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Na^+-STIMULATED ATPase ACTIVITIES IN KIDNEY BASAL-LATERAL PLASMA MEMBRANES

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In the present work we demonstrate the existence of a Na^+-stimulated, Mg^{2+}-dependent ATPase activity, which is insensitive to ouabain, and which is 100% inhibited by 1.5 mM ethacrynic acid in freshly prepared, basal-lateral enriched, plasma-membrane fractions obtained from guinea-pig kidney cortex slices (which are mainly made up by proximal tubules). This ATPase activity has characteristics similar to those described previously in a microsomal fraction of the guinea-pig kidney cortex by a procedure which required ageing of the preparation for more than two weeks (Proverbio, F., Condrescu-Guidi, M. and Whittembury, G. (1975) Biochim. Biophys. Acta 394, 281–292), it does not seem to be due to some partially modified activity of the Mg^{2+}-ATPase, the $(Na^+ + K^+)$- or the Ca^{2+}-ATPase activities present in this tissue. It seems to be due to the activity of another system, which is located on the basal-lateral membrane of the kidney tubular cells.

Introduction

In a recent paper, Proverbio et al. [1] have described two forms of Na^+-stimulated ATPase activity which were present in aged microsomal fractions from guinea-pig kidney cortex. One was the well known ouabain-sensitive, $(Na^+ + K^+)$-stimulated ATPase activity [2–6], the other was an ouabain-insensitive, Na^+-stimulated ATPase activity, which was preferentially sensitive to ethacrynic acid. Both ATPase activities paralleled some of the conditions that should be fulfilled by the energy sources responsible for the two modes of Na^+ extrusion which have been described in guinea-pig kidney cortex slices: (a) an ouabain-sensitive one, which exchanges intracellular Na^+ for extracellular K^+ [7–12], and (b) an ouabain-insensitive mode, which extrudes Na^+ accompanied by Cl^- and water [8,10–13]. However, the Na^+-stimulated ATPase activity was only uncovered if the preparations of the cortex kidney microsomal fractions were aged for 12–15 days [1]. If this ATPase activity has any physiological role it is necessary to demonstrate first, that the system is present at the basal-lateral plasma membranes of the tubular kidney cells and second, that the ATPase activity is present, not only in aged, but also in fresh preparations.

In the present work we have prepared a subcellular fraction enriched in basal-lateral plasma membranes. The enrichment and distribution of the Na^+-stimulated ATPase activity, in all the fractions obtained during the preparative procedure, are compared to that of the $(Na^+ + K^+)$-stimulated ATPase, a marker for basal-lateral plasma membranes [25,28], and to the distribution of glucose-6-phosphatase, a marker for endoplasmic reticulum [14–16]; acid phosphatase, a marker for lysosomes [14]; 5′-nucleotidase, a marker for brush border membranes [17] and succinate dehydrogenase, a marker for mitochondria [18].

It was found that the Na^+-stimulated ATPase activity, present in fresh preparations, parallels the distribution of the $(Na^+ + K^+)$-ATPase activity, indicating that it is also related to basal-lateral plasma membranes.

Materials and Methods

Preparation of fractions enriched in basal-lateral plasma membranes

Outermost slices of kidney cortex (rich in proximal tubules) from healthy adult guinea-pigs, weighing about 500 g were obtained as previously described [9]. The slices were homogenized at 4°C with 8 strokes at 2500 rev./min in an Eberbach homogenizer with a Teflon pestle, in 3 vols. of a medium containing: 250 mM sucrose, 20 mM Tris-HCl (pH 7.2) and 2 mM EDTA. All steps were done at 4°C. Fractions enriched in plasma membranes were prepared using a slight modification of the method developed by Fitzpatrick et al. [19] for rat kidney as follows.

The homogenate (H) was filtered through two sheets of gauze to remove clumps and centrifuged for 10 min at 1 475 $\times g$. The supernatant (S) was discarded and the pellet resuspended in 3 vol. of 2 M sucrose (density 1.252) and rehomogenized with three strokes at 2 500 rev./min in the same homogenizer used before. The resuspension was centrifuged for 10 min at 13 300 $\times g$. The pellet (P_1) was discarded and the supernatant was taken back to isotonicity by adding 7 vol. of cold distilled water and then recentrifuged for 15 min at 35 000 $\times g$. The supernatant of this centrifugation (S_1) was discarded. The resulting pellet consisted of three layers: a lower, brown pellet, rich in mitochondria (P_2), an intermediate layer (P_{3MIT}) and an upper, pink layer (P_3) rich in plasma membranes. The upper layer (P_3) was removed by swirling the sucrose-Tris-EDTA medium over the pellet, and after two strokes of the homogenizer it was recentrifuged and resuspended twice in the same medium. All fractions were frozen and kept at –20°C.

