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Na^+-ATPase is a different entity from the ($Na^+ + K^+$)-ATPase in rat kidney basolateral plasma membranes

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In this work, we present evidence in agreement with the hypothesis that there exist two Na^+-stimulated ATPase activities in basolateral plasma membranes from rat kidney proximal tubular cells: (1) ($Na^+ + K^+$)-ATPase activity, which is inhibited by ouabain and by treating the membranes with trypsin, is insensitive to furosemide and reaches maximal activity upon treatment with SDS at an SDS/protein ratio of 1.6; (2) the Na^+-ATPase activity, which is insensitive to ouabain and to trypsin treatment, is inhibited by furosemide and reaches maximal activity upon treatment with SDS at an SDS/protein ratio of 0.4.

Introduction

Besides the ($Na^+ + K^+$)-ATPase, a K^+-independent, Mg^{2+}-dependent, Na^+-stimulated ATPase activity has been demonstrated in aged microsomal fractions from guinea-pig kidney cortex [1] and in rat whole kidney [2]. Recently, a similar Na^+-stimulated ATPase activity has been shown to be present in basolateral plasma membranes from guinea-pig [3] and rat [4] kidney proximal tubular cells. This ATPase activity (usually about 10% of that of ($Na^+ + K^+$)-ATPase) has the following characteristics. 1. It is stimulated by Na^+ and by Li^+, but not by other cations. 2. The Na^+ stimulation is independent of the anion accompanying Na^+. 3. It is insensitive to even 10 mM ouabain. 4. It is specifically inhibited by furosemide and triflocin and preferentially inhibited (over the ($Na^+ + K^+$)-ATPase) by ethacrynic acid. 5. It is modulated by micromolar quantities of Ca^{2+}. 6. It has a K_m for Na^+ of 8 mM [4,5]. Furthermore, working with inside-out vesicles from basolateral plasma membranes of rat kidney proximal tubular cells, we have been able to demonstrate an active Na^+ flux which is K^+-independent, ouabain-insensitive, Ca^{2+}-modulated and inhibited by furosemide. This active Na^+ flux proceeds in the presence of Mg^{2+} and ATP, which is hydrolyzed during the Na^+ movement [6,7].

In spite of these characteristics, which in general are very different from the characteristics of the ($Na^+ + K^+$)-ATPase, it is still subject to debate whether the Na^+-ATPase does exist as a separate entity from the classical ($Na^+ + K^+$)-ATPase, or whether it is just an expression of the last system.

In this work, we present evidence in agreement with the hypothesis that the ouabain-sensitive ($Na^+ + K^+$)-ATPase and the ouabain-insensitive Na^+-ATPase are two different entities.

Materials and Methods

The preparation of outermost slices of rat kidney cortex, the extraction of basolateral plasma membranes, membrane treatment with SDS

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(sodium dodecyl sulfate), membrane treatment with trypsin, the Na^+-ATPase, the $(Na^+ + K^+)$-ATPase assay, the inorganic phosphate determination and the protein determination were carried out as described in the previous paper [8].

NaI treatment used in the experiments of Table II

The original method of Nakao et al. [9] and Matsui and Schwartz [10] modified by Ebel et al. [11] was used. The membrane fractions were suspended in 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 6 M NaI/10 mM EDTA/80 mM Tris/5 mM $MgCl_2$ (pH 8.5). After 10 min at 0°C, the suspension was diluted by adding 1 mM EDTA to a final NaI concentration of 1.2 M. The suspension was centrifuged for 30 min at $30000 \times g$, and the resulting pellet was washed with 0.25 M sucrose/20 mM Tris-HCl (pH 7.2). Finally, the pellet was resuspended in the same solution, frozen at −20°C and stored for 24 h before use.

Na^+-ATPase assay

Na^+-ATPase activity was measured by a modification of a previously described method [3,4]. 180 μl incubation medium containing (final concentration) 50 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 100 mM NaCl, 2 mM Tris-ATP, 7 mM ouabain, in the presence or absence of 2 mM furosemide, were preincubated at 37°C for 5 min. The reaction was started by adding 20 μl of the membrane suspension (0.1 mg/ml). In all cases, the assays were carried out for 10 min. The reaction was stopped and the inorganic phosphate was determined as described in the previous paper [8]. All samples were run in quadruplicate. The activity was expressed as nmol P_i liberated per mg protein per min, after subtraction of a blank run in parallel without the membrane suspension, which was added after adding the stop solution. Na^+-ATPase activity was calculated as the difference between the P_i liberated in the absence or presence of 2 mM furosemide which, as already shown [4,5], inhibits 100% the activity of the Na^+-ATPase.

Chemicals

ATP, ouabain (strophanthin-G), porcine trypsin (T-0134) and trypsin inhibitor were purchased from the Sigma Chemical Company. SDS was obtained from Bio-Rad Laboratories; NaI was purchased from Mallinkcrodt Co., furosemide was generously provided by Medicamentos York S.A., Caracas.

