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HIGH SODIUM DIET AND Na^+-STIMULATED ATPase ACTIVITIES IN BASOLATERAL PLASMA MEMBRANES FROM RAT KIDNEY PROXIMAL TUBULAR CELLS

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Abstract—The ouabain-insensitive, Na^+-stimulated ATPase activity of kidney proximal tubular cells from rats fed a high Na^+ diet for 4 months was increased approximately 70% when compared with control (normal diet) rats. The higher ATPase activity was not due to a change in the affinity of the system toward ATP, Mg^{2+} or Na^+. This increase in Na^+-ATPase activity may be due to either a higher number of pumps or to a higher turnover rate of the enzyme or both. The oubain-sensitive, Na^+,K^+-stimulated ATPase activity, on the other hand, did not change with the high sodium diet. These results can be taken as evidence that the Na^+,K^+-ATPase and the Na^+-ATPase of basolateral plasma membranes of proximal tubular cells from rat kidney are two different entities.

Two mechanisms of cellular Na^+ extrusion have been demonstrated to occur in kidney cortex slices (rich in proximal tubules) from several mammals, including rats [1–5]: (1) exchange of Na^+ for K^+, and (2) extrusion of Na^+ accompanied by Cl^- and water. Two types of Mg^{2+}-dependent, Na^+-stimulated ATPase activity have also been shown to be present in basolateral plasma membranes of cells from guinea pig and rat kidney cortex slices [6, 7]: (1) the ouabain-sensitive, Na^+,K^+-ATPase, and (2) the ouabain-insensitive, Na^+-ATPase. The Na^+,K^+-ATPase is considered to generate the energy supply for the extrusion of Na^+ exchanged for K^+. The Na^+-ATPase is considered to generate the energy supply for the extrusion of Na^+ accompanied by Cl^- and water [6–11]. Even though the two ATPase systems have very different characteristics [9, 10], the existence of the Na^+-ATPase is still controversial.

Changes in the Na^+ intake may well produce differential changes in renal Na^+ transport mechanisms and, consequently, changes in Na^+-ATPase activities. With this in mind, we fed 25-day-old rats an isotonic NaCl solution. After 4 months, we found that the Na^+-ATPase activity of basolateral plasma membranes of kidney proximal tubular cells from the experimental rats was about twice the activity of control rats. The Na^+, K^+-ATPase activity, on the other hand, was similar in both groups.

MATERIALS AND METHODS

Sprague–Dawley rats (25 days old) were separated into two groups: a control group was given tap water to drink and an experimental group was given a solution of 0.9% NaCl to drink. Every month, some of the control and the experimental rats were killed, and basolateral plasma membranes from their kidney proximal tubular cells were prepared and assayed for ATPase activity.

Isolation of basolateral plasma membranes. The basolateral plasma membrane enriched fractions were prepared as follows. Three-month-old, healthy male Sprague–Dawley rats were anesthetized with ether and immediately decapitated. The kidneys were removed, decapsulated, and collected in a medium containing 250 mM sucrose/20 mM Tris–HCl (pH 7.2)/0.5 mM dithiothreitol/0.2 mM phenylmethylsulfonylfluoride (sucrose/Tris/DTT/PMSF medium), at 4°. The outermost slices of the kidney cortex (which are rich in proximal tubules [12]) were homogenized and centrifuged at 4°, using a modification of the method of Kinsella *et al.* (originally developed for dog kidney cortex) [13]. The slices were homogenized at 4°, with eight strokes at 2500 rpm in an Eberbach homogenizer with a tight-fitting Teflon pestle, in 3 vol./g of tissue of sucrose/Tris/DTT/PMSF medium. DTT and PMSF were used to avoid possible oxidation of the ATPases and to inhibit proteases respectively. These agents were not used originally by Kinsella *et al.* [13]. The homogenate (usually 240 mg protein) was spun at 1000 *g* for 10 min and the supernatant fraction was saved. The pellet was resuspended with sucrose/Tris/DTT/PMSF medium and recentrifuged at 1000 *g* for 10 min. The resulting supernatant fraction was combined with the previous supernatant. This mixture was spun at 9500 *g* for 10 min. The supernatant and the soft, lighter upper portion of the pellet were combined and spun at 48,000 *g* for 20 min. The soft, lighter upper portion of the pellet was resuspended in a solution of 25 mM Tris–HCl (pH 7.2)/100 mM mannitol/2 mM $CaCl_2$/1 mM $MgCl_2$/1 mM $MnCl_2$ to a final protein concentration of 4–6 mg/ml. This membrane suspension was kept on ice for 90 min and then spun at 1400 *g* for 12 min. The pellet was resuspended with the same solution (Tris/mannitol/