5 ml of the P_3 fraction were put on the top of 10 ml of a discontinuous sucrose gradient, consisting from bottom to top of 5 ml of sucrose solution of density 1.1562 and 5 ml of sucrose solution of density 1.1266. After centrifugation for 50 min at 25 500 $\times g$, five different fractions were separated: the upper supernatant, fraction S_3; the first interfase, fraction P_4; the next supernatant was divided into an upper fraction, S_4 and a lower fraction, S_5. The lower pellet corresponds to fraction P_5. These fractions were washed and resuspended in the sucrose-Tris-EDTA medium and frozen and kept at –20°C.

Determination of enzyme activities

ATPase activities (EC 3.6.1.3). Required amounts of the different suspensions were preincubated for 5 min at the prescribed incubation temperature in the presence of (final concentrations); 50–150 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$ and when required, 100 mM NaCl, 20 mM KCl and 1 mM ouabain. $CaCl_2$, other salts and ethacrynic acid were also used when required. The concentration of Tris was varied to adjust the osmolarity of the media. The final volume was 1 ml. The reaction was started by adding the Na^+-free Tris-ATP (2 mM final concentration) to the medium. After 15 min, the reaction was stopped by the addition of 1 ml of ice cold 6% $HClO_4$ to the incubation tubes. The samples were chilled and centrifuged and the liberated orthophosphate (P_i) was determined in the deproteinized solution [20]. Preliminary experiments demonstrated that, under our incubation conditions a linear relationship existed between the liberation of phosphate and the time of incubation. All samples were run in triplicate. ATPase activity is expressed as nmol of P_i produced per mg of protein per min, after subtraction of a blank run in parallel without the membrane suspension, which was added after the $HClO_4$. The protein content of the original suspensions was measured by means of Folin reagent [21]. Aliquots of the fractions were run all at pH 7.0, with the required amounts of Tris-HCl. Some in the presence of only 5 mM $MgCl_2$. Others in the presence of 5 mM $MgCl_2$, 100 mM NaCl. Others in the presence of 5 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl. The ATPase activity observed in the presence of Mg^{2+} alone is referred to as Mg^{2+}-ATPase activity. The difference in activity between ($Mg^{2+} + Na^+$)-ATPase and Mg^{2+}-ATPase will be referred to as Na^+-ATPase. The difference in activity between ($Mg^{2+} + Na^+ + K^+$)-ATPase and ($Mg^{2+} + Na^+$)-ATPase will be referred to as ($Na^+ + K^+$)-ATPase.

Determination of the Na^+-stimulated ATPase activity. In the present work we did confirm that ageing of the fractions at 0–4°C for about 2 weeks brings up a Na^+-stimulated, Mg^{2+}-dependent ATPase activity. In addition, we found that if after its preparation at pH 7.2, the membrane fraction is resuspended at pH 7.8, the Na^+-stimulated, Mg^{2+}-dependent activity, shows up in freshly prepared fractions, when the assay is performed at pH 7.0. In consequence this procedure was used to assay for the Na^+-ATPase activity.

Other phosphatases. The determination of other phosphatases was carried out at 37°C in 1 ml volume for all the supernatant and pellets described above. The reactions were terminated by the addition of 1 ml of ice cold 6% $HClO_4$. The following methods have been used: acid phosphatase (EC 3.1.3.2) was determined using β-glycerophosphate at pH 5.4 as substrate, according to Hübscher and West [22], 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 [23]. 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 phosphatases [23].

Other enzymes: Succinate dehydrogenase (EC 1.3.99.1) was determined following the succinate-dependent reduction of potassium ferricyanide according to the method of King [24].

Treatments

NaI treatment. The original method of Nakao et al. and Matsui and Schwartz modified by Ebel et al. [25] was utilized: The membrane fractions were suspended in a solution of 1 mM Tris-EDTA (pH 7.5) to a final protein concentration of 2–4 mg/ml. The suspension was mixed with an equal volume of a solution containing: 6 M NaI; 10 mM EDTA; 80 mM Tris and 5 mM $MgCl_2$ at pH 8.5. After 10 min at 0°C the suspension was diluted by adding the 1 mM EDTA solution to a final NaI concentration of 1.2 M. The suspension was centrifuged for 30 min at 30 000 $\times g$, and the resulting pellet washed with the 1 mM EDTA solution. Finally, the pellet was resuspended in the same medium, frozen at −20°C and stored for 24 h before use.

Deoxycholate treatment. The method of Jørgensen and Skou [26] was followed. Briefly, the membrane fractions suspended in the sucrose-Tris medium (pH 7.8), containing 2–4 mg/ml proteins, were treated with deoxycholate and EDTA, to obtain concentrations of 0.1% deoxycholate and 1 mM EDTA, at room temperature, for 30 min. The fractions were assayed for enzyme activities, immediately after treatment.