Results

As indicated in our previous paper [8], the $(Na^+ + K^+)$-ATPase activity of the basolateral plasma membrane fraction prepared for these studies is enriched by about 33-fold when compared with the activity in homogenates. These membranes are practically 100% vesiculated. The Na^+-ATPase activity in this fraction is also enriched about 30-fold when compared with the activity in homogenates (6.7 nmol P_i/mg protein per min, in homogenates treated with SDS at the optimal SDS/protein ratio). The yield of Na^+-ATPase activity is 13 ± 1%. The membrane vesicles were treated with SDS, at different SDS/protein ratios, and then assayed for Na^+- and $(Na^+ + K^+)$-ATPase activities. The results of these experiments, expressed as percent of the maximal activity in each case, are shown in Fig. 1. An interest-

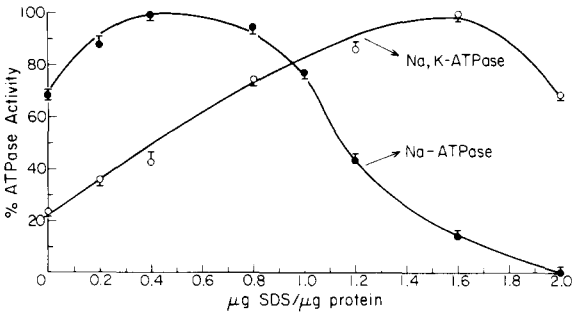

Fig. 1. Effect of the treatment of basolateral plasma membranes with SDS on the activity of the $(Na^+ + K^+)$-ATPase (white circles) and the Na^+-ATPase (black circles). At the end of the treatment, 2 mM EDTA was added to the treatment medium. The ATPase assay was carried out in the following medium (mM): NaCl, 100; $MgCl_2$, 5; Tris-ATP, 2; Tris-HCl, 50, and, when required, KCl, 20; ouabain, 7; furosemide, 2; and $CaCl_2$, 0.05. The $(Na^+ + K^+)$-ATPase activity was determined from the difference in P_i released in the presence of K^+ with or without ouabain. The Na^+-ATPase activity was determined from the difference in P_i released in the presence or absence of furosemide. Values are expressed as means ± S.E. ($n = 6$).

TABLE I

EFFECT OF THE TREATMENT WITH SDS OR TRYPSIN ON THE Na^+- AND ($Na^+ + K^+$)-ATPase ACTIVITIES OF BASOLATERAL PLASMA MEMBRANE FRACTIONS

The Na^+-ATPase and ($Na^+ + K^+$)-ATPase activities were measured in membranes treated with SDS at an SDS/protein ratio of 0.4 and 1.6, respectively. Values are expressed as means ± S.E. ($n = 6$), in nmol P_i released per mg protein per min.

Membrane treatment	Na^+-ATPase	($Na^+ + K^+$)-ATPase
SDS	198 ± 10	1906 ± 67
Trypsin + SDS	187 ± 8	687 ± 18
SDS + trypsin	190 ± 9	6 ± 2

TABLE III

EFFECT OF OUABAIN OR FUROSEMIDE ON THE Na^+- AND ($Na^+ + K^+$)-ATPase ACTIVITIES OF BASOLATERAL PLASMA MEMBRANE FRACTIONS

In all the cases, the membranes were treated with SDS before the ATPase assays using the optimal SDS/protein ratio. Ouabain concentration, 7 mM. Furosemide concentration, 2 mM. Values are expressed as means ± S.E. ($n = 6$), in nmol P_i liberated per mg protein per min.

Incubation conditions	Na^+-ATPase	($Na^+ + K^+$)-ATPase
Control	196 ± 11	1895 ± 71
+ Ouabain	202 ± 13	23 ± 15
+ Furosemide	11 ± 7	1799 ± 59

ing point can be drawn from this figure: the Na^+-ATPase shows approx. 70% of its maximal activity in membranes not treated with SDS; it reaches its maximal activity in membranes treated at an SDS/protein ratio of 0.4. This is not the case for the ($Na^+ + K^+$)-ATPase: this activity is only 23% of its maximum in membranes not treated with SDS; it reaches its maximal activity in membranes treated at an SDS/protein ratio of 1.6. At this ratio, the Na^+-ATPase activity is almost totally inhibited.

It has been shown that trypsin inhibits the expression of the ($Na^+ + K^+$)-ATPase only when the cytoplasmic side of the cell membranes is exposed to its action [12,13]. Our membranes were treated with trypsin and then assayed for Na^+ and ($Na^+ + K^+$)-ATPase activities. The results of these experiments are shown in Table I. The first row shows the ATPase activities of membranes treated with the optimal SDS/protein ratio, in each case, before the assay. When the membranes were treated with trypsin and then with SDS (second row), there was practically no change in the activity of the Na^+-ATPase, while the ($Na^+ + K^+$)-ATPase activity was inhibited by approx. 70%. Finally, when the membranes were treated with SDS and then with trypsin, the Na^+-ATPase activity was still maximal, while the ($Na^+ + K^+$)-ATPase activity was totally inhibited.