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$CaCl_2/MgCl_2/MnCl_2$) and re-extracted again at the same speed. The final pellet was resuspended in a medium with 25 mM Tris–HCl (pH 7.2)/100 mM mannitol to a final protein concentration of 2–4 mg/ml. A 5-ml aliquot of this final suspension was layered over a discontinuous sucrose gradient comprised of 10 ml of 893 mM sucrose and 10 ml of 250 mM sucrose/20 mM Tris–HCl (pH 7.2) (sucrose/Tris). The following steps were essentially different from those used by Kinsella *et al.* [13]. The gradient was centrifuged at 48,000 *g* for 20 min. At the end of the centrifugation period, the sucrose gradient was carefully removed by aspiration, and the soft, lighter upper portion of the pellet was resuspended in the sucrose/Tris medium. This final suspension was dialyzed for 30 min at 4° against 100 vol. of the sucrose/Tris medium and then re-dialyzed overnight at 4° against 100 vol. of the same fresh medium. At the end of the dialysis period, this final membrane preparation was assayed for Na^+,K^+-ATPase activity.

SDS/BSA pretreatment. The above final membrane suspension (250 μl; 0.1 mg protein/ml) was treated with sodium dodecyl sulfate (SDS), in the presence of 1% bovine serum albumin (BSA), 25 mM imidazole (pH 7.0) and 2 mM EDTA. After incubation for 20 min at 37°, the SDS-treated membranes were assayed for ATPase activity. The SDS/protein ratio during the pretreatment was 1.6 or 0.4 for the Na^+,K^+- or the Na^+-ATPase assay respectively. These ratios were found to be the best for optimal activity in each case.

ATPase assays. The Na^+,K^+-ATPase activity (EC 3.6.1.3) was measured by a modification of a previously described method [6–10]. A 180-μl aliquot of the incubation medium containing (final concentrations) 50 mM Tris–HCl (pH 7.0), 5 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 2 mM Tris–ATP and, when required, 7 mM ouabain was preincubated at 37° for 5 min. The reaction was started by adding 20 μl of the membrane suspension (0.1 mg protein/ml) and continued for the prescribed time. The reaction was stopped and the inorganic phosphate was determined following the method of Baginski *et al.* [14], modified by Ottolenghi [15], Brotherus *et al.* [16], Forbush [17] and ourselves. At the end of the incubation period, 300 μl of an ice-cold solution of 2.8% ascorbic acid, 0.48 N HCl, 0.48% ammonium molybdate, and 2.8% SDS was added, and the test tubes were placed on ice. After 10 min at 0°, 500 μl of a solution of 2% sodium arsenite, 2% sodium citrate and 2% acetic acid was added, and the test tubes were rewarmed to 37° for 10 min. At the end of the rewarming period the absorbance was determined at 705 nm (16 ± 2 absorbance/μmole P_i in the above volume). All samples were run in quadruplicate. The activity was expressed as nmoles of P_i liberated per mg of protein per min, after subtraction of a blank run in parallel without the membrane suspension, which was added after the ascorbic acid/HCl/ammonium molybdate/SDS solution. The protein content of the original suspension was determined by the Coomassie blue method [18, 19]. Na^+,K^+-ATPase activity was calculated as the difference between the P_i liberated in control tubes and that liberated in the presence of 7 mM ouabain.

The Na^+-ATPase activity was determined in a similar way, in the same incubation medium, without K^+, and with the addition of 50 μM Ca^{2+}. A 2 mM concentration of ouabain was always present. The assays were run in the presence or absence of 2 mM furosemide in the incubation medium. The Na^+-ATPase activity was calculated as the difference between the P_i liberated in the absence and presence of furosemide. This agent was shown to inhibit completely the expression of the Na^+-ATPase [7, 10].

Chemicals. ATP, ouabain (strophanthin-G), SDS and EDTA were purchased from the Sigma Chemical Co., St. Louis, MO. Furosemide was a gift from Medicamentos York S.A., Caracas.