Chemicals

β-Glycerophosphate, glucose 6-phosphate, AMP, ATP, ouabain (strophanthin-G), EGTA, EDTA, were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. Ethacrynic acid, was generously provided by Merck, Sharp and Dohme, Rahway, NJ, U.S.A.

Results

Preparation of fractions enriched in basal-lateral plasma membranes

Table I shows the protein recoveries, the specific activities, the calculated relative specific activities and the total recoveries of several marker enzymes in the different fractions. $(Na^+ + K^+)$-ATPase, a recognized marker for the basal-lateral plasma membranes [25, 28], is enriched preferentially in fraction P_3, by a factor of 6.33 when compared with the homogenate, since there is a recovery of 32.3% of this activity, while this fraction contains only 5.1% of the proteins. Since there is still some contamination of other membranes, as shown by the recoveries of glucose-6-phosphatase (endoplasmic reticulum); acid phosphatase (lysosomes) and 5′-nucleotidase (brush border membranes), fraction P_3 was centrifuged on a discontinuous sucrose gradient (see methods). Table II shows protein recoveries, specific activities, calculated relative specific activities and recoveries of marker enzymes in the different fractions prepared from P_3.

It is clear that fraction P_5 is the richest in $(Na^+ + K^+)$-ATPase activity. This is further demonstrated in Table III. Notice that when this fraction is treated with NaI, 95% of the ATPase activity is $(Na^+ + K^+)$-ATPase and only 5% is Mg^{2+}-ATPase activity. These enrichment factors compare reasonably well with those obtained by others for basal-lateral plasma membrane preparations [25,28]. Fig. 1 shows the enzyme content of P_5 as related to the initial homogenate (H). Notice that the enrichment factor of the $(Na^+ + K^+)$-ATPase, is very similar to that of the Na^+-ATPase. Both ATPase activities are enriched by a factor of about 8. All other enzymatic markers have a specific activity lower than the homogenate. Therefore the degree of enrichment of the Mg^{2+}-dependent ATPase activity (which is also contained in mitochondrial and microsomal fractions) is due, in all probability, to the enrichment of the basal-lateral plasma membranes. It may also be an expression of the Ca^{2+}-ATPase activity, which can also be stimulated by Mg^{2+} [27,28]. Table II also shows that the percent of recovery of the Na^+-stimulated and of the $(Na^+ + K^+)$-

TABLE I

ENZYME ACTIVITIES AND RECOVERIES IN KIDNEY CORTEX HOMOGENATE (H) AND SUBCELLULAR FRACTIONS

The different fractions named as indicated in Materials and Methods. Abbreviations: S.A. specific activity, defined as nmol of liberated P_i/mg protein per min for the phosphatases and ΔUA/mg protein per min for the succinate dehydrogenase. R.S.A. relative specific activity, calculated as the ratio of specific activity of any particular fraction to that of the homogenate. R%, percentage of total activity of the homogenate recovered in the fraction. In this and in the following tables, the values are expressed as the mean ± S.E. In the present table (n = 8).

