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Angiotensin II activates the ouabain-insensitive Na^+-ATPase from renal proximal tubules through a G-protein

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Abstract

Angiotensin II (AG II) stimulates the ouabain-insensitive, furosemide- sensitive Na^+-ATPase present in the basolateral membrane of pig renal proximal tubules in a dose dependent manner. Maximum effect was obtained with 10^{-8} M AG II, which corresponded to an activity 134% higher than control. Half of the maximum effect was observed between 10^{-11} M and 10^{-10} M, corresponding to physiological hormone levels. Saralasin, an AG II peptide analogue receptor antagonist, abolished the phenomenon, demonstrating that AG II interacts with specific sites in pig proximal tubules. The AG II stimulatory effect was also prevented by dithiothreitol (DTT), a reducing compound, and by 10 nM losartan, a non-peptide antagonist highly specific for AT_1 receptors, characterizing AG II binding to AT_1 receptors. GTPγS, a non-hydrolysable GTP analogue, increased by 159% the enzyme activity as compared to the control values. The simultaneous addition of 10^{-5} M GTPγS and 10^{-8} M AG II did not have additive effects. Furthermore, the stimulatory action of AG II was completely abolished by 0.1 μM GDPβS, a non-hydrolysable GDP analogue. Two μg ml^{-1} pertussis toxin, an inhibitor of G_i-protein, did not modulate the AG II stimulatory effect. On the other hand, the Na^+-ATPase activity was enhanced 100% in the presence of cholera toxin and 85% in the presence of both AG II and cholera toxin. Taken together, these data suggest that AG II activates the Na^+-ATPase activity through AT_1 receptors coupled to a pertussis-insensitive and cholera-sensitive G-protein.

Keywords: Na^+-ATPase; Angiotensin II; Furosemide; Proximal tubule; G protein

1. Introduction

The renin–angiotensin system plays a crucial role in the regulation of extracellular volume through indirect and direct effects on renal sodium excretion [1]. It has been described that angiotensin II (AG II) directly modulates Na^+ reabsorption in different segments of the nephron, including the proximal tubule [2–7]. Micropuncture and microperfusion studies performed in rat and rabbit renal proximal tubules demonstrated that AG II modulates sodium reabsorption in a dose dependent and biphasic manner [2,3]. Physiological doses of AG II, between 10^{-12} M and 10^{-10} M, stimulate sodium reabsorption, whereas higher AG II concentrations, between 10^{-7} M and 10^{-5} M, are inhibitory. However, the mechanism involved is not well understood.

AG II effects are due to its binding to specific receptors on the plasma membrane of the target tissues, which were characterized and cloned [8,9]. In renal tissue, they have been classified as AT_1, AT_2, and non-AT_1/non-AT_2 (also designated as AG III/

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AG IV) according to their pharmacological and molecular characteristics [8,9]. The AT_1 receptors are the most abundant type found in the adult kidney, and were described as a protein of about 65 kDa with seven transmembrane domains and four cysteine residues, being coupled to a G-protein and susceptible to the reducing power of thiols [8]. The AT_2 receptors, on the other hand, are detected mainly in foetal tissues representing only 10% of AG II receptors in the adult kidney and are activated by thiols [10]. It is relevant that AG II receptors are present on the brush-border and basolateral membranes of the proximal tubule [11,12].

The activity of the ATPases and the process of Na^+ reabsorption are correlated. There is a prominent expression of Na^+,K^+-ATPase in the renal proximal tubule cells, which provides the energy gradient that supplies the transcellular sodium reabsorption in this segment of the nephron. In the past decade, a second Na^+-ATPase, which is insensitive to ouabain and sensitive to furosemide, was described in several animal tissues [13–16]. This Na^+-ATPase transports Na^+ against an electrochemical gradient and is not stimulated by K^+. In proximal tubule cells this enzyme is localized in the basolateral membrane and is involved with the extrusion of sodium along with chloride and water [13]. In spite of several papers published on this enzyme, its physiological role remains to be elucidated. It was first suggested that the Na^+-ATPase could be involved in cell volume regulation [13,15]. Recently, our laboratory proposed that this enzyme involved in the regulation of Na^+ reabsorption in the proximal tubule is the primary active transport target for natriuretic compounds [17,18]. Munday and colleagues [19] showed previously that 10^{-12} M AG II stimulates sodium and fluid reabsorption via the ouabain-insensitive Na^+-ATPase in rat kidney slices.

