotor of a Beckman L5-65 ultracentrifuge the material banding at 36% sucrose (basolateral membranes) was collected.

In some experiments, mitochondria were also recovered during the isolation of the basolateral membranes. All steps were carried out at 0–4°C. After the first centrifugation at $6000 \times g$ and after the top loosely packed layer has been recovered as noted above, the yellow-brown middle layer of the pellet (mitochondria) was separated from the dark bottom layer (lysosomes) by gentle swirling of the tube with 3 ml of buffer 2. The resuspended mitochondrial layer was homogenized using five strokes in a Dounce homogenizer (A pestle) and the volume was adjusted to 50 ml with ST buffer. The suspension was centrifuged at $10\,000 \times g$ for 10 min (brake off). The supernatant fluid was discarded. The tube was gently shaked with 5 ml of buffer 2 to remove the top loosely packed layer and only the middle yellow brown layer was collected by repeating the procedure used after the centrifugation at $6000 \times g$.

Unless otherwise stated, the basolateral membranes and the mitochondria were diluted 1 : 30 in a medium containing 100 mM KCl, 50 mM sucrose and 10 mM Tris-Hepes, pH 7.4. Membranes and mitochondria were sedimented by centrifugation for 10 min at $22\,000 \times g$ and resuspended in this same medium at a protein concentration of 10 to 20 mg/ml.

Approximately 10 mg of basolateral membranes were obtained from the kidneys of one rabbit which corresponded to 12–15% recovery of the total $(Na^+ + K^+)$-ATPase of the starting homogenate.

Transport studies

Uptake of ^{22}Na or D-[^{14}C]glucose into the

basolateral vesicles was measured by a Millipore filtration technique using HAMK 0.45 μm filters, as reported [10]. All incubations were performed at 37°C and membranes were preincubated at this temperature for 20 min in a shaking water bath. Unless otherwise specified, incubations were started by adding 5 μl of a suspension of membranes (40–100 μg of protein) to 100 μl of incubation medium preequilibrated to 37°C, followed by immediate mixing and incubation in a shaking water bath. Uptake was terminated by addition of 0.8 ml of an ice-cold solution containing 150 mM NaCl and 10 mM Tris-Hepes buffer, pH 7.4 (wash buffer). The membranes were immediately collected on a prewetted filter and washed with 5 ml of cold wash buffer. Uptake of [^{3}H]TPMP$^+$ into the membrane vesicles or mitochondria was assayed using the above mentioned procedure, except that Millipore Cellotate filters (EHWP, 0.5 μm) were used, as nonspecific retention of radioactivity was reduced about 50-fold, and that filters were washed with 10 instead of 5 ml of cold wash buffer. The concentration of [^{3}H]TPMP$^+$ was 20 μM. In most experiments using [^{3}H]TPMP$^+$, ^{22}Na was included in the incubation medium. Similar results were obtained for ^{22}Na uptake with the two procedures. For each experiment the composition of solutions used to incubate the membranes is given in the figure legends. All incubatin solutions contained 200 mM sucrose, 4 mM NaCl, 2 mM $MgCl_2$ and 10 mM Tris-Hepes buffer, pH 7.4, unless stated otherwise. When present, ATP was at a final concentration of 4 mM. All incubation solutions were filtered through 0.45 μm Millipore filters (SLHA 025 05) prior to use. The radioactivities of ^{3}H, ^{22}Na, or ^{14}C remaining on the filters were measured in 10 ml of Aquasol using a SL 4000 Intertechnique Liquid Scintillation Counter. Background retention of radioactivity was substracted from experimental values [10]. All incubations were performed at least in triplicate with three different membrane preparations. Although absolute uptake of solutes varied from one preparation to another, comparable results were obtained with the different preparations, and, therefore, only results of single experiment are presented.

The quality of the isolated basolateral membranes was evaluated by marker enzyme analysis. Alkaline phosphatase and ($Na^+ + K^+$)-ATPase activities were assayed as recently described [10]. Succinate dehydrogenase activity was assayed by the method of Pennington [11] as modified by Porteous and Clark [12]. Protein was determined by the method of Lowry et al. [13].