		Fractions							Total recovery (%)
		H	S	P_1	S_1	P_2	P_{3MT}	P_3	
Protein	R%	100	47.9 ± 1.7	20.4 ± 1.0	15.0 ± 0.8	9.6 ± 0.6	7.1 ± 0.5	5.1 ± 0.7	105.1
Mg^{2+}-ATPase	S.A.	118.0 ± 5.2	80.9 ± 2.8	146.9 ± 8.0	67.7 ± 5.4	234.8 ± 16.4	202.8 ± 13.2	233.2 ± 7.7	
	R.S.A.	1	0.68	1.24	0.57	1.99	1.72	1.98	
	R%	100	32.8 ± 1.2	25.4 ± 1.7	8.6 ± 0.6	19.1 ± 1.5	12.2 ± 1.1	10.1 ± 1.1	108.2
$(Na^+ + K^+)$-ATPase	S.A.	59.9 ± 5.7	36.6 ± 3.3	75.8 ± 7.2	28.4 ± 2.6	66.1 ± 6.6	87.7 ± 4.1	379.4 ± 24.6	
	R.S.A.	1	0.61	1.27	0.47	1.10	1.46	6.33	
	R%	100	29.2 ± 2.9	25.8 ± 3.4	7.1 ± 0.7	10.6 ± 1.8	10.4 ± 0.6	32.3 ± 1.3	115.4
5′-Nucleotidase	S.A.	13.0 ± 1.4	10.2 ± 1.7	11.3 ± 1.1	12.0 ± 1.7	13.9 ± 1.3	16.5 ± 3.2	25.0 ± 1.1	
	R.S.A.	1	0.78	0.87	0.92	1.07	1.27	1.92	
	R%	100	37.6 ± 4.6	17.7 ± 1.5	13.8 ± 1.2	10.3 ± 0.8	9.0 ± 0.2	9.8 ± 0.6	98.2
Glucose-6-phosphatase	S.A.	48.5 ± 2.4	57.9 ± 2.9	36.4 ± 2.3	33.3 ± 3.1	10.1 ± 9.8	39.6 ± 2.1	57.1 ± 2.5	
	R.S.A.	1	1.19	0.75	0.69	0.21	0.82	1.18	
	R%	100	57.2 ± 1.3	15.3 ± 0.8	10.3 ± 0.6	2.0 ± 0.2	5.8 ± 0.5	6.0 ± 0.3	96.6
Acid phosphatase	S.A.	3.4 ± 0.1	3.1 ± 0.2	2.6 ± 0.1	4.4 ± 0.2	0.43 ± 0.04	3.18 ± 0.06	3.08 ± 0.3	
	R.S.A.	1	0.91	0.76	1.29	0.13	0.93	0.91	
	R%	100	43.7 ± 2.4	15.6 ± 0.3	19.4 ± 1.7	1.2 ± 0.1	6.6 ± 0.8	4.6 ± 0.5	91.1
Succinate dehydrogenase	S.A.	0.27 ± 0.02	0.04 ± 0.001	0.41 ± 0.01	0.16 ± 0.01	0.66 ± 0.02	0.53 ± 0.02	0.15 ± 0.01	
	R.S.A.	1	0.15	1.52	0.59	2.44	1.96	0.56	
	R%	100	7.1 ± 0.4	30.9 ± 0.4	8.9 ± 0.6	23.5 ± 0.8	13.9 ± 0.2	2.8 ± 0.2	87.1
Na^+-ATPase	S.A.	6.06 ± 0.60	2.61 ± 0.45	7.96 ± 0.94	4.42 ± 0.57	6.61 ± 0.84	9.14 ± 1.02	32.82 ± 3.03	
	R.S.A.	1	0.43	1.31	0.73	1.09	1.51	5.41	
	R%	100	20.6 ± 2.0	26.8 ± 2.1	10.9 ± 1.3	10.5 ± 1.2	10.7 ± 0.6	27.6 ± 2.6	107.1

TABLE II

ENZYME ACTIVITIES AND RECOVERIES OF SUBCELLULAR FRACTIONS FROM THE DISCONTINUOUS DENSITY GRADIENT

S.A., specific activity; R.S.A., relative specific activity; R%, percentage of total activity. R.S.A. and R% refer to the P_3 fraction. For details see the legend of Table I ($n = 8$).

		Fractions					Total recovery (%)
		P_3	S_3	P_4	S_4	S_5	P_5	
Protein	R%	100	2.2 ± 0.13	37.8 ± 1.67	15.0 ± 0.78	19.3 ± 0.34	23.2 ± 1.06	97.5
Mg^{2+}-ATPase	S.A.	233.2 ± 7.7	97.6 ± 9.4	157.3 ± 6.5	319.7 ± 3.1	209.4 ± 4.0	290.4 ± 8.5	
	R.S.A.	1	0.42	0.67	1.37	0.90	1.24	
	R%	100	0.92 ± 0.09	25.5 ± 2.20	20.6 ± 2.2	17.3 ± 0.13	28.9 ± 2.39	93.22
$(Na^+ + K^+)$-ATPase	S.A.	379.4 ± 24.6	44.0 ± 4.4	249.0 ± 8.5	391.0 ± 10.4	398.6 ± 9	477.7 ± 12.1	
	R.S.A.	1	0.12	0.66	1.03	1.05	1.26	
	R%	100	0.26 ± 0.01	24.8 ± 2.6	15.5 ± 2.1	20.3 ± 0.66	29.2 ± 1.4	90.06
5'-Nucleotidase	S.A.	25.0 ± 1.1	39.0 ± 0.32	35.7 ± 1.4	19.7 ± 0.51	19.4 ± 2.1	13.3 ± 0.51	
	R.S.A.	1	1.56	1.43	0.79	0.78	0.53	
	R%	100	3.4 ± 0.25	54.0 ± 1.9	11.8 ± 0.54	15.0 ± 1.1	12.3 ± 0.90	96.5
Glucose-6-phosphatase	S.A.	57.1 ± 2.5	49.4 ± 1.3	77.6 ± 2.8	32.3 ± 2.5	31.3 ± 1.0	35.1 ± 1.8	
	R.S.A.	1	0.87	1.36	0.57	0.54	0.61	
	R%	100	1.9 ± 0.17	51.4 ± 3.9	8.5 ± 0.56	10.5 ± 0.64	14.3 ± 0.69	86.6
Acid phosphatase	S.A.	3.08 ± 0.30	13.6 ± 0.80	3.01 ± 0.31	3.28 ± 0.17	1.93 ± 0.12	1.00 ± 0.09	
	R.S.A.	1	4.41	0.98	1.06	0.63	0.32	
	R%	100	9.7 ± 0.61	36.9 ± 3.4	16.0 ± 1.10	12.1 ± 0.55	7.5 ± 0.50	82.2
Succinate dehydrogenase	S.A.	0.151 ± 0.01	non detectable	0.157 ± 0.004	0.048 ± 0.004	0.100 ± 0.004	0.165 ± 0.005	
	R.S.A.	1	0	1.04	0.32	0.66	1.09	
	R%	100	0	39.3 ± 4.10	4.8 ± 0.38	12.8 ± 1.20	25.4 ± 2.25	82.3
Na^+-ATPase	S.A.	32.83 ± 3.03	0.00	23.18 ± 2.57	36.70 ± 5.00	37.83 ± 4.67	46.50 ± 3.34	
	R.S.A.	1	0.00	0.71	1.12	1.15	1.42	
	R%	100	0.00	26.7 ± 2.1	16.8 ± 1.7	22.2 ± 1.6	32.9 ± 2.0	98.6