Based on the hypothesis that AG II regulates proximal sodium transport, the purpose of this study was to investigate the modulation of Na^+-ATPase and Na^+,K^+-ATPase activities by AG II, as well as the possible signal transduction pathway involved.

Our results show that AG II stimulates the Na^+-ATPase activity through the AT_1 receptor but does not change the Na^+,K^+-ATPase activity.

2. Materials and methods

2.1. Materials

ATP, ouabain, furosemide, azide, EGTA, mannitol, angiotensin II, pertussis toxin, cholera toxin, saralasin (Sar^1Ile^8-AG II) and GTPγS were purchased from Sigma Chemical Co., St. Louis, MO, USA. Percoll was purchased from Pharmacia Biotech, Uppsala, Sweden. All chemicals reagents were of the highest purity available. [^{32}P]P_i was obtained from the Institute of Energetic and Nuclear Research, São Paulo, SP, Brazil.

All solutions were prepared with deionized glass-distilled water. [γ-^{32}P]ATP was prepared as described by Maia and coworkers [20].

2.2. Preparation of cortex homogenates and isolated basolateral membrane

Cortex homogenates and basolateral membranes were prepared from adult pig kidney. The kidneys were obtained from a commercial slaughter house immediately after the death of the animals, and maintained at 4°C in a solution containing (mM): 250 sucrose, 10 Hepes–Tris (pH 7.6), 2 EDTA and 1 phenylmethanesulfonyl fluoride (PMSF) [17,18]. Thin slices were removed from the cortex (cortex-corticis) with a scalpel. After dissection, slices were homogenized in the same cold solution with a Teflon and glass homogenizer. The homogenate was centrifuged for 10 min at 3000 rpm in a Sorvall centrifuge using a SS-34 rotor at 4°C. The supernatant was collected and stored at −4°C. The fraction containing the basolateral membrane was isolated by the Percoll gradient method [21]. The membrane preparation was resuspended in 250 mM sucrose to a final concentration between 20–30 mg of protein ml^{-1} and was stored at −4°C.

2.3. Measurement of ATPase activity

Except as noted under Section 3, the composition of the standard assay medium (0.2 ml) contained: 10 mM $MgCl_2$; 5 mM [γ-^{32}P]ATP; 20 mM Hepes–Tris (pH 7.0); 5 mM azide; 1 mM EGTA; and 90 mM NaCl, for measuring of the Na^+-ATPase

activity. The final osmolality was adjusted with mannitol to 300 mosmol kg^{-1}.

The ATPase activity was measured according to the method described by Grubmeyer and Penefsky [22]. The reaction was started by the addition of cortex homogenate or isolated basolateral membrane to a final protein concentration of 0.3–0.5 mg ml^{-1}. The reaction was stopped after 30 min by the addition of charcoal activated by HCl (0.1 N). The [^{32}P]P_i released was measured in an aliquot of the supernatant obtained after centrifugation of the charcoal suspension for 20 min at 3000 rpm in a clinical centrifuge. Spontaneous hydrolysis of [γ-^{32}P]ATP was measured simultaneously in tubes where protein was added after the acid. The Na^+-ATPase activity was calculated by the difference between the [^{32}P]P_i released in the absence and in the presence of 2 mM furosemide, both in the presence of 1 mM ouabain [23,24]. The Na^+,K^+-ATPase activity was calculated by the difference between the [^{32}P]P_i released in the absence and in the presence of 1 mM ouabain [25]. The Na^+,K^+-ATPase activity measured in isolated basolateral membrane is five times higher when compared to the activity found in cortex homogenate. Protein concentrations were determined by the Folin phenol method [26] using bovine serum albumin as a standard. Each experiment was performed in an independent preparation of basolateral membrane or cortex homogenate. The data were analysed by two-way analysis of variance (ANOVA), considering the treatments as factors. The significance of the differences was verified by the Bonferroni *t*-test. Statistical analysis was performed using absolute values and the results were expressed as percentage of the control.