Chemicals

The [^{3}H]TPMP$^+$, 3.59 Ci/mmol, was from New England Nuclear, unlabeled TPMP$^+$ was purchased from K and K laboratories (Plainview, NY, U.S.A.). ^{22}Sodium, 55 mCi/mg was obtained from Commissariat à l'Energie Atomique SACLAY, France. Vanadium-free Tris-ATP and Tris-ADP, ouabain, oligomycin, and atractyloside were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Valinomycin was from Calbiochem. (San Diego, CA, U.S.A.). Ammonium vanadate (NH_4VO_3) was from Fluka, A.G. (Buchs, Switzerland). All other chemicals were of the highest purity available. As oligomycin and valinomycin were added in 95% ethanol, control suspensions received equivalent volumes of ethanol. The concentration of ethanol in the incubation medium did not exceed 1.2%.

Statistical analyses were performed by group *t*-tests. Results are expressed as means ± standard deviation (S.D.).

Results

Purity of the membrane preparations

The purity of the basolateral membranes was assessed by measuring the activities of 'marker' enzymes. It can be seen in Table I that ($Na^+ + K^+$)-ATPase, the basolateral marker, was enriched 12.3 ± 3.12-fold in the final membrane preparation as compared with the starting homogenate. In contrast, the specific activities of succinate dehydrogenase (a mitochondrial marker) and of alkaline phosphatase (a brush border marker) were enriched only 0.93 ± 0.16 and 1.20 ± 0.18-fold, respectively, compared with the original homogenate indicating little contamination by these subcellular components.

For mitochondria, the enrichment in succinate dehydrogenase specific activity (mitochondria/homogenate) averaged 5.67 ± 1.26-fold, while for ($Na^+ + K^+$)-ATPase and alkaline phosphatase the

TABLE I

ENZYMATIC ACTIVITIES IN BASOLATERAL MEMBRANES

Specific activities are expressed as μmol/min per mg protein. Numbers of preparations are shown in parentheses.

Enzyme		Specific activity	
		Homogenate	Basolateral membrane
$(Na^+ + K^+)$-ATPase	(12)	0.108 ± 0.029	1.32 ± 0.43
Alkaline phosphatase	(4)	0.185 ± 0.06	0.23 ± 0.07
Succinate dehydrogenase	(10)	0.037 ± 0.007	0.035 ± 0.011

ratios were less than unity; 0.64 ± 0.43 and 0.36 ± 0.11, respectively (five preparations).

Distinction between transport and binding

As illustrated in Fig. 1, uptake of Na^+ was found to be inversely related to medium osmolarity, indicating that Na^+ entered an interior space. Extrapolation of the line to infinite osmolarity revealed, however, a small binding component (0.2 nmol/mg protein), which would account for only 10% of the total uptake measured under conditions to evaluate transport (i.e. with 200 to 250 mosmol). Additional evidence, albeit indirect, that Na^+ uptake represented transport was obtained from experiments comparing on the same membrane preparation the equilibrium volume of distribution of 4 mM ^{22}Na to that of 4 mM D[^{14}C]glucose that is known to have, in basolateral vesicles, a negligible, if any, binding component [14,15]. We found that in the presence of 200 mM sucrose the equilibrium volume of distribution of ^{22}Na (0.60 ± 0.16 μl/mg protein) was identical to that of D-[^{14}C]glucose (0.58 ± 0.29) ($n = 6$).

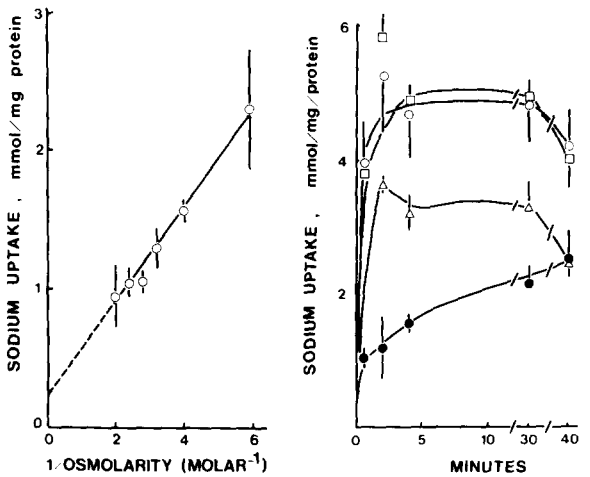

Fig. 1. Effect of medium osmolarity on Na^+ uptake. Renal basolateral membranes were washed twice in a medium containing 300 mM sucrose and 10 mM Tris-Hepes, pH 7.4. They were then incubated for 40 min in a medium containing 4 mM ^{22}Na, 10 mM Tris-Hepes, pH 7.4, and sufficient sucrose to give final concentrations indicated. Means ± S.D., $n = 4$.