TABLE III

EFFECT OF THE TREATMENT WITH NaI ON THE ATPase ACTIVITY OF P_5 FRACTIONS

The NaI treatment of fractions P_5 carried out as indicated under Materials and Methods. Ouabain concentration, 1 mM; Mg^{2+}, 5 mM; Na^+, 100 mM and K^+, 20 mM. $n = 8$.

Conditions	ATPase activity (nmol P_i liberated/mg protein per min), incubation medium					
	Mg^{2+} + ouabain	$Mg^{2+} + Na^+$ + ouabain	$Mg^{2+} + Na^+ + K^+$ + ouabain	$Mg^{2+} + Na^+ + K^+$	ΔNa^+	$\Delta Na^+ + K^+$
Control	310 ± 6	373 ± 3	370 ± 4	827 ± 27	+63 ± 7	+457 ± 27
NaI treated	42 ± 3	40 ± 2	43 ± 2	894 ± 43	− 2 ± 4	+851 ± 43

stimulated ATPase activities in fraction P_5 is much higher than the recovery of the other enzymes.

We can see from Table I and II, that the relative specific activities as well as the percentages of recovery of the Na^+-stimulated ATPase and of the ($Na^+ + K^+$)-stimulated ATPase are very similar through all the

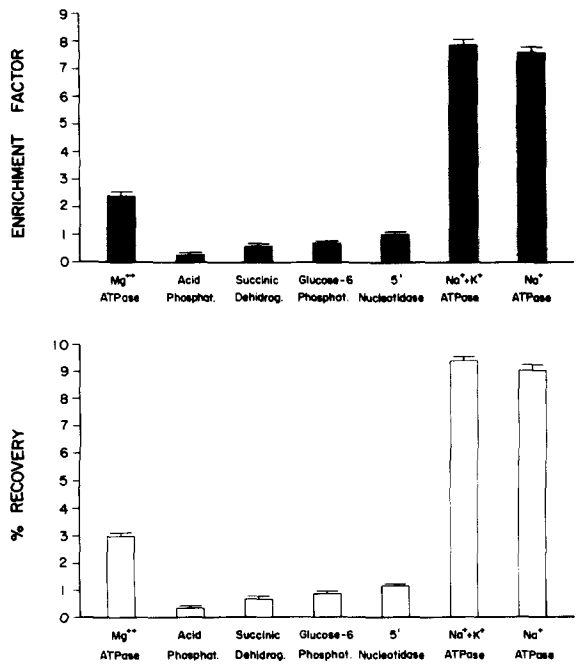

Fig. 1. Enrichment factor (specific activity of each enzyme in fraction P_5/specific activity homogenate) (top pannel), and percentage of recovery of the different enzyme markers referred to the homogenate (lower pannel). The values are expressed as the mean ± S.E. (n = 8). Data taken from Tables I and II.

different fractions obtained during the preparation of P_5. The regression coefficients for the relative specific activities and for the percentages of recovery between the Na^+ and the ($Na^+ + K^+$)-ATPase activities of the different fractions until P_3 and until P_5 were 0.99 both for the relative specific activities and for the percentages of recovery.

A further enrichment of the specific activity of the ($Na^+ + K^+$)-ATPase was achieved by treating fraction P_5 with NaI. The results of this treatment are shown in Table III: the ($Na^+ + K^+$)-stimulated ATPase activity of fraction P_5 was enriched by this treatment by a factor of 1.86, that is, a specific activity 14-times higher than that of the homogenate. The recovery of proteins from the NaI treatment was 26% and the ratio ($Na^+ + K^+$)-ATPase/Mg^{2+}-ATPase, became 19.8, indicating, as said before, that for this fraction the ATPase activity in the presence of Mg^{2+} is 95% ($Na^+ + K^+$)-stimulated, and only 5% is Mg^{2+}-dependent, ouabain insensitive. The Na^+-stimulated ATPase activity, on the other hand, becomes totally inhibited after the NaI treatment.