3. Results

3.1. Effect of AG II on the Na^+-ATPase and Na^+,K^+-ATPase activities

To verify the AG II modulation on the second sodium pump, its activity was measured in the absence and in the presence of different concentrations of the hormone (10^{-11} M to 10^{-6} M) in isolated basolateral membrane and cortex homogenate preparations (Fig. 1). In both preparations AG II enhanced the Na^+-ATPase activity in a dose dependent manner. The enzyme activity increased from 4.28 ± 0.21 to 10.00 ± 1.38 nmol $^{32}P_i$ min^{-1} mg^{-1} in basolateral membrane, and from 1.10 ± 0.26 to

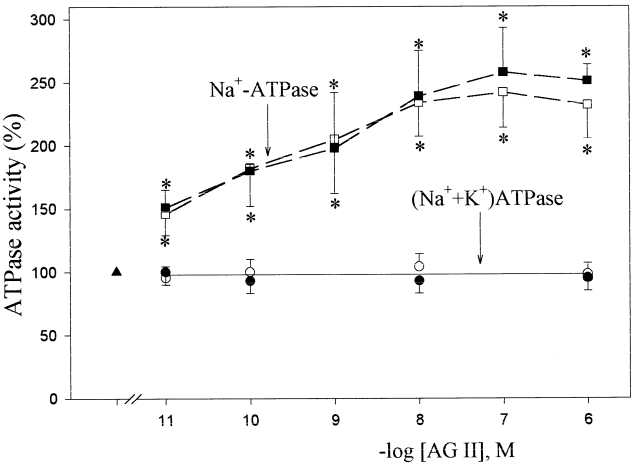


Fig. 1. Dependence of Na^+-ATPase (■) and Na^+,K^+-ATPase (●) activities in cortex homogenate (■,●) and isolated basolateral membrane (□,○) of renal proximal tubule on AG II concentration. ATPase activity was measured as described in Section 2 ($n = 7$). The AG II concentration was increased from 10^{-11} M to 10^{-6} M. Results are expressed as percentage of the control (▲). *Statistically significant when compared to control ($P < 0.05$).

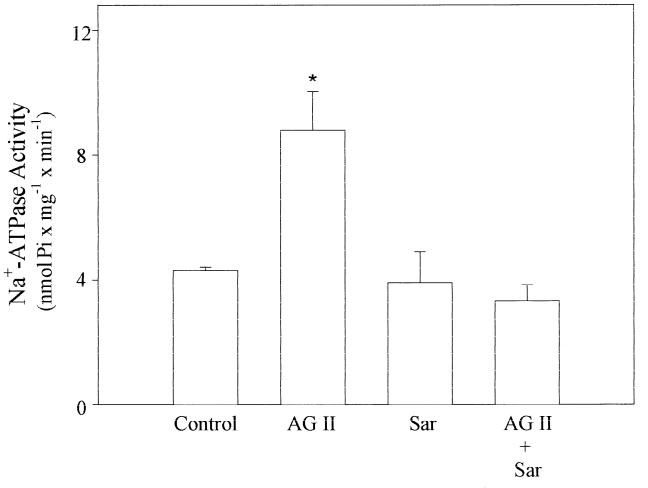


Fig. 2. Inhibition of the effect of AG II on Na^+-ATPase activity in isolated basolateral membrane of renal proximal tubule by 10^{-9} M saralasin (Sar). ATPase activity was measured as described in Section 2 ($n = 6$). 10^{-8} M AG II was added where indicated. Results are expressed as absolute values of the enzyme activity. *Statistically significant when compared to control ($P < 0.05$).