Fig. 2. Time course of effects of ATP and inhibitors on 4 mM Na^+ uptake. A 5 μl aliquot of suspension of membranes was added to 200 μl of 200 mM buffered sucrose containing either ATP (○), ATP + 1 mM vanadate (△), ATP + 2 mM ouabain (□), or no ATP (●). Means ± S.D., $n = 4$.

Effects of ATP, vanadate and ouabain on the uptake of Na^+

As illustrated in Fig. 2, addition of ATP in the presence of Mg^{2+} to the outside of K^+-loaded membrane vesicles stimulated Na^+ uptake about 2- to 5-fold, indicating that the membrane of the basolateral vesicle is everted relative to its orientation in vivo. Further evidence for the inside-out orientation of the vesicles was obtained with two inhibitors considered to have opposite sidedness of action. Previous studies with red cell ghosts and giant squid axons have indicated that ouabain acts only on the external surface of the membrane [16, 17] whereas vanadate inhibits the red cell Na^+ pump from the cytoplasmic side [18]. As illustrated in Fig. 2, the ATP-dependent uptake of Na^+ was progressively abolished by external vanadate, while ouabain had no effect. When, however, ouabain was internally trapped during formation of the vesicles, this agent inhibited completely the ATP-dependent Na^+ uptake (Table III).

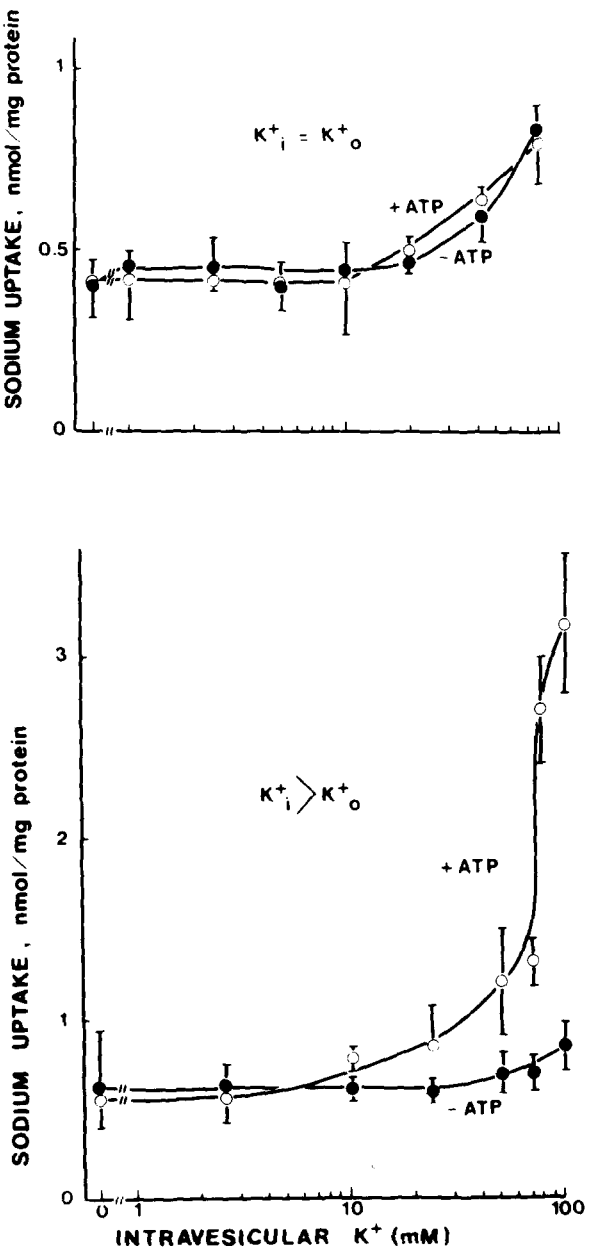

Fig. 3. Effects of K^+ on Na^+ uptake. Upper panel: $[K^+]_i = [K^+]_o$. Vesicles were preincubated for 30 min at 37°C in 10 mM Tris-Hepes, pH 7.4, in absence or presence of various amounts of KCl plus the necessary sucrose to maintain a total of 260 mosmol. Each vesicle sample was then incubated for 30 s into an identical medium containing in addition 4 mM $^{22}NaCl$, 2 mM $MgCl_2$, with (○) or without (●) ATP. Lower panel: $[K^+]_i > [K^+]_o$. Vesicles were preincubated as noted above. They were then incubated in 260 mM sucrose, 10 mM Tris-Hepes, 4 mM $^{22}NaCl$, 2 mM $MgCl_2$ with (○) or without (●) 4 mM ATP. Incubation was for 30 s. Means ± S.D., $n = 4$.