Membrane treatment with 3 M NaI did not affect the ($Na^+ + K^+$)-ATPase activity (Table II). On the other hand, the Na^+-ATPase activity was totally inhibited by this treatment.

Table III shows the behavior of the two ATPase systems toward ouabain and furosemide. The membranes were treated with SDS and then assayed for Na^+- and ($Na^+ + K^+$)-ATPase activities. It should be noted that 7 mM ouabain in the incubation medium inhibits totally the ($Na^+ + K^+$)-ATPase activity, without affecting the Na^+-ATPase activity. 2 mM furosemide in the incubation medium, on the other hand, completely inhibits the Na^+-ATPase activity without affecting the ($Na^+ + K^+$)-ATPase activity.

TABLE II

EFFECT OF THE TREATMENT WITH NaI ON THE Na^+- AND ($Na^+ + K^+$)-ATPase ACTIVITIES OF BASOLATERAL PLASMA MEMBRANES FRACTIONS

The Na^+-ATPase and ($Na^+ + K^+$)-ATPase activities were measured in membranes treated with SDS at an SDS/protein ratio of 0.4 and 1.6, respectively. Values are expressed as means ± S.E. ($n = 6$), in nmol P_i liberated per mg protein per min.

Membrane treatment	Na^+-ATPase	($Na^+ + K^+$)-ATPase
SDS	203 ± 11	1980 ± 65
NaI + SDS	3 ± 7	2012 ± 57

Discussion

This paper presents evidence that clearly differentiates the ouabain-insensitive Na^+-ATPase from the ouabain-sensitive ($Na^+ + K^+$)-ATPase. Thus, complementing the criteria of the different

characteristics already mentioned in the introduction, it was found that:

(1) They react differently toward different concentrations of SDS: the Na^+-ATPase reaches maximal activity at an SDS/protein ratio of 0.4, while the ($Na^+ + K^+$)-ATPase reaches maximal activity at an SDS/protein ratio of 1.6, a ratio at which the Na^+-ATPase is strongly inhibited (Fig. 1).

(2) Digestion of the ($Na^+ + K^+$)-ATPase by trypsin from the cytoplasmic side of the membranes results in the loss of activity of the enzyme [8,9]. When our membrane preparation was treated with trypsin and then with SDS (Table I), there was approx. 30% of the maximal ($Na^+ + K^+$)-ATPase activity. When the membranes were treated with SDS and then with trypsin, the ($Na^+ + K^+$)-ATPase was totally inactivated. In the first case, trypsin digested the cytoplasmic face of the inside-out vesicles (approx. 70%), inhibiting the ($Na^+ + K^+$)-ATPase activity of these vesicles. Since the right-side-out vesicles (approx. 30%) do not expose the cytoplasmic portion of the ($Na^+ + K^+$)-ATPase, trypsin treatment of these vesicles did not affect the ($Na^+ + K^+$)-ATPase activity. When the right-side-out vesicles were opened with the SDS treatment, they showed their ($Na^+ + K^+$)-ATPase activity. In the second case, the SDS treatment opened all the vesicle populations, allowing trypsin to digest the ($Na^+ + K^+$)-ATPase present in all the membranes and, therefore, the ($Na^+ + K^+$)-ATPase activity was totally inhibited. This was not the case for the Na^+-ATPase activity; its expression is maximal when the membranes are treated with trypsin either before or after the SDS treatment (Table I). These results indicate that, as for the Ca^{2+}-ATPase [14–17], trypsin does not affect the Na^+-ATPase activity.

(3) Treatment of the membranes with NaI did not affect the ($Na^+ + K^+$)-ATPase activity (Table II). This was not the case for the Na^+-ATPase: its activity was totally inhibited by the NaI treatment. This result is similar to that already shown for basolateral plasma membranes from guinea-pig kidney proximal tubular cells [3].

(4) As already demonstrated for other preparations [4,5], the Na^+- and the ($Na^+ + K^+$)-ATPase activities showed different behavior toward ouabain and furosemide. Thus, 7 mM ouabain in the incubation medium totally inhibited the ($Na^+ + K^+$)-ATPase activity without affecting the Na^+-ATPase activity. On the other hand, 2 mM furosemide completely inhibited the Na^+-ATPase activity without affecting the ($Na^+ + K^+$)-ATPase activity (Table III).

To conclude, the present results constitute strong evidence for the existence, in basolateral plasma membranes from rat kidney proximal tubular cells, of two Na^+-stimulated ATPase systems: the ouabain-sensitive, furosemide-insensitive ($Na^+ + K^+$)-ATPase and the ouabain-insensitive, furosemide-sensitive Na^+-ATPase.

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