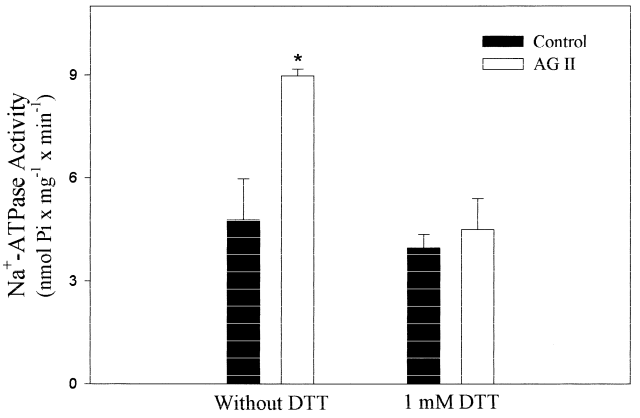


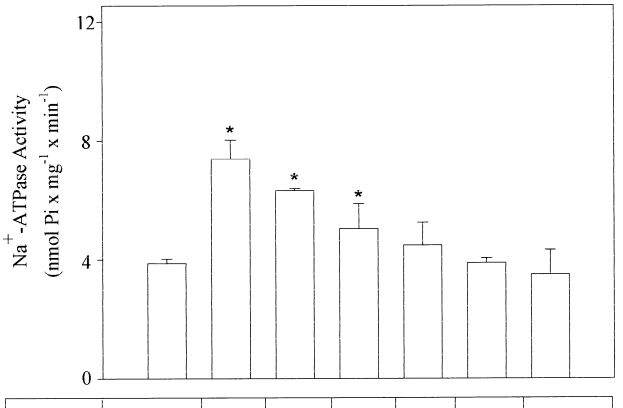
Fig. 3. Modulation of the effect of AG II on Na^+-ATPase activity in isolated basolateral membrane of renal proximal tubule by 1 mM dithiothreitol (DTT). ATPase activity was measured as described in Section 2 ($n=5$). 10^{-8} M AG II was added where indicated. Results are expressed as absolute values of the enzyme activity. *Statistically significant when compared to control ($P<0.05$).

2.63 ± 0.76 nmol $^{32}P_i$ min^{-1} mg^{-1} in cortex homogenate, corresponding to an increase of 134% and 139%, respectively. The maximum stimulatory action was observed at 10^{-8} M AG II. Since the effects in isolated basolateral membrane and in cortex homogenate were similar, it is reasonable to conclude that all signal transduction pathway elements activated by AG II during the stimulation of Na^+-ATPase were preserved in the isolated basolateral membrane.

The Na^+,K^+-ATPase activity in isolated basolateral membrane and cortex homogenate was not changed by increasing AG II concentrations, from 10^{-11} to 10^{-6}, as shown in Fig. 1.

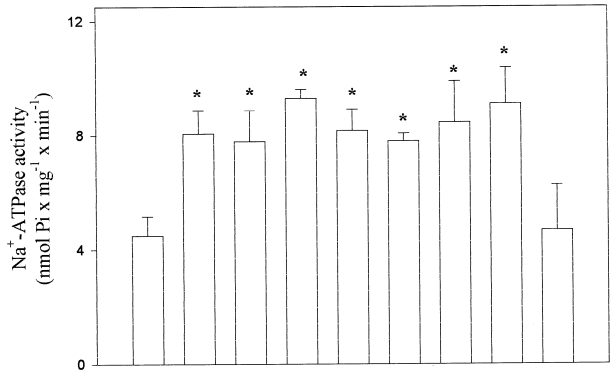
3.2. Characterization of the receptor involved in the stimulation of the Na^+-ATPase activity by AG II