The data in Fig. 2 also show that the final level of uptake of Na^+ in the absence of ATP is identical to that attained in the presence of ATP plus vanadate, suggesting that equilibrium is established. Since the external concentration of Na^+ is 4 mM, the apparent equilibrium volume of distribution for Na^+ averaged 0.64 ± 0.11 μl/mg membrane protein. During the first 30 min of incubation, the uptake of Na^+ in the presence of ATP was about 2-fold greater than the equilibrium value, suggesting that Na^+ transport occurred against its concentration gradient.

Effects and sidedness of effects of K^+ on Na^+ uptake

Two approaches were used to determine the K^+ requirement for Na^+ transport. In the first series of experiments the basolateral membrane vesicles were loaded with various concentrations of K^+ and they were diluted 21-fold with K^+-free incubation medium. As shown in Fig. 3 (bottom), intravesicular K^+, i.e. K^+ at the plasma or external membrane surface, stimulated progressively ATP-dependent Na^+ uptake in the range of 10 to 100 mM KCl. In the second series of experiments

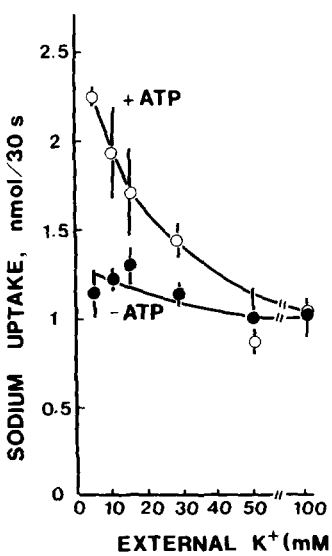

Fig. 4. Effect of external K^+ on 4 mM Na^+ uptake. The membranes were preincubated for 30 min at 37°C in a medium containing 100 mM KCl, 50 mM sucrose and 10 mM Tris-Hepes, pH 7.4. They were then incubated for 30 s in presence of the indicated concentrations of KCl in presence (○) or absence (●) of ATP. Means ± S.D., $n = 4$.

(Fig. 3, top), the vesicles were equilibrated with various concentrations of K^+ and incubated into an identical medium such that $[K^+]_i = [K^+]_o$. Under these conditions, K_i^+ did not stimulate ATP-dependent uptake of Na^+.

The differential effects of K_i^+ under non gradient and gradient conditions may be explained, at least partly, by the inhibitory action of K_o^+, i.e. K^+ cytoplasmic on the activity of the sodium pump. That it is the case is illustrated in Fig. 4. The ATP-dependent Na^+ uptake by K^+-loaded vesicles is progressively inhibited when K_o^+ is increased from 5 to 20 mM. These results are consistent with previous work by Knight and Welt [19] who have shown that intracellular K^+ acts as an inhibitor of the sodium pump in human erythrocytes particularly, when the internal Na^+ concentration is low.

Effects of ionophores on membrane potential and Na^+ transport

In the experiments carried out to evaluate the relationships between Na^+ movements and membrane potential, [^{3}H]TPMP$^+$ was included in the incubation media. Recent studies have shown that accumulation of this lipophilic cation can reliably be used to estimate relative changes of membrane potential in biological systems not amenable to microelectrode recordings (for review, see Ref. 20). Valinomycin in the presence of outwardly directed K^+ diffusion gradient was used to modify the membrane potential. As illustrated in Fig. 5, however, only in the absence of ATP was the expected modification of the membrane potential achieved as indicated by the 2-fold stimulation of TPMP$^+$ uptake in the presence of the K^+-ionophore. The effect of valinomycin, however, is lower than the recently observed in brush border membrane vesicles [21]. The unexpected and important finding of these studies was that addition of MgATP to the outside of vesicles promoted a strong hyperpolarization which was evidenced by the 5-fold increase in TPMP$^+$ uptake. This ATP effect was inhibited almost completely by valinomycin.