RESULTS

The basolateral plasma membrane fractions from control (normal diet) and experimental (high Na^+ diet) rat kidneys were tested routinely for several enzymatic membrane markers. These fractions were enriched in Na^+-ATPase and Na^+,K^+-ATPase (33-fold enrichment when compared with the activity of homogenates, for both groups of rats). These enzymes have been demonstrated to be specific markers of basolateral plasma membranes [6, 7]. All the other tested enzymatic markers (5′-nucleotidase/brush border membranes; glucose-6-phosphatase/endoplasmic reticulum membranes; succinic dehydrogenase/mitochondrial membranes; acid phosphatase/lysosomal membranes) had specific activities similar to, or lower than, the specific activity of the homogenate (data not shown). Accordingly, these fractions were enriched in basolateral plasma membranes, with low contamination by brush-border; endoplasmic reticulum; mitochondrial or lysosomal membranes.

After 4 months of a high Na^+ diet, the Na^+-ATPase activity of experimental rats showed marked variations. Therefore, all the experiments were carried out with rats loaded with Na^+ during 4 months.

Table 1 shows the Na^+- and the Na^+,K^+-ATPase activities of basolateral plasma membranes of proximal tubular cells from control and experimental rat kidneys. The Na^+,K^+-ATPase activity was similar for both groups. This was not the case for the Na^+-ATPase activity, which was approximately 70% higher for the experimental rats.

Figure 1 illustrates the behavior of the Na^+-ATPase of control and experimental rats as the Mg^{2+}: ATP concentrations of the incubation medium (panel A) and the Na^+ concentrations (panel B) were varied. In previous experiments it had been found that the optimal Mg^{2+}: ATP ratio for both control and experimental rats was 2.5:1 (mM:mM). This was the Mg^{2+}: ATP ratio used in all the experiments. The ouabain-insensitive Na^+-ATPase activities, expressed as percent of maximal activity in each case (control and experimental rats), behaved similarly as the Mg^{2+}: ATP concentration (Fig. 1, panel A) was changed. The maximum activity was reached, in both cases, at a concentration of Mg^{2+}: ATP of 5:2 (mM:mM). The K_m, for both groups, calculated by means of the Woolf derivative of the Lineweaver–Burk transformation of the Michaelis–Menten equation, was 1.2:0.48

Table 1. Na^+ and Na^+,K^+-ATPase activities in basolateral plasma membranes from kidney proximal tubular cells of control (normal diet) and experimental (high Na^+ diet) rats

Diet	Na^+-ATPase activity [nmoles P_i liberated · (mg protein)$^{-1}$ · min^{-1}]	Na^+,K^+-ATPase activity [nmoles P_i liberated · (mg protein)$^{-1}$ · min^{-1}]
Control	189 ± 10	1952 ± 82
High sodium	318 ± 19	2013 ± 76

SDS treatment of the membranes and ATPase activity assays were carried out as indicated under Materials and Methods. Values are expressed as mean ± S.E. (N = 6).