Brown and Douglas [10,11] had previously documented that AG II receptors are present in both luminal and basolateral membrane of the proximal tubules. To characterize AG II interaction with basolateral membrane receptors from pig renal proximal tubules, we used saralasin, an AG II analogue peptide receptor antagonist [27]. Data illustrated in Fig. 2 show that the AG II stimulatory effect on the Na^+-ATPase activity in isolated basolateral membrane was completely abolished by 10^{-9} M saralasin. Furthermore, 10^{-9} M saralasin alone did not change the enzyme activity. These data indicate that AG II stim-



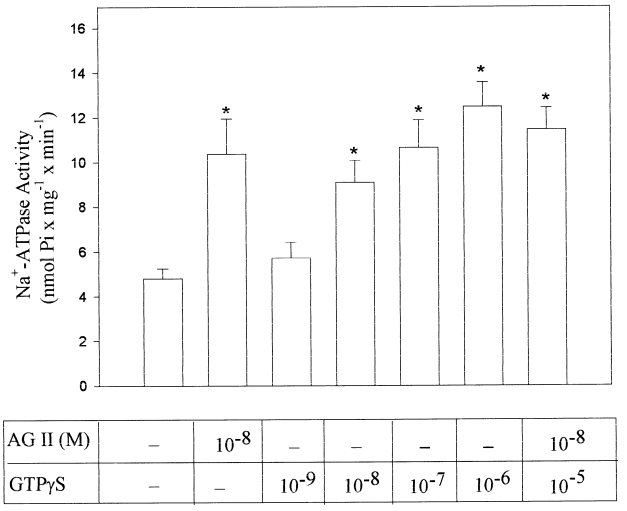
AG II (M)	–	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}
Los (M)	–	–	10^{-10}	10^{-9}	10^{-8}	$2x10^{-8}$	10^{-7}

Fig. 4. Reversal of the effect of AG II on Na^+-ATPase activity in isolated basolateral membrane of renal proximal tubule by losartan (Los). Losartan concentration was increased from 10^{-10} M to 10^{-7} M. 10^{-8} M AG II was added where indicated. ATPase activity was measured as described in Section 2 ($n=6$). Results are expressed as absolute values of the enzyme activity. *Statistically significant when compared to control ($P<0.05$).



AG II (M)	–	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	–
PD (nM)	–	–	1	3	10	20	30	50	30

Fig. 5. Modulation of the effect of AG II on Na^+-ATPase activity in isolated basolateral membrane of renal proximal tubule by PD 123319. The concentration of PD 123319 was increased from 40 nM to 2 μM. Where indicated 10^{-8} M AG II was added. ATPase activity was measured as described in Section 2 ($n=5$). Results are expressed as absolute values of the enzyme activity. *Statistically significant when compared to control ($P<0.05$).



AG II (M)	–	10^{-8}	–	–	–	–	10^{-8}
GTPγS	–	–	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}

Fig. 6. Modulation of the effect of AG II on Na^+-ATPase activity in isolated basolateral membrane of renal proximal tubule by GTPγS. GTPγS concentration was increased from 10^{-9} M to 10^{-5} M. 10^{-8} M AG II was added where indicated. ATPase activity was measured as described in Section 2 ($n=4$). Results are expressed as absolute values of the enzyme activity. *Statistically significant when compared to control ($P<0.05$).

ulates the Na^+-ATPase activity through its interaction with specific plasma membrane receptors.

