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Spectrophotometric assay of renal ouabain-resistant Na^+-ATPase and its regulation by leptin and dietary-induced obesity✪

Jerzy Bełtowski✉, Anna Jamroz-Wiśniewska, Jarosław Nazar and Grażyna Wójcicka

Department of Pathophysiology, Medical University, Lublin, Poland

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Apart from Na^+,K^+-ATPase, a second sodium pump, Na^+-stimulated, K^+-independent ATPase (Na^+-ATPase) is expressed in proximal convoluted tubule of the mammalian kidney. The aim of this study was to develop a method of Na^+-ATPase assay based on the method previously used by us to measure Na^+,K^+-ATPase activity (*Acta Biochim Polon.*; 2002, 49: 515–27). The ATPase activity was assayed as the amount of inorganic phosphate liberated from ATP by isolated microsomal fraction. Na^+-ATPase activity was calculated as the difference between the activities measured in the presence and in the absence of 50 mM NaCl. Na^+-ATPase activity was detected in the renal cortex (3.5 ± 0.2 μmol phosphate/h per mg protein), but not in the renal medulla. Na^+-ATPase was not inhibited by ouabain or an H^+,K^+-ATPase inhibitor, Sch 28080, but was almost completely blocked by 2 mM furosemide. Leptin administered intraperitoneally (1 mg/kg) decreased the Na^+,K^+-ATPase activity in the renal medulla at 0.5 and 1 h by 22.1% and 27.1%, respectively, but had no effect on Na^+-ATPase in the renal cortex. Chronic hyperleptinemia induced by repeated subcutaneous leptin injections (0.25 mg/kg twice daily for 7 days) increased cortical Na^+,K^+-ATPase, medullary Na^+,K^+-ATPase and cortical Na^+-ATPase by 32.4%, 84.2% and 62.9%, respectively. In rats with dietary-induced obesity, the Na^+,K^+-ATPase activity was higher in the renal cortex and medulla by 19.7% and 34.3%, respectively, but Na^+-ATPase was not different from control. These data indicate that both renal Na^+-dependent ATPases are separately regulated and that up-regulation of Na^+-ATPase may contribute to Na^+ retention and arterial hypertension induced by chronic hyperleptinemia.

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✉Correspondence to: Jerzy Bełtowski, Department of Pathophysiology, Medical University of Lublin, Jaczewskiego 8, 20-090 Lublin, Poland; phone: (48 81) 742 5837; fax: (48 81) 742 5828; e-mail: patfiz@asklepios.am.lublin.pl

Abbreviation: CV, coefficient of variation.

Na^+,K^+-ATPase, located in the basolateral membranes of renal tubular cells, drives active Na^+ reabsorption throughout the nephron and is involved in the regulation of extracellular fluid volume and blood pressure (Feraille & Doucet, 2001). Apart from Na^+,K^+-ATPase, a second sodium pump, so called Na^+-ATPase, is expressed in proximal convoluted tubule of the mammalian kidney. This enzyme also actively extrudes Na^+, however, this is not coupled with K^+ transport. Unlike Na^+,K^+-ATPase, Na^+-ATPase does not require K^+ for its hydrolytic activity and is not sensitive to ouabain, but is inhibited by loop diuretics such as furosemide and ethacrynic acid (Whittembury & Proverbio, 1970; Proverbio *et al.,* 1975).

Due to its key role in systemic sodium balance, the regulation of renal Na^+,K^+-ATPase has been extensively studied. In contrast, little is known about the mechanisms which control Na^+-ATPase activity, even though it may transport up to 15% of sodium reabsorbed in the proximal tubule. Taking into account that this nephron segment reabsorbs about 60–70% of filtered Na^+ load, Na^+-ATPase is responsible for the transport of about 10% of filtered sodium, i.e. 10-fold more than the amount finally excreted in urine. Thus, changes in Na^+-ATPase activity could markedly modulate renal sodium handling.

Recently, we have developed a method of renal Na^+,K^+-ATPase assay based on non-isotopic, spectrophotometric determination of inorganic phosphate liberated from ATP. Furthermore, we extended this method to the measurement of ouabain-sensitive H^+,K^+-ATPase, whose activity is about 1/6 of that of Na^+,K^+-ATPase (Bełtowski & Wójcicka, 2002). In the present study we adopted this method for the measurement of renal ouabain-resistant Na^+-ATPase. In addition, to recognize whether this enzyme is regulated, we investigated the effect of an adipose tissue hormone, leptin, as well as of experimental obesity on its activity. Both leptin and dietary-induced obesity have been previously demonstrated to modulate renal Na^+,K^+-ATPase (Hussain *et al.,* 1999; Sweeney *et al.,* 2000; Bickel *et al.,* 2001; Bełtowski *et al.,* 2002; 2004).