Determination of the Na^+-stimulated ATPase activity

We have investigated the presence of a Na^+-stimulated ATPase activity in our fresh preparations and we have studied its distribution in the different fractions. As mentioned in the introduction, Proverbio et al. [1] had observed an ouabain-resistant, Na^+-ATPase activity, after ageing the microsomal preparations for about 2 weeks. As said before, in the present work, we did confirm that ageing of the fractions at 0–4°C for about 2 weeks brings up a Na^+-stimulated, Mg^{2+}-dependent ATPase activity. In addition,

TABLE IV

ATPase ACTIVITY OF P_3 FRACTIONS RESUSPENDED AT pH 7.8 OR 7.2

Assays performed at pH 7 in the presence of 1 mM ouabain. Mg^{2+} concentration, 5 mM; Na^+, 100 mM. (n = 13). n.s., not significant.

Resuspension pH	Additions	ATPase activity (nmol P_i liberated/mg protein per min), incubation medium			
		Mg^{2+}	$Mg^{2+} + Na^+$	ΔNa^+	
7.8	None	218 ± 3	248 ± 3	+30	$P < 0.001$
7.2	None	225 ± 3	222 ± 3	− 3	n.s.
7.8	25 μM Ca^{2+}	223 ± 2	255 ± 3	+32	$P < 0.001$
7.2	25 μM Ca^{2+}	222 ± 4	252 ± 5	+30	$P < 0.001$

two approaches have been successful to show a Na^+-stimulated, ouabain insensitive ATPase activity in the fresh preparations. One, consists in resuspending the membranes in Tris at pH 7.8. The second approach consists in resuspending the membranes at the usual pH of 7.2 and in running the assays in the presence of micromolar quantities of Ca^{2+}. In both cases, the ATPase activities were run in an incubation medium at pH 7.0. The results of the two approaches are compared in Table IV. Notice that the membranes resuspended at pH 7.2 also show the Na^+-stimulated activity the same day of preparation only upon addition of 25 μM Ca^{2+} to the incubation medium. Addition of Ca^{2+} did not show any appreciable effect on the membranes resuspended at pH 7.8, either in the presence, or in the absence of Na^+. Table V shows that chelation of Ca^{2+} by addition of 0.5 mM EGTA to the incubation medium, did not show any effect on the Na^+-stimulated ATPase activity observed in membrane fractions resuspended at pH 7.8. These results indicate that stimulation of the ATPase activity by

TABLE V

EFFECT OF EGTA ON THE ATPase ACTIVITY OF P_3 FRACTIONS RESUSPENDED AT pH 7.8

Assays performed in the presence of 1 mM ouabain. Mg^{2+} concentration, 5 mM; Na^+, 100 mM. n = 6.

Additions	ATPase activity (nmol P_i liberated/mg protein per min), incubation medium			
	Mg^{2+}	$Mg^{2+} + Na^+$	ΔNa^+	
None	230 ± 4	252 ± 3	+22	$P < 0.01$
0.5 mM EGTA	232 ± 3	259 ± 3	+27	$P < 0.01$

TABLE VI

EFFECT OF INCREASING Ca^{2+} CONCENTRATIONS ON THE ATPase ACTIVITY OF P_3 FRACTIONS RESUSPENDED AT pH 7.2 AND 7.8

Assays performed in the presence of 1 mM ouabain. Mg^{2+} concentration, 5 mM; Na^+, 100 mM. Calcium concentrations refer to added and not to free calcium concentration.

Added calcium (μM)	ATPase activity (nmol P_i liberated/mg protein per min), incubation medium		
	Mg^{2+}	$Mg^{2+} + Na^+$	ΔNa^+
A. Fractions resuspended at pH 7.2 (n = 9)			
0	223 ± 3	225 ± 2	+ 2[a]
5	223 ± 2	236 ± 3	+13[b]
10	216 ± 3	241 ± 2	+25[c]
25	218 ± 4	251 ± 4	+33[c]
50	216 ± 4	247 ± 3	+31[c]
100	214 ± 1	237 ± 2	+23[c]
B. Fractions resuspended at pH 7.8 (n = 6)			
0	230 ± 3	258 ± 2	+28[c]
25	235 ± 4	266 ± 3	+31[c]
50	241 ± 3	274 ± 4	+33[c]
100	242 ± 5	273 ± 5	+31[b]

[a] P not significant.
[b] $P < 0.01$.
[c] $P < 0.001$.