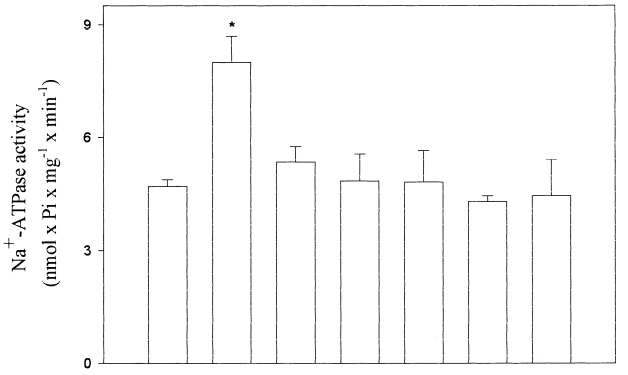
Additional information concerning the type of receptor mediating the activation of Na^+-ATPase by AG II was obtained with the use of the thiol dithiothreitol (DTT). Initially, the isolated basolateral membrane fraction was incubated with 1 mM DTT for 10 min. After the pre-incubation, Na^+-ATPase activity was measured in the presence of 1 mM DTT. In this condition, Na^+-ATPase activity was 3.96 ± 0.40 nmol $^{32}P_i$ min^{-1} mg^{-1} in the absence of the hormone, and 4.78 ± 1.19 nmol $^{32}P_i$ min^{-1} mg^{-1} in the presence of 10^{-8} M AG II (Fig. 3). To rule out the possibility that the lack of AG II stimulatory effect on the Na^+-ATPase could be due to pre-incubation with DTT, a simultaneous experiment was run in which the enzyme was pre-incubated in the absence of DTT and its activity was also measured. Under this situation, the enzyme activity increased from 4.50 ± 0.90 nmol $^{32}P_i$ min^{-1} mg^{-1}, in the absence of AG II, to 8.97 ± 0.20 nmol $^{32}P_i$ min^{-1} mg^{-1}, in the presence of 10^{-8} M AG II.

One of the differences among the AG II receptors is the sensitivity to losartan, a non-peptide antagonist. Our data illustrated in Fig. 4 show that the stimulatory effect of AG II was abolished by losartan in a dose-dependent manner. Complete reversal of the effect of AG II was obtained in the presence of 10^{-8} M losartan. The ATPase activity decreased from 7.38 ± 0.64 nmol $^{32}P_i$ min^{-1} mg^{-1}, in the presence of AG II, to 4.48 ± 0.77 nmol $^{32}P_i$ min^{-1} mg^{-1}, in the presence of AG II plus losartan, a level similar to that obtained in the absence of both drugs. The addition of 10^{-8} M losartan alone did not change the Na^+-ATPase activity. Furthermore, PD 123319, a specific antagonist of AT_2 receptor [28], did not change the stimulatory effect of AG II on the Na^+-ATPase activity (Fig. 5).

Taken together, these data indicate that the AG II stimulatory effect on the Na^+-ATPase is mediated by its interaction with AT_1 receptors present in the basolateral membrane of renal proximal tubule.

3.3. Coupling of AG II receptors to G-protein during activation of Na^+-ATPase activity

Signal transduction pathways activated by AG II are, in general, coupled to G-proteins [27]. To verify the involvement of a G-protein in the activation of the Na^+-ATPase by AG II, a non-hydrolysable GTP



AG II (M)	–	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	–
GDPβS (μM)	–	–	0.1	1.0	5.0	100.0	200.0

Fig. 7. Modulation of the effect of AG II on Na^+-ATPase activity in isolated basolateral membrane of renal proximal tubule by GDPβS. GDPβS concentration was increased from 0.1 μM to 100 μM. Where indicated, 10^{-8} M AG II was added. ATPase activity was measured as described in Section 2 ($n=4$). Results are expressed as absolute values of the enzyme activity. *Statistically significant when compared to control ($P<0.05$).