MATERIALS AND METHODS

Reagents. The specific inhibitor of H^+,K^+-ATPases, 2-methyl 8-(phenylmethoxy)imidazol(1,2-α) pyridine-3-acetonitrile (Sch 28080) was kindly provided by the Schering-Plough Research Institute (Kenilworth, NJ, U.S.A.). Recombinant human leptin was purchased from Calbiochem-Novabiochem (San Diego, CA, U.S.A.). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Animals and tissue collection. All studies were performed on adult male Wistar rats weighing 200–250 g. The study protocol was approved by the Bioethics Committee of the Medical University of Lublin.

The rats were anaesthetized with pentobarbital (50 mg/kg i.p.). The abdominal cavity was opened and 5 ml of 0.9% NaCl was infused into the aorta distally to the renal arteries to remove erythrocytes from the kidney. The kidneys were excised and the animals sacrificed by a lethal dose of pentobarbital. ATPases were assayed in the microsomal fraction isolated from the renal cortex and medulla. The procedure of isolation was described in detail previously (Bełtowski & Wójcicka, 2002).

Na^+-ATPase assay. Na^+-stimulated ATPase activity (Na^+-ATPase) was assayed by measuring the amount of inorganic phosphate (P_i) liberated from ATP during the incubation of the microsomal fraction in the presence of appropriate activators. Until otherwise stated, basal activity was assayed in a solution (1 ml) containing: 10 mM $MgCl_2$, 50 mM Tris/HCl (pH 7.4), 50 mM choline chloride, 25 μM $CaCl_2$ and 2 mM ouabain. Calcium was added to block Na^+-inhibited ATPase which is also present in the rat kid-

ney and could interfere with the Na^+-stimulated ATPase assay (el Mernissi *et al.*, 1991). After adding 50 μg of microsomal protein, the incubation mixture was prewarmed at 37°C for 10 min and then 5 mM of Tris-ATP was added. For the measurement of Na^+-stimulated ATPase, choline chloride was replaced with 50 mM NaCl. After 30 min, the enzymatic reaction was terminated by adding 0.35 ml of ice-cold 1 M $HClO_4$. Then, P_i was assayed as previously described (Bełtowski & Wójcicka, 2002). The absorbance of a blank sample which did not contain microsomal protein was subtracted to correct for spontaneous ATP breakdown. Na^+-ATPase activity was calculated as the difference between the activities assayed in the presence and in the absence of 50 mM NaCl and was expressed in μmole of P_i liberated by 1 mg of microsomal protein during 1 h (μmol/h per mg protein). Protein concentration in the microsomal fraction was assayed by the method of Lowry *et al.* (1951).

Kinetic calculations. The effect of Na^+ concentration on Na^+-ATPase was examined by measuring its activity in the presence of 1, 2, 5, 10, 20, 30, 40 or 50 mM Na^+. The relationship between Na^+ concentration and enzyme activity was fitted to the following equation:

$$v = V_{max} \times [Na^+]/([Na^+] + K_{0.5}),$$

where: v is actual enzyme activity, V_{max} is maximal activity at saturating sodium concentration, $[Na^+]$ is actual sodium concentration, $K_{0.5}$ is the dissociation constant for Na^+.

The inhibition of Na^+-ATPase by furosemide and ethacrynic acid was analyzed according to the following formula:

$$v = V_{max}/(1 + [I]/K_i)$$

where v is the activity measured at a given concentration of inhibitor [I], V_{max} is the maximal activity and K_i is the inhibition constant. The kinetic parameters were calculated by linear least square regression after Lineweaver-Burk transformation of the above relationships:

$$1/v = (1/[Na^+]) \times (K_{0.5}/V_{max}) + 1/V_{max}$$

and:

$$1/v = [I] \times 1/(V_{max} \times K_i) + 1/V_{max}$$

$K_{0.5}$ and K_i were calculated separately for each animal and the values reported in the text are means ± S.E.M. from 8 rats.