In contrast, valinomycin had no effect on Na^+ uptake measured in the absence or presence of external ATP. Thus, under these conditions, ATP-dependent Na^+ transport is independent of the membrane potential. The change in membrane potential induced by ATP can be estimated using the Nernst equation, as reported [22]. At equilibrium, i.e. after a 4 min incubation period, 18 pmol of TPMP$^+$ were accumulated per mg protein in the absence of ATP. Since the external con-

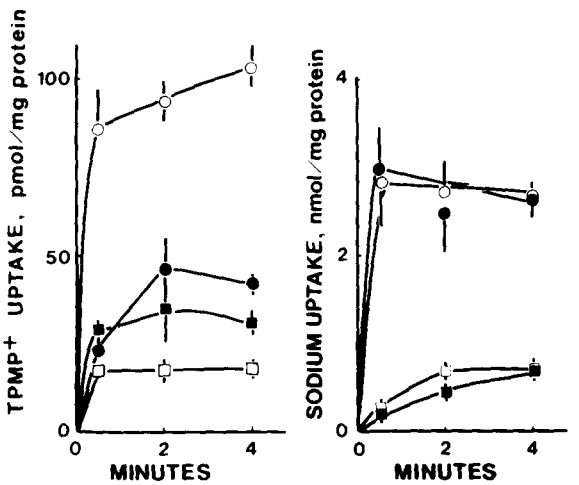

Fig. 5. Effects of valinomycin on uptakes of TPMP$^+$ and Na^+. After isolation, basolateral membranes were washed twice in a medium containing 50 mM K_2SO_4, 50 mM sucrose, and 10 mM Tris-Hepes, pH 7.4, by centrifugation at 22000 × *g* for 10 min at 2°C. The membranes were preincubated in same medium for 20 min at 37°C. They were then incubated in 200 mM sucrose, 4 mM ^{22}NaCl, 2 mM $MgCl_2$, 20 μM [^{3}H]TPMP$^+$, 10 mM Tris-Hepes, pH 7.4 with (○, ●) or without (□, ■) ATP, Closed symbols (●, ■) indicate inclusion of valinomycin (24 μg/mg protein) in the medium. Means ± S.D., $n = 4$.

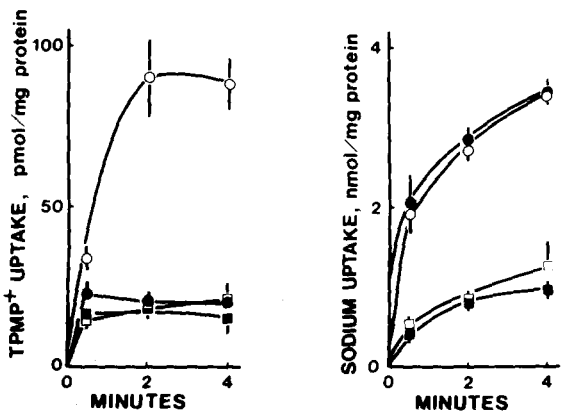

Fig. 6. Effects of dinitrophenol on uptakes of TPMP$^+$ and Na^+. Potassium chloride-loaded membrane vesicles were incubated as described in the legend to Fig. 5 except that 100 μM dinitrophenol (●, ■) replaced valinomycin.

centration of $TPMP^+$ was 20 μM, 1 mg protein represented an intravesicular space of 0.9 μl. Taking this internal volume, it can be calculated that ATP induced a −45 mV (inside negative) potential across these membranes. Fig. 6 demonstrates that the hyperpolarization that occurred upon ATP addition was completely inhibited within 2 min by the protonophore dinitrophenol, which is known to have no effect on the $(Na^+ + K^+)$-ATPase activity [23]. Identical results (not shown) were obtained with the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Again, the lack of effect of membrane potential on Na^+ uptake was confirmed.

Characteristics of the ATP-induced intravesicular negativity

The 3-min uptake of $TPMP^+$ measured in the presence of ATP was found to be inversely related to medium osmolarity and, thus, directly related to the intravesicular space (not shown). Extrapolation of the regression line to infinite osmolarity revealed, however, a binding component of 34 pmol/mg protein which would account for only 16% of the total uptake measured under conditions to evaluate transport (i.e. with 250 mosmol). Moreover, addition of 100 μM dinitrophenol 3 min after ATP addition resulted after a 1-min incubation in 80% loss of the accumulated $TPMP^+$ (not shown). Thus, it seems likely that the described effects of ATP, ionophores and various inhibitors on $TPMP^+$ uptake mainly reflect changes of transport of $TPMP^+$ across the basolateral membrane rather than alterations in binding. As illustrated in Table II hyperpolarization showed nucleotide specificity in that only ATP is effective to increase the uptake of the potential indicator. The results in Table II also show a similar nucleotide specificity for Na^+ uptake.