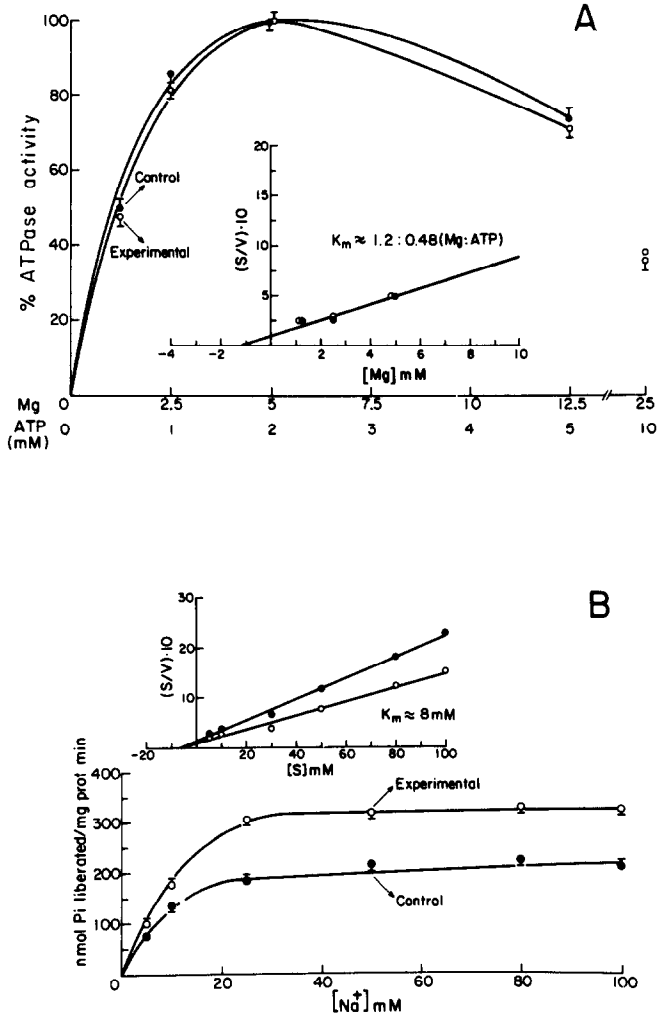

Fig. 1. (A) Effect of increasing the Mg^{2+} : ATP concentrations at fixed ratios of 2.5 : 1 in the incubation medium on the ouabain-insensitive, Na^+-ATPase activity of basolateral plasma membranes from kidney proximal tubular cells of control (100% ATPase activity: 194 ± 7 nmoles P_i · (mg protein)$^{-1}$ · min^{-1}) and experimental (100% ATPase activity: 330 ± 21 nmoles P_i · (mg protein)$^{-1}$ · min^{-1}) rats. (B) Effect of increasing Na^+ concentration (as NaCl) on the Na^+-ATPase activity of control and experimental basolateral plasma membranes. In both cases (panels A and B), assays were performed in the presence of 50 μM Ca^{2+} and 2 mM ouabain. Mg concentration: 5 mM; and ATP, 2 mM. Values are expressed as mean ± S.E. (N = 6).

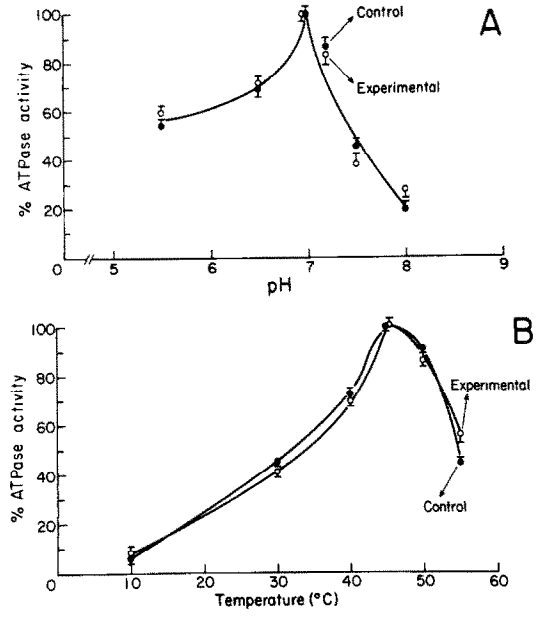

Fig. 2. Effect of pH (A) and temperature (B) of the incubation medium on the ouabain-insensitive, Na^+-ATPase activity of basolateral plasma membranes from control and experimental rats. One hundred percent ATPase activities in panel A: 176 ± 12 nmoles $P_i \cdot (mg\ protein)^{-1} \cdot min^{-1}$ (control) and 325 ± 16 nmoles $P_i \cdot (mg\ protein)^{-1} \cdot min^{-1}$ (experimental). One hundred percent ATPase activities in panel B: 270 ± 23 nmoles $P_i \cdot (mg\ protein)^{-1} \cdot min^{-1}$ (control) and 480 ± 27 nmoles $P_i \cdot (mg\ protein)^{-1} \cdot min^{-1}$ (experimental). In both cases (panels A and B), assays were performed in the presence of 50 μM Ca^{2+} and 2 mM ouabain. Mg^{2+} concentration; 5 mM; ATP, 2 mM; and NaCl, 100 mM. Values are expressed as mean ± S.E. (N = 6).

(mM:mM) for Mg^{2+}:ATP. Panel B of Fig. 1 illustrates the ouabain-insensitive Na^+-ATPase activities for both groups of rats as a function of the Na^+ concentration of the incubation medium. The V_{max} (as shown in Table 1) of the Na^+-ATPase of experimental rats was about 70–80% higher than the V_{max} of control rats. In both cases, the optimum activities were reached at about 30 mM Na^+, and the K_m, as shown in the insert, was about 8 mM.