To study the reproducibility of the Na^+-ATPase assay, samples from 5 animals were assayed in 5 replicates simultaneously to calculate the intra-assay coefficient of variation (CV), as well as on 5 different days to calculate the inter-assay CV. The recovery of inorganic phosphate was studied by adding 64, 320 or 640 nmol (each in triplicate) of KH_2PO_4 to the incubation mixture during the enzyme assay. The sensitivity was determined as 2 standard deviations of 10 independently assayed blank samples.

Na^+,K^+-ATPase assay. Na^+,K^+-ATPase activity was measured in the presence of 100 mM NaCl and 20 mM KCl and was calculated as the difference between the activities assayed in the absence and in the presence of 2 mM ouabain; both samples containing 0.2 mM Sch 28080 to block the ouabain-sensitive H^+,K^+-ATPase (Bełtowski & Wójcicka, 2002). To examine whether the Na^+-stimulated ATPase contributes to Na^+,K^+-ATPase activity in our assay, additional samples from each homogenate were run in the presence of inhibitors of Na^+-stimulated ATPase, furosemide or ethacrynic acid, each at a concentration of 2 mM.

Acute leptin administration. Leptin (1 mg/kg) or the same dose of bovine serum albumin (BSA) was injected intraperitoneally and the animals were sacrificed after 0.5, 1 or 2 h (Bełtowski *et al.*, 2002). ATPases were assayed as described above.

Chronic leptin administration. Three groups of rats were used in these studies: 1) control group, fed standard rat chow (62% calories from carbohydrates, 26% protein and 12% fat) *ad libitum*, 2) leptin-treated group, which received leptin injections (0.25 mg/kg twice daily sc for 7 days), 3) pair-fed group, in which the food intake was adjusted to the leptin group. This dose of leptin raises its plasma concentration to the level observed in rodent obesity (Bełtowski *et al.,* 2004).

Induction of obesity. The effect of experimental obesity on renal Na^+-stimulated ATPase was studied in two separate groups of animals. One group of rats was fed standard laboratory chow *ad libitum*. The second group received a "cafeteria diet" containing standard chow combined 1:1 with a liquid diet consisting of equal amounts of sucrose, glucose, whole milk powder and soybean powder. The caloric composition of this diet (60% carbohydrates, 26% protein, 16% fat) was similar to standard chow. Both groups were fed their respective diets for 4 weeks.

Statistics. Data are presented as mean ± S.E.M. from 8 animals in each group. Statistical significance was evaluated by unpaired or paired Student's *t*-test or by ANOVA followed by Duncan's multiple range test for comparison of 2 or > 2 groups, respectively. $P < 0.05$ was considered significant.

RESULTS

Na^+-stimulated ATPase in renal tissue and its sensitivity to inhibitors

The basal ATPase activity measured in the absence of either Na^+ or K^+ was 8.1 ± 0.4 μmol P_i/h per mg protein in the renal cortex and 10.8 ± 0.6 μmol P_i/h per mg protein in the renal medulla. In the renal cortex, addition of 50 mM NaCl to the incubation medium stimulated this basal activity by 43.2% (Fig. 1). In contrast, Na^+ did not stimulate ATPase activity in the renal medulla. These data are consistent with the exclusive expression of Na^+-stimulated ATPase in the proximal convoluted tubule contained in the renal cortex. Therefore, all other studies were confined to the renal cortex.

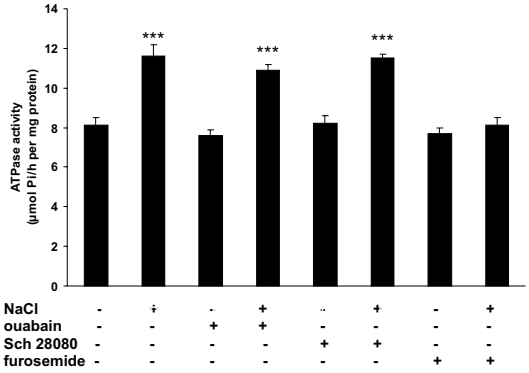

Figure 1. Na^+-stimulated ATPase activity in the renal cortex and its sensitivity to inhibitors.

ATPase was assayed in isolated microsomal fraction. Enzyme activities are expressed in μmole of inorganic phosphate liberated by 1 mg of microsomal protein during 1 h. Basal activity was assayed in the presence of 10 mM $MgCl_2$, 50 mM Tris/HCl (pH 7.4), 50 mM choline chloride and 25 μM $CaCl_2$. To measure Na^+-stimulated activity, choline chloride was replaced with 50 mM NaCl. In separate samples, ouabain (2 mM), Sch 28080 (0.2 mM) or furosemide (2 mM) was added to the incubation medium. ***$P < 0.001$; compared to basal activity by paired Student's *t*-test.