TABLE II

NUCLEOTIDE SPECIFICITY FOR HYPERPOLARIZATION OF BASOLATERAL VESICLES AND FOR Na^+ UPTAKE

Potassium-loaded vesicles were incubated for 30 s in 200 mM sucrose, 2 mM $MgCl_2$, 10 mM Tris-Hepes (pH 7.4), 4 mM ^{22}Na and 20 μM [^{3}H]TPMP. The nucleotides (4 mM) were added as indicated. Means ± S.D., $n = 4$.

Nucleotide	$TPMP^+$ uptake (pmol/mg protein)	Sodium uptake (nmol/mg protein)
None	14.16 ± 1.71	0.72 ± 0.09
ATP	42.01 ± 2.94	2.14 ± 0.13
ADP	16.20 ± 3.92	0.80 ± 0.08
AMP	12.50 ± 2.66	0.83 ± 0.07
GTP	16.20 ± 2.87	0.86 ± 0.08

TABLE III

EFFECTS OF INTRAVESICULAR OUABAIN ON THE UPTAKE OF $TPMP^+$ AND Na^+

After isolation, basolateral membranes were diluted 50-fold in a medium containing 100 mM KCl, 50 mM sucrose, 10 mM Tris-Hepes (pH 7.4), with or without 2 mM ouabain. Control and ouabain-treated vesicles were centrifuged at 22000 × *g* for 10 min at 2°C and resuspended into their corresponding medium at a concentration of about 10 mg/ml. Control and ouabain-loaded vesicles were then incubated for 30 s in 200 mM sucrose, 2 mM $MgCl_2$, 10 mM Tris-Hepes (pH 7.4), 4 mM ^{22}Na, 20 μM [^{3}H]$TPMP^+$ in the presence or absence of ATP.

Incubation conditions	$TPMP^+$ uptake (pmol/mg protein)	Sodium uptake (nmol/mg protein)
Control vesicles		
0 ATP	16.0 ± 2.16	0.81 ± 0.08
4 mM ATP	62.5 ± 7.01	2.31 ± 0.61
Ouabain-loaded vesicles		
0 ATP	16.5 ± 4.79	0.97 ± 0.31
4 mM ATP	62.7 ± 11.32	0.93 ± 0.13

To determine whether the effect of ATP on transmembrane potential was distinct from $(Na^+ + K^+)$-ATPase activity we compared the effects of the nucleotide on the uptake of $TPMP^+$ and Na^+ by basolateral vesicles either control or in which the specific $(Na^+ + K^+)$-ATPase inhibitor ouabain was internally trapped. As shown in Table III, the ATP-dependent uptake of Na^+ was completely abolished in ouabain-loaded membrane vesicles, while uptake of $TPMP^+$ was unaffected by ouabain.

To determine whether the hyperpolarization caused by ATP resulted from mitochondrial contamination of the basolateral preparation we compared the effects of ATP and ATP plus various inhibitors on the uptake of the potential indicator in either basolateral membranes or mitochondria isolated from the same homogenate. The relevant data are summarized in Table IV. This table shows that addition of ATP stimulated to the same ex-

TABLE IV

COMPARISON OF EFFECTS OF ATP AND VARIOUS INHIBITORS ON $TPMP^+$ UPTAKE BY BASOLATERAL MEMBRANES AND MITOCHONDRIA

Basolateral membrane vesicles and mitochondria suspended in 100 mM KCl, 50 mM sucrose, 10 mM Tris-Hepes (pH 7.4), were incubated for 2 min in 200 mM sucrose, 2 mM $MgCl_2$, 4 mM NaCl, 10 mM Tris-Hepes (pH 7.4) and 20 μM [^{3}H]$TPMP^+$. The test substances were added as indicated.