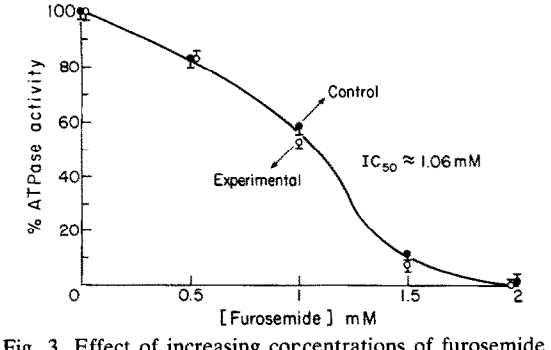

Fig. 3. Effect of increasing concentrations of furosemide on the Na^+-ATPase activity of basolateral plasma membranes from control (100% ATPase activity: 195 ± 9 nmoles $P_i \cdot (mg\ protein)^{-1} \cdot min^{-1}$) and experimental (100% ATPase activity: 315 ± 17 nmoles $P_i \cdot (mg\ protein)^{-1} \cdot min^{-1}$) rats. Assays were performed in the presence of 50 μM Ca^{2+} and 2 mM ouabain. Mg^{2+} concentration; 5 mM; ATP, 2 mM; and NaCl, 100 mM. Values are expressed as mean ± S.E. (N = 6).

Figure 2 illustrates the behavior of the Na^+-ATPase of control and experimental rats as the pH (panel A) and the temperature (panel B) of the incubation medium were changed. For both variables, the behaviors were very similar. The optimum pH value, in both cases, was around 7. The optimum temperature, in both cases, was 45°.

Finally, we studied the sensitivity of the Na^+-ATPase, for control and experimental rats, to furosemide. The results are shown in Fig. 3. The sensitivity of Na^+-ATPase from both groups of rats varied similarly in different concentrations of furosemide. In both cases, the Na^+-ATPase activity was inhibited totally at a concentration of 1.5 mM. The concentration for 50% inhibition (IC_{50}) for control and experimental rats was 1.06 mM.

DISCUSSION

Basolateral plasma membranes of kidney proximal tubular cells from rats chronically loaded with Na^+ for 4 months showed an interesting change in their Na^+-stimulated ATPase activities. Whereas the ouabain-sensitive Na^+,K^+-ATPase activity did not change, the activity of the ouabain-insensitive Na^+-ATPase was enhanced about 70% (Table 1). The selective increase of the Na^+-ATPase activity can be explained in several ways: (1) changes in the affinities of the system to its ligands, (2) changes in the behavior of the system toward the incubation conditions, (3) presence of more Na^+-ATPases in the basolateral plasma membranes of the experimental rats, and/or (4) increase in the turnover of Na^+-ATPase by the experimental treatment. The first two possibilities can be disregarded, since the results were not due to a change in the affinity of the enzyme to its ligands (Fig. 1) or to a change in the behavior of the system toward the incubation conditions (Fig. 2).

It is very difficult to find a physiological explanation for our results. Administration of saline to different animals is known to increase the rate of sodium excretion, as a consequence of decreased proximal tubular sodium reabsorption [20–22]. Two hypotheses have been raised to try to explain this phenomenon: (a) production of a natriuretic factor, which, by inhibiting the Na^+,K^+-ATPase activity, would inhibit Na^+ reabsorption [23]; (b) increase in the passive component of Na^+ backflux from the peritubular side to the lumen [24].

A decreased proximal tubular sodium reabsorption would be expected to be associated with a concomitant decrease in Na^+-ATPase activity. The unexpected and selective increase in Na^+-ATPase activity found in this work can be explained by arguing that chronic inhibition of the Na^+,K^+-ATPase activity "*in vivo*", and hence of the Na^+,K^+-pump by a possible natriuretic factor, could drive the kidney proximal tubular cells of the experimental rats to produce more Na^+-pumps or to increase the turnover of the already existent Na^+-pumps to try to compensate for the inhibition of the Na^+,K^+-pump.

The selective increase of the Na^+-ATPase activity

as a consequence of a chronic Na^+ loading clearly differentiates it from the Na^+,K^+-ATPase, which did not show any increase as a consequence of the experimental treatment. This is a very important point. There are still serious doubts regarding the existence of the Na^+-ATPase. Similar to the Na^+,K^+-ATPase, the Na^+-ATPase is stimulated by Na^+, and both systems are located at the same membrane (basolateral plasma membrane) [6, 7]. These characteristics, together with the fact that the activity of the Na^+-ATPase is only a small fraction of the activity of the Na^+,K^+-ATPase (8–10%), are usually taken as an indication that the Na^+-ATPase activity is just a partial expression of the Na^+,K^+-ATPase. Our results demonstrate that this is not the case. The Na^+-ATPase activity can be selectively increased without changing the Na^+,K^+-ATPase activity. This selective increase is a demonstration that they are two different enzymes.

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