Addition of 2 mM ouabain to the incubation mixture tended to reduce the ATPase activity measured both in the absence and in the presence of Na^+, however, this effect did not reach the level of statistical significance. The difference between these two activities, i.e. Na^+-ATPase activity, was similar in the absence and in the presence of ouabain (3.5 ± 0.2 and 3.3 ± 0.3 μmol P_i/h per mg protein, respectively). Sch 28080 (0.2 mM) had no effect on either the basal or Na^+-stimulated ATPase activity. In contrast, 2 mM furosemide completely abolished the Na^+-stimulated activity (Fig. 1). Also 2 mM ethacrynic acid decreased the Na^+-ATPase activity to undetectable values (not shown). Thus, the Na^+-stimulated ATPase activity can be calcu-

lated in two ways: (1) as the difference between the activities measured in the presence and in the absence of Na^+; (2) as the difference between the activities measured in the absence and in the presence of 2 mM furosemide, both samples containing Na^+ ions. Despite its insensitivity to ouabain, in subsequent experiments Na^+-stimulated ATPase was assayed in the presence of 2 mM of this inhibitor to eliminate any possible contribution of Na^+,K^+-ATPase.

Table 1 presents Na^+-stimulated ATPase activity measured in the presence of compounds known to inhibit other ATPases. An inhibitor of mitochondrial ATPase, sodium azide (2 mM), as well as two inhibitors of V-type H^+-ATPase, bafilomycin A1 (1 μM) and *N*-ethylmaleimide (2 mM) had no effect on Na^+-stimulated ATPase activity. These data indicate that the Na^+-stimulated ATPase activity is not the expression of Na^+,K^+-ATPase, H^+,K^+-ATPase, mitochondrial ATPase, or H^+-ATPase. In contrast, 1 μM sodium orthovanadate, an inhibitor of all P-type ATPases, completely inhibited the Na^+-stimulated ATPase activity (Table 1).

Table 1. Na^+-ATPase activity in the renal cortex measured in the presence of various activators and inhibitors.

Na^+-ATPase was calculated as the difference between the activities assayed in the presence and in the absence of 50 mM NaCl, both containing 2 mM ouabain. In separate samples, NaCl was replaced by equimolar concentration of other salts.

Activator	Inhibitor	Na^+-ATPase activity (μmol P_i/h per mg protein)
NaCl	–	3.3 ± 0.3
NaCl	Sodium azide (2 mM)	3.2 ± 0.2
NaCl	Bafilomycin A1 (1 μM)	3.5 ± 0.4
NaCl	*N*-ethylmaleimide (2 mM)	3.7 ± 0.5
NaCl	Sodium orthovanadate (1 μM)	0.4 ± 0.3
KCl	–	2.2 ± 0.4
KCl	Sch 28080 (0.2 mM)	0.2 ± 0.3
KCl	Furosemide (2 mM)	2.3 ± 0.5
RbCl	–	2.0 ± 0.4
RbCl	Sch 28080 (0.2 mM)	0.3 ± 0.2
RbCl	Furosemide (2 mM)	2.1 ± 0.5
LiCl	–	2.8 ± 0.3
LiCl	Sch 28080 (0.2 mM)	2.6 ± 0.3
LiCl	Furosemide (2 mM)	0.1 ± 0.4
CsCl	–	0.3 ± 0.3
NH_4Cl	–	0.2 ± 0.4
Na_2SO_4	–	3.4 ± 0.4
NaBr	–	3.6 ± 0.3
Sodium lactate	–	3.5 ± 0.4
Sodium acetate	–	3.7 ± 0.4
Sodium pyruvate	–	3.0 ± 0.2

Next, we investigated whether other monovalent cations mimic the effect of sodium. For this purpose, NaCl was replaced with KCl, RbCl, LiCl, CsCl or NH_4Cl. KCl stimulated ATPase activity by 2.2 ± 0.4 μmol/h per mg protein. However, this activity was most likely accounted for by ouabain-resistant H^+,K^+-ATPase, because it was inhibited by 0.2 mM Sch 28080 but not by furosemide (Table 1). Similarly, RbCl caused stimulation of ATPase activity which was also sensitive to Sch 28080 but not to furosemide. It is well known that Rb^+ can be transported by Na^+,K^+-ATPase and H^+,K^+-ATPases instead of potassium (Féraille & Doucet, 2001), thus it can readily stimulate the ouabain-resistant H^+,K^+-ATPase. LiCl increased ATPase activity and this Li^+-stimulated activity was abolished by 2 mM furosemide but not by Sch 28080, suggesting that it is accounted for by Na^+-ATPase. Also previous studies demonstrated that Li^+ could activate renal ouabain-resistant Na^+-ATPase (Proverbio & Del Castillo, 1982). Neither CsCl nor NH_4Cl had any stimulatory effect on cortical ATPase activity (Table 1).