Expt. No.	Additions	n	$TPMP^+$ uptake, pmol/mg protein		Succinate dehydrogenase (μmol/min per mg protein)	
			Basolaterals	Mitochondria	Basolaterals	Mitochondria
1	None	4	26.3 ± 5.4 [a]	19.7 ± 6.5 [a]	0.065	0.283
	ATP	4	104.0 ± 0.5	91.0 ± 16.9		
	ATP + 0.1 mM Atractyloside	4	33.6 ± 2.8 [a]	24.6 ± 2.0 [a]		
2	None	4	16.0 ± 2.1 [a]	10.7 ± 1.7 [a]	0.047	0.197
	ATP	4	62.5 ± 7.0	44.0 ± 7.3		
	ATP + 0.5 mM NH_4VO_3	4	76.7 ± 16.3	28.7 ± 5.1 [a]		
3	None	4	19.5 ± 2.6 [a]	15.1 ± 2.1 [a]	0.054	0.257
	ATP	8	94.5 ± 9.5	126.2 ± 13.6		
	ATP + 10 μg/ml oligomycin	4	29.5 ± 4.0 [a]	21.1 ± 5.0 [a]		
	ATP + 1 mM NH_4VO_3	4	116.3 ± 11.6	101.0 ± 6.5 [a]		
	ATP + 1.5 mM NH_4VO_3	4	125.2 ± 16.5 [a]	74.8 ± 2.8 [a]		
4 [b]	None	4	15.4 ± 2.8 [a]	13.2 ± 7.3 [a]	0.029	0.29
	ATP	8	76.9 ± 8.0	113.8 ± 17.0		
	ATP + 2 mM NH_4VO_3	4	83.8 ± 7.7	39.4 ± 6.0 [a]		
	ATP + 2 mM NaN_3	4	23.7 ± 5.3 [a]	13.4 ± 6.7 [a]		

[a] $P < 0.01$ vs. respective values at 4 mM external ATP.

[b] In these experiments NaCl was omitted.

tent the uptake of $TPMP^+$ in both preparations incubated under identical conditions. For mitochondria, these results confirm observations by others [24,25] that the matrix of energized mitochondria concentrated $TPMP^+$ to a large extent, due to the potential difference, inside negative, across the mitochondrial membrane. The magnitude of the ATP-dependent uptake of $TPMP^+$ into the mitochondria present as a contaminant in the basolateral preparation was estimated by calculating the ATP-dependent uptake of the potential indicator/succinate dehydrogenase ratio for the two preparations. For each paired experiment this ratio was 3.2- to 6.1-fold higher ($P < 0.01$) with the basolateral membrane vesicles than with the mitochondrial fraction: mean ratio from the four experiments were 1423 ± 492 and 300 ± 114 with the basolateral vesicles and the mitochondria, respectively. These results suggest that the hyperpolarizing effect of ATP observed with the basolateral preparations could not be entirely attributed to mitochondrial contamination. In line with this observation is the finding that vanadate at concentrations ranging from 0.5 to 2 mM inhibits the effect of ATP on membrane potential only in mitochondria: with 2 mM vanadate the ATP-dependent uptake of $TPMP^+$ is inhibited by 74.6 ± 6.8%, $P < 0.001$. In contrast, vanadate stimulated the ATP-dependent uptake of $TPMP^+$ in the basolateral membrane vesicles by 11 to 40%, although the changes were not significant in all experiments. From the data in Table IV, it seems that stimulation of $TPMP^+$ uptake into basolateral vesicles by vanadate requires medium Na^+ but this possibility was not investigated. On the other hand, atractyloside, azide, and oligomycin caused almost complete inhibition of the ATP-dependent uptake of $TPMP^+$ in both preparations.

Discussion

The primary purpose of this study was to characterize Na^+ transport linked to ($Na^+ + K^+$)-

ATPase by using basolateral membrane vesicles, isolated from rabbit renal cortex, which were oriented inside-out. We have not assessed the proportion of inside-out vesicles present in our preparation but Kinne and Schwartz [7] have estimated that in approx. 40% of the rat renal basolateral vesicles the membrane is oriented inside-out. Recently, however, Del Castillo and Robinson [26] have presented evidence that all the basolateral membrane vesicles isolated from guinea-pig intestine are oriented inside-out.

The present experiments (Figs. 3 and 4) indicated clearly that the ATP-dependent uptake of Na^+ by inside-out vesicles required absolutely the presence of intravesicular K^+, i.e. K^+ at the plasma or external membrane surface, while K^+ in the bathing medium, i.e. K^+ at the cytoplasmic surface, inhibits Na^+ uptake. These results confirm qualitatively, in the basolateral membranes, the side-specific effects of K^+ previously reported by others in red cells [19,27]. The influence of intravesicular K^+ on the ATP-dependent transport of Na^+ into inside-out basolateral vesicles (Fig. 3) was also consistent with the conclusion of others that there was an absolute requirement for K^+ at the peritubular membrane of the proximal tubule for fluid absorption [28,29], and that active Na^+ transport in the kidney is mediated predominantly, if not exclusively, by $(Na^+ + K^+)$-ATPase [1,2].

The lack of effect of transmembrane potential on the activity of the Na^+ pump in basolateral membrane vesicles (Figs. 5 and 6) is in qualitative agreement with the observations of Hodgkin and Keynes [30] and Brinley and Mullins [31,32] on squid axon, of Thomas [33] on snail neuron, and of Marmor [34] on mollusc neuron. These data suggest that the basolateral sodium pump functions as a constant current source at least over change in membrane potential from 0 to about -30 or -40 mV.