TABLE VII

EFFECT OF 1.5 mM ETHACRYNIC ACID (E.A.) ON THE ATPase ACTIVITY OF P_3 FRACTIONS RESUSPENDED AT pH 7.8

Assays performed in the presence or absence of 25 μM Ca^{2+}. Ouabain concentration, 1 mM; Mg^{2+}, 5 mM; Na^+, 100 mM and K^+, 20 mM. $n = 8$.

Additions		ATPase activity (nmol P_i liberated/mg protein per min), incubation medium				
Ca^{2+} (μM)	E.A. (mM)	Mg^{2+} + ouabain	Mg^{2+} + Na^+ + ouabain	Mg^{2+} + Na^+ + K^+	ΔNa^+	$\Delta Na^+ + K^+$
0	0	219 ± 2	245 ± 2	595 ± 8	+26 ± 3	+350 ± 8
0	1.5	131 ± 4	126 ± 3	277 ± 4	− 5 ± 5	+151 ± 5
25	0	233 ± 3	266 ± 1	545 ± 10	+33 ± 3	+279 ± 10
25	1.5	129 ± 3	121 ± 3	244 ± 4	− 8 ± 4	+123 ± 5

Na^+ is not related to a Ca^{2+}-stimulated ATPase activity. Other experiments, (data not shown), indicate that in the absence of Mg^{2+}, 25 μM of added Ca^{2+}, alone, is totally unable to produce any ATPase activity either in the absence or in the presence of Na^+.

The effect of increasing the concentration of the added Ca^{2+} to the incubation medium, on the Na^+-stimulated ATPase of membranes resuspended at pH 7.2 or 7.8, is shown in Table VI: there is an optimal activation at 25 μM of added Ca^{2+} for the membranes resuspended at pH 7.2. This is not the case, however, when the membranes were resuspended at pH 7.8, in which case, the Na^+-stimulated activity is about the same, and maximal, either at 0, or at 25 or at 100 μM of added Ca^{2+}. The apparent K_a for the added Ca^{2+} activation, for membranes resuspended at pH 7.2, was calculated to be on the order of 5 μM.

The Na^+-stimulated ATPase activity described for aged microsomal fractions of this same tissue, was known to be 100% inhibited by 1.5 mM ethacrynic acid [1]. Table VII shows the effect of 1.5 mM ethacrynic acid on the ATPase activity of fractions resuspended at pH 7.8, in the presence or absence of 25 μM Ca^{2+}. In both cases, while there was an inhibition of about 40% on the Mg^{2+}-ATPase and 57% on the ($Na^+ + K^+$)-ATPase, the Na^+-stimulated activity was totally inhibited. Notice how the 25 μM of added Ca^{2+}, inhibits the ($Na^+ + K^+$)-ATPase activity by about 20%. To further demonstrate that the Na^+-stimulated ATPase activity is not an expression of a Ca^{2+}-stimulated ATPase, ethacrynic acid was used in measurements of this latter enzyme. The Ca^{2+}-ATPase activity was assayed as already described for this tissue [27,28], i.e. in the presence of 5 mM Ca^{2+} and without Mg^{2+} in the incubation medium. The results are shown in Table VIII: we can see that there is no Na^+ stimulation under these conditions and that the Ca^{2+}-ATPase is inhibited about 45% by the presence of 1.5 mM ethacrynic acid.

Despite the observation that the Na^+-stimulated ATPase activity is totally inhibited by 1.5 mM ethacrynic acid, while the ($Na^+ + K^+$)-ATPase activity is inhibited about 57%, the observation that NaI treatment inhibits the Na^+- but not the ($Na^+ + K^+$)-ATPase activity, could still suggest that the Na^+-

TABLE VIII

EFFECT OF 1.5 mM ETHACRYNIC ACID ON THE ATPase ACTIVITY OF P_3 FRACTIONS ASSAYED IN THE PRESENCE OF 5 mM Ca^{2+} WITH OR WITHOUT 100 mM Na^+ IN THE ABSENCE OF Mg^{2+} ($n = 4$)

Additions	ATPase activity (nmol P_i liberated/mg protein per min), incubation medium	
	Ca^{2+}	Ca^{2+} + Na^+
None	129 ± 4	113 ± 11
Ethacrynic acid	68 ± 8	64 ± 3
Inhibition (%)	47	43

TABLE IX

EFFECT OF THE TREATMENT WITH DEOXYCHOLATE ON THE ATPase ACTIVITY OF P_3 FRACTIONS RESUSPENDED AT pH 7.8

The deoxycholate (DOC) treatment of fractions P_3 was carried out as indicated under Materials and Methods. Ouabain concentration, 1 mM; Mg^{2+}, 5 mM; Na^+, 100 mM and K^+, 20 mM. $n = 6$.