Subsequently, we examined whether the anion accompanying Na^+ has any effect on Na^+-activated ATPase. Both inorganic (Na_2SO_4, NaBr) and organic (Na lactate, pyruvate or acetate) salts had comparable stimulatory effects on ATPase activity (Table 1). Independently of the accompanying anion, Na^+-stimulated activity was abolished by 2 mM furosemide (not shown).

Characteristics of Na^+-stimulated ATPase assay

To verify the method of Na^+-ATPase assay, we first determined the relationship between incubation time and the measured activity. We measured enzyme activity after 10, 20, 30, 40, 50, 60 and 70 min of incubation. Within this range, the relationship between Na^+-stimulated activity and incubation time was very good (r^2 = 0.91, $P < 0.001$). To investigate the effect of the amount of microsomal protein used for enzyme assay, we added 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 μg protein to the reaction mixture and incubated it for 30 min. The linearity was also excellent (r^2 = 0.95, $P < 0.001$). Thus, the conditions used in the initial studies (50 μg protein and 30-min incubation time) are well within the linearity range. The intra- and inter-assay CVs for Na^+-ATPase were 2.4% and 4.8%, respectively. The recovery of inorganic phosphate was 105 ± 3%, 96 ± 2% and 84 ± 4% for 64, 320 and 640 nmol of P_i added to the incubation medium, respectively. It should be noted that 64 and 320 nmol P_i correspond to the ATPase activities of 2.6 and 12.8 μmol P_i/h per mg protein. Thus, the recovery is satisfactory within the range of P_i liberated by Na^+-ATPase. The detection limit of the assay was 0.31 μmol/h per mg protein.

Kinetic analysis of Na^+-ATPase

The renal cortex Na^+-ATPase was activated by sodium in a concentration-dependent manner with $K_{0.5}$ of 12.3 ± 1.1 mM (Fig. 2A). Furosemide induced a concentration-dependent decrease in the enzyme activity with half-maximal inhibition observed at 0.93 ± 0.05 mM (Fig. 2B). Ethacrynic acid caused a similar inhibition with K_i of 0.98 ± 0.09 mM (Fig. 2C).

Effect of Na^+-ATPase inhibitors on Na^+,K^+-ATPase activity

To examine whether furosemide and ethacrynic acid, which at 2 mM almost completely block Na^+-ATPase, have also any effect on Na^+,K^+-ATPase, we assayed its activity in the presence of these inhibitors. The Na^+,K^+-ATPase activity measured in the presence of 2 mM furosemide was lower than in the absence of this drug by 9.4% ($P < 0.05$) in the renal cortex and by 8.1% ($P < 0.05$) in the renal medulla. Ethacrynic acid at the same concentration decreased the cortical and