About the marked hyperpolarization of the transmembrane potential specifically induced by external ATP (Figs. 5 and 6 and Table II) the present experiments demonstrated that the ATP effect was resistant to internal ouabain (Table III) or external vanadate (Table IV), while these agents inhibited ATP-dependent Na^+ transport. Conversely, the results in Fig. 6 show that dinitrophenol had no effect on ATP-dependent Na^+ uptake, whereas this protonophore completely blocked the ATP-induced hyperpolarization. Thus, it seems reasonable to conclude that the capacity of ATP to hyperpolarize the basolateral membrane is not linked to $(Na^+ + K^+)$-ATPase activity.

Two lines of argument suggest that the effect of ATP on membrane potential was not due to mitochondrial contamination of the basolateral preparation. First, the capacity of ATP to generate an interior negative in basolateral membrane vesicles and in mitochondria is unrelated to the respective content of these preparations in the mitochondrial enzyme marker succinate dehydrogenase. Second, 2 mM vanadate suppressed by 75% the effect of ATP in mitochondria, while this agent had not inhibitory effect in the basolateral preparation (Table IV). The observations that vanadate, usually considered as a plasma membrane inhibitor, suppressed the effect of ATP on membrane potential in mitochondria but not in basolateral vesicles and that a typical mitochondrial inhibitor, such as oligomycin, inhibited the hyperpolarization in both preparations are apparently distrubing. It has been shown, however, that vanadate exerts an uncoupling effect [35] and an inhibitory effect on respiration and phosphorylation [36] in mitochondria. Conversely, it is well established that several non-mitochondrial ATPases are also sensitive to oligomycin [37,38].

The effect of ATP on membrane potential was probably not due to lysosomal contamination. Indeed, the lysosomal proton pump [39] differs from the purported basolateral proton pump in that it is directed inwardly and operates electroneutrally. In addition, the lysosomal pump is not inhibited by valinomycin.

Recently, Murer et al. [40] and Kinne-Saffran et al. [41] have reported the existence in rat renal brush border membrane vesicles of an ATP-driven, probably electrogenic proton pump. It is unlikely, however, that our results may be explained by the activity of this brush border proton pump. Indeed, there is a major difference in the experimental technique. Brush border membrane vesicles are predominantly right side out and proton extrusion was observed only with ATP-loaded vesicles whereas the basolateral vesicles hyperpolarized immediately after external addition of ATP. Also, the

brush border proton pump functions in the presence of oligomycin [41] while this inhibitor prevented the ATP-induced hyperpolarization in the basolateral membrane vesicles.

We suggest, therefore that the hyperpolarization caused by ATP reflects predominantly, if not exclusively, an event that occurred across the basolateral membrane, but which is distinct from ($Na^+ + K^+$)-ATPase activity. The observations that protonophores such as dinitrophenol (Fig. 6) or FCCP (not shown) inhibit the ATP effect suggest that the hyperpolarization induced by ATP reflects the activity of an electrogenic proton pump. This view is strengthened by the finding (Fig. 5) that alteration of membrane conductance by valinomycin abolishes almost completely the potential induced by the purported ATP-driven proton pump. The present data, however, do not provide any evidence that the development of an inside-negative membrane potential after ATP addition is associated with alkalinization of the vesicle interior.

In conclusion, with inside-out basolateral vesicles it has been possible to confirm, at least qualitatively, observations by others, mainly obtained in red cells (for reviews, see Refs. 42–44) for the effects and the sidedness of interaction of K^+, ouabain and vanadate on the sodium pump. We were also able to show, in agreement with previous work by other in nerve, that the activity of the sodium pump is largely independent of membrane potential. These findings suggest that inside-out renal basolateral vesicles should be useful to characterize further ATP-driven transport processes. In particular, the unexpected finding that ATP specifically hyperpolarizes the basolateral membrane by a process sensitive to protonophores suggest the presence of a basolateral electrogenic proton pump which deserve further studies.

Acknowledgments

We thank Miss C. Priol for expert technical assistance, Miss M. Bizien and F. Carlier for secretarial assistance. We thank Dr. C. Amiel for many valuable discussions during the course of this study. This investigation was supported by the Institut National de la Santé et de la Recherche Médicale, grants 815050 and 825032, and by the Faculté de Médecine Xavier Bichat.

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