Incubation medium	ATPase activity (nmol P_i liberated/mg protein per min)	
	−DOC	+DOC
Mg^{2+} + ouabain	234 ± 4	253 ± 5
$Mg^{2+} + Na^+$ + ouabain	261 ± 3	284 ± 4
$Mg^{2+} + Na^+ + K^+$	601 ± 22	634 ± 20
ΔNa^+	+ 27 ± 5	+ 31 ± 6
$\Delta Na^+ + K^+$	+340 ± 22	+350 ± 20

ATPase activity is due to a partial activity of the $(Na^+ + K^+)$-ATPase, which stems from inside-out vesicles, that are permeable to ions but impermeable to ouabain. The system could work with endogenous K^+, present in the membrane preparations, and it would be insensitive to ouabain, which could not exert its inhibiting action, because of the inside-out arrangement of the membranes. If this were the case, the Na^+-stimulated, ouabain-insensitive ATPase activity should disappear if the vesicles are opened by treating them with a small amount of detergent. Results of such an experiment are shown in Table IX: membrane samples were separated in two groups and one of them was treated with deoxycholate, following the method of Jørgensen and Skou [26]. The values of the Mg^{2+}-, Na^+-, and $(Na^+ + K^+)$-ATPase activities are very similar for the treated and untreated membranes, indicating that this suggestion is untenable.

Discussion

Evidence that there are two Na^+-stimulated ATPase activities associated with basal-lateral membranes of proximal tubular cells is presented in this paper. One is the $(Na^+ + K^+)$-stimulated, ouabain-sensitive activity. The other is the Na^+-stimulated ouabain-insensitive activity.

Both systems show the same distribution pattern through all the different fractions of the preparation (Tables I, II; Fig. 1), which is not the case for the other enzyme markers. The observation that P_3 and particularly P_5 become highly enriched in the specific activities of the enzymes $(Na^+ + K^+)$-ATPase and Na^+-ATPase, while the specific activity of the other enzymatic markers tested is lower than the activity of the homogenate (Fig. 1), indicates that both enzymes are associated with the basal-lateral membranes, to which the $(Na^+ + K^+)$-ATPase system is associated.

The present work demonstrated that the ouabain-insensitive Na^+-stimulated ATPase can be activated by three procedures: either by resuspending the membranes in a medium at pH 7.8 (Table IV), or by addition to the incubation medium of micromolar quantities of Ca^{2+} or (as for the microsomal fractions [1]) by ageing the preparations (data not shown). We do not have yet a clear cut explanation of why these maneuvers unmask the Na^+-ATPase activity. Further investigations are under progress to try to explain this particular behaviour of the Na^+-stimulated ATPase system. The possibility is not excluded that Ca^{2+} could be involved in some kind of control mechanism of this system.

The identity of the Na^+-ATPase system seems to be clear. It does not seem to be a modification of the basal, Mg^{2+}-dependent ATPase activity, since both systems show a different behaviour towards ethacrynic acid (Table VII), or towards the pH of the resuspension medium (Table IV) or towards the presence of Ca^{2+} in the incubation medium (Table IV). Its differentiation from the $(Na^+ + K^+)$-ATPase does not need K^+ in the incubation medium; it is insensitive to 1 or even 10 mM ouabain, which on the other hand inhibits totally the $(Na^+ + K^+)$-ATPase. The Na^+-ATPase is totally inhibited by 1.5 mM ethacrynic acid, which only causes an inhibition of about 50% of the $(Na^+ + K^+)$-ATPase activity (Ref. 1 and Table VII). NaI inhibits the Na^+- but not the $(Na^+ + K^+)$-ATPase activity (Table III). Both systems can be demonstrated after treating the membranes with deoxycholate (Table IX), indicating that the Na^+-ATPase is not just a partial activity of the $(Na^+ + K^+)$-ATPase from inside-out vesicles impermeable to ouabain. Finally, the Na^+-stimulated system can be activated by the addition of 25 μM Ca^{2+} to the incubation medium (Table IV), which concomitantly produces

about 21% of inhibition of the $(Na^+ + K^+)$-ATPase activity (Table VII). The differentiation of the Na^+-stimulated activity from the Ca^{2+}-ATPase activity is clear: the Ca^{2+}-ATPase system can be demonstrated in this tissue in the presence of high Ca^{2+} concentrations (mM range), and Na^+ does not exert any stimulation of it (Refs. 27 and 28 and Table VIII). Even more, ethacrynic acid only inhibits about 45% of the Ca^{2+}-ATPase (Table VIII), while it inhibits 100% of the Na^+-ATPase (Table VII). Finally, the Na^+ stimulation can be shown in the presence of 0.5 mM EGTA in the incubation medium (Table V), while Ca^{2+} alone in the μM range did not activate any ATPase system either in the absence or in the presence of Na^+ (data not shown).

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