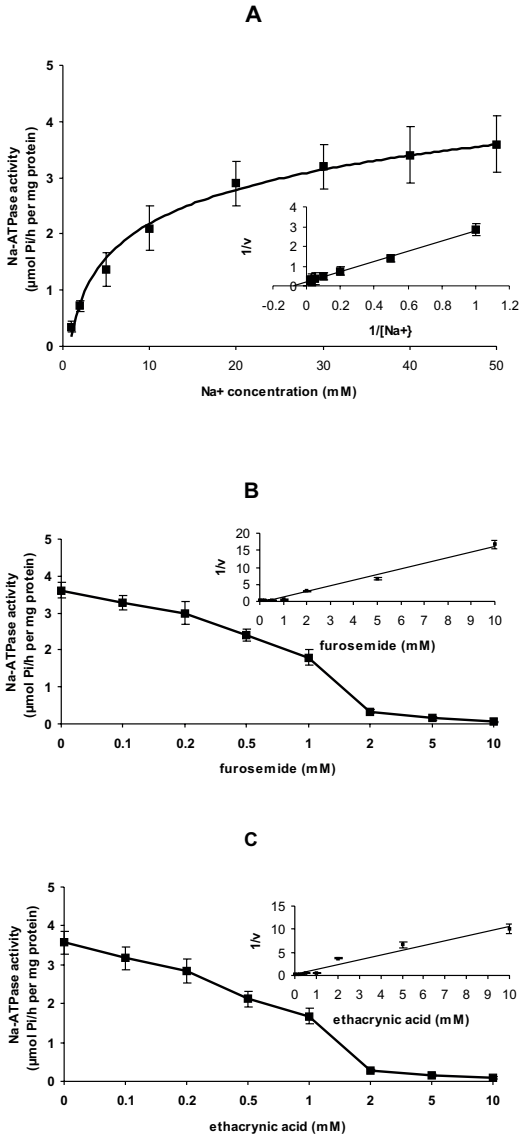

Figure 2. The effect of sodium concentration (A), furosemide (B) and ethacrynic acid (C) on Na^+-ATPase activity in the renal cortex.

A. Na^+-ATPase was calculated as the difference between the activities measured in the presence of 1, 2, 5, 10, 20, 30, 40 or 50 mM NaCl and in the absence of this salt, both samples containing 2 mM ouabain. **B** and **C**. Na^+-ATPase was calculated as the difference between the activities measured in the presence and in the absence of 50 mM NaCl, both containing 0.1, 0.2, 0.5, 1, 2, 5 or 10 mM of inhibitor. Insets: plots of Lineweaver-Burk transformation of Michaelis-Menten equation (**A**. relationship between 1/[Na^+] and 1/v; **B** and **C**. relationships between inhibitor concentration and 1/v). Points represent means ±S.E.M. from 8 animals and lines are the best fit of means to the respective equations (see Methods).

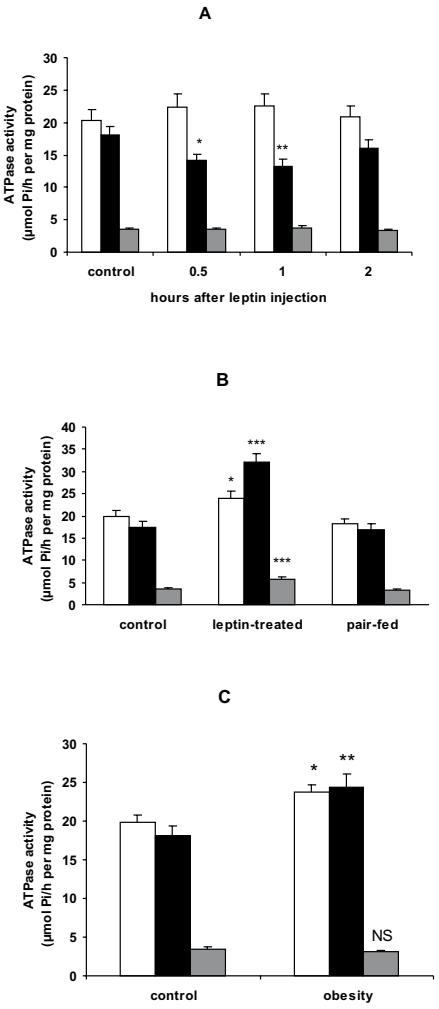

Figure 3. Regulation of renal Na^+,K^+-ATPase and Na^+-ATPase by leptin and experimental obesity.

A. Acute effect of leptin. Leptin was administered intraperitoneally at a dose of 1 mg/kg. The animals were anesthetized and the kidneys were obtained after 0.5, 1 or 2 h after injection. **B**. Chronic effect of leptin. Control group was fed standard chow *ad libitum*. Leptin-treated group received leptin injections (0.25 mg/kg twice daily s.c. for 7 days). In pair-fed group, the amount of food served was adjusted to the amount consumed by the leptin-treated group. Control and pair-fed animals received bovine serum albumin (0.25 mg/kg twice daily s.c. for 7 days). **C**. Effect of dietary-induced obesity. Control group was fed standard chow *ad libitum*. Another group received "cafeteria diet" for 4 weeks (see Methods for details). Na^+,K^+-ATPase was assayed in the renal cortex (open bars) and medulla (closed bars) and Na^+-ATPase activity was measured in the renal cortex (grey bars). NS – not significant, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to control by ANOVA and Duncan's test (A and B) or by unpaired Student's *t*-test (C).

medullary Na^+,K^+-ATPase activity by 36.9% and 37.9%, respectively (both $P < 0.001$).

Acute effect of leptin on renal Na^+-ATPase

Leptin decreased the Na^+,K^+-ATPase activity in the renal medulla at 0.5 and 1 h by 22.1% and 27.1%, respectively. The Na^+,K^+-ATPase activity in the renal cortex did not change following leptin administration. In addition, leptin had no effect on renal cortical Na^+-ATPase (Fig. 3A).

Effect of chronic hyperleptinemia on renal Na^+-ATPase

The Na^+,K^+-ATPase activity was higher in leptin-treated than in control rats by 32.4% in the renal cortex and by 84.2% in the renal medulla (Fig. 3B). The Na^+-ATPase activity in the renal cortex was by 62.9% higher in animals receiving leptin. In contrast, pair-feeding had no effect on either Na^+-ATPase or Na^+-ATPase. Thus, hyperleptinemia causes a coordinate up-regulation of both renal sodium pumps.

Renal Na^+-ATPase in experimental obesity

After 4 weeks of feeding, body mass of "cafeteria diet"-fed rats was by 34.3% higher than in control animals (356 ± 21 g *vs* 265 ± 12 g, $P < 0.001$). The Na^+,K^+-ATPase activity in the obese rats was higher by 19.7% and 34.3% in the renal cortex and medulla, respectively. In contrast, Na^+-ATPase in the renal cortex was similar in the lean and obese groups (Fig. 3C).

DISCUSSION

Na^+-ATPase activity is detected in renal tissue of various mammalian species including guinea-pig (Proverbio *et al.*, 1975; Del Castillo *et al.*, 1982), rat (Marin *et al.,* 1985; 1990; Proverbio *et al.,* 1986; el Mernissi *et al.,* 1991) and pig (Caruso-Neves *et al.,* 2000a; 2002). The enzyme is involved in active tubular Na^+ reabsorption (Proverbio & Del Castillo, 1981; Del Castillo *et al.,* 1982) and its activity is modulated by dietary sodium intake (Obando *et al.,* 1987; Di Campo *et al.,* 1990; 1991), however, the mechanisms regulating Na^+-ATPase are poorly understood. In fact, currently only one group investigates hormonal regulation of this enzyme using isolated basolateral membranes of the pig kidney (Caruso-Neves *et al.,* 1997, and later papers of these authors). In this broken cell preparation, the intracellular signalling machinery is undoubtedly incomplete. Using this approach, it has been demonstrated that Na^+-ATPase is regulated by natriuretic and antinatriuretic mediators such as angiotensin II, angiotensin (1-7), adenosine, bradykinin and atrial natriuretic peptide (Caruso-Neves *et al.*, 1997; 1999; 2000a; 2004; Rangel *et al.,* 1999).

Recently, we have developed a new model to study the regulation of renal Na^+,K^+-ATPase (Bełtowski *et al.,* 1998). In this model, an investigated mediator is administered *in vivo* by local infusion and then Na^+,K^+-ATPase activity is assayed in isolated microsomal fraction. The advantage of this approach is that the mediator of interest acts on intact renal tissue with preserved physicochemical features of the microenvironment, e.g. pH and oxygen tension, which profoundly affect the regulation of renal ATPases (Kiroytscheva *et al.,* 1999). In addition, in contrast to studies on intact cells, enzyme activity is measured directly so it is not affected by the rate of Na^+ entry to the cell. In the present study we adopted our method for the assay of renal ouabain-resistant Na^+-stimulated ATPase. Several lines of evidence indicate that the activity measured by us is in fact due to this enzyme. This activity: (1) is confined to the renal cortex, consistently with the exclusive localization of Na^+-ATPase in proximal convoluted tubule, (2) is stimulated by either Na^+ or Li^+ but not by other cations, (3) is equally stimulated by different Na^+ salts irrespectively of the accompanying anion, (4) is insen-

sitive to ouabain or Sch 28080, (5) is inhibited by either furosemide or ethacrynic acid, (6) the $K_{0.5}$ for Na^+ and K_i values for the inhibitors are within the range reported by other authors.

Previously, we have demonstrated that if Sch 28080 is not added to the incubation medium, the ouabain-sensitive H^+,K^+-ATPase is active during Na^+,K^+-ATPase assay and falsely overestimates its activity (Bełtowski & Wójcicka, 2002). However, it is unlikely that Na^+-ATPase activity interferes with our Na^+,K^+-ATPase assay. Na^+,K^+-ATPase is measured in the presence of EGTA which inhibits Na^+-ATPase by chelating Ca^{2+} (Proverbio *et al.,* 1982; el Mernissi *et al.,* 1991). Moreover, even if Na^+-ATPase is active during Na^+,K^+-ATPase assay, it is not inhibited by ouabain and thus should be equally active in samples measured in the absence and in the presence of this inhibitor. Therefore, Na^+-ATPase could increase the total and the ouabain-resistant activity but will not affect the difference between them used as a measure of Na^+,K^+-ATPase. Although furosemide decreased Na^+,K^+-ATPase activity, this is accounted for by inhibition of Na^+,K^+-ATPase itself rather than by eliminating the contribution of Na^+-ATPase, because the effect was observed also in the renal medulla which does not contain Na^+-ATPase. Previous studies have demonstrated that millimolar concentrations of furosemide decrease renal Na^+,K^+-ATPase activity by about 10% (Del Castillo *et al.,* 1982). In addition, ethacrynic acid caused a more marked inhibition of Na^+,K^+-ATPase, which could not be accounted for only by blocking Na^+-ATPase.

Leptin is a recently described hormone secreted by white adipose tissue which is involved in the regulation of energy balance. Leptin receptors are expressed in many tissues including cardiovascular system and the kidney (Margetic *et al.,* 2002). Acutely administered leptin has natriuretic activity (Jackson & Li, 1997; Villarreal *et al.,* 1998; Jackson & Herzer, 1999), partially accounted for by the inhibition of Na^+,K^+-ATPase in the renal medulla (Sweeney *et al.,* 2000; Bełtowski *et al.,* 2002). The mechanisms regulating Na^+-ATPase are in part parallel to those which regulate Na^+,K^+-ATPase. For example, cyclic AMP and protein kinase C stimulate both pumps in the proximal tubule (Féraille *et al.,* 1995; Carranza *et al.,* 1998; Caruso-Neves *et al.,* 2000b; Rangel *et al.,* 2001), whereas cyclic GMP and prostaglandin E_2 decrease their activity (Jabs *et al.,* 1989; Bełtowski *et al.,* 1998; Caruso-Neves *et al.,* 2004; Lopes *et al.,* 2004). Angiotensin II has a dose-dependent effect on both ATPases with stimulation observed at low and inhibition at high concentrations (Bharatula *et al.,* 1998; Caruso-Neves *et al.,* 1999), whereas atrial natriuretic peptide (ANP) reduces the activity of both pumps (Bełtowski *et al.,* 1998; Caruso-Neves *et al.,* 2004). Herein we demonstrate that leptin has no effect on Na^+-ATPase activity. It remains to be established whether this results from different signalling mechanisms regulating both pumps or from the exclusive localization of leptin receptors in the renal medulla.

Obesity is often accompanied by arterial hypertension, which is partially accounted for by abnormal renal Na^+ handling (Hall, 1997; 2003). Plasma leptin level is increased in obese subjects, and hyperleptinemia may be involved in the pathogenesis of obesity-associated hypertension (Hall *et al.,* 2001; Rahmouni & Haynes, 2004). Recently, we have shown that experimental hyperleptinemia decreases natriuresis, up-regulates renal Na^+,K^+-ATPase and increases blood pressure (Bełtowski *et al.,* 2004). Here we demonstrate that Na^+-ATPase activity is also increased in this model. Thus, both sodium pumps may contribute to abnormal Na^+ retention and blood pressure elevation in leptin-treated rats. In contrast, Na^+-ATPase activity was unchanged in dietary-induced obesity. Interestingly, blood pressure is normal in our obese rats but is increased in lean hyperleptinemic animals (Bełtowski *et al.,* 2002; 2004). Thus, Na^+-ATPase may play an

important role in the regulation of sodium balance and blood pressure.

In conclusion, we described a reliable method for the measurement of renal ouabain-resistant, furosemide-sensitive Na^+-stimulated ATPase activity. Acutely administered leptin had no effect on Na^+-stimulated ATPase. The Na^+-ATPase activity is increased in rats with experimental hyperleptinemia, which may contribute to Na^+ retention and arterial hypertension in this model. In contrast, the Na^+-ATPase activity is unchanged in dietary-induced obesity. These data indicate that renal Na^+-ATPase and Na^+,K^+-ATPase are differentially regulated under physiologic and pathologic conditions.

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