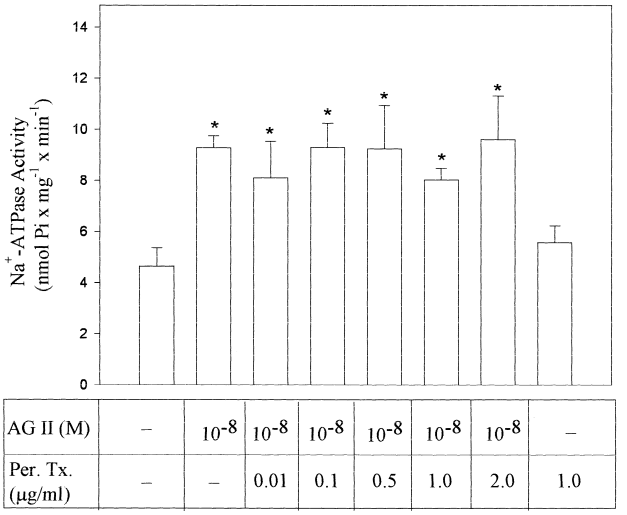


Fig. 8. Modulation of the effect of AG II on Na^+-ATPase activity in isolated basolateral membrane of renal proximal tubule by pertussis toxin (Per. Tx.). Pertussis toxin concentration was increased from 0.01 μg ml^{-1} to 2.0 μg ml^{-1}. 10^{-8} M AG II was added where indicated. ATPase activity was measured as described in Section 2 ($n=4$). Results are expressed as absolute values of the enzyme activity. *Statistically significant when compared to control ($P<0.05$).

analogue, GTPγS, and a non-hydrolysable GDP analogue, GDPβS, was used. The results presented in Fig. 6 show that the increase in GTPγS concentration (from 10^{-9} M to 10^{-5}) raised, in a dose-dependent manner, the Na^+-ATPase activity from 4.80 ± 0.43 to 12.47 ± 1.36 nmol $^{32}P_i$ min^{-1} mg^{-1}, corresponding to an activity 159% higher than the control. Similar results were obtained with AG II alone, as mentioned before. Maximal effect was obtained in the presence of 10^{-6} M GTPγS. In addition, the concomitant use of 10^{-8} M AG II and 10^{-5} M GTPγS increased the enzyme activity 2.4 times above control levels (11.46 ± 0.97 nmol $^{32}P_i$ min^{-1} mg^{-1}). Since AG II and GTPγS stimulatory effects were not additive it might be possible that they act in the same transduction pathway to modulate the Na^+-ATPase activity. In addition, data illustrated in Fig. 7 demonstrate the reversal of AG II stimulatory effect on the enzyme activity by a non-hydrolysable GDP analogue, GDPβS. The Na^+-ATPase activity in the presence of 10^{-8} M AG II raised from 4.70 ± 0.18 nmol $^{32}P_i$ min^{-1} mg^{-1} (control) to 7.99 ± 0.70 nmol $^{32}P_i$ min^{-1} mg^{-1}. Besides, the simultaneous addition of 10^{-8} M AG II and different concentrations of GDPβS (from 0.1 μM to 100.0 μM), abolished the hormone action. In this situation, the enzyme activity was 4.29 ± 0.16 nmol $^{32}P_i$ min^{-1} mg^{-1}, in the presence of both 10^{-8} M AG II and 100 μM GDPβS (values statistically similar to the control). It is relevant that 200 μM GDPβS has no effect on the Na^+-ATPase activity.

It has been proposed that the effect of AG II on fluid reabsorption in the proximal tubule is mediated by cyclic adenosine 3′,5′-monophosphate (cAMP) [28]. The level of cAMP depends on modulation of the adenylyl cyclase by stimulatory G-proteins (G_s) and inhibitory G-proteins (G_i). One of the principal characteristics of G_i-protein is its sensitivity to pertussis toxin, whereas the G_s protein is sensitive to cholera toxin [29]. To verify the possible involvement of the G_i-protein in the stimulatory effect of AG II on the Na^+-ATPase activity, pertussis toxin was used. Fig. 8 describes the data obtained with experiments designed to verify the modulation of 10^{-8} M AG II effect on the Na^+-ATPase activity by different concentrations of pertussis toxin. Increase in pertussis concentration from 0.1 μg ml^{-1} to 2.0 μg ml^{-1} did not change the 10^{-8} M AG II stimulatory effect on the Na^+-ATPase activity. Furthermore, 1 μg ml^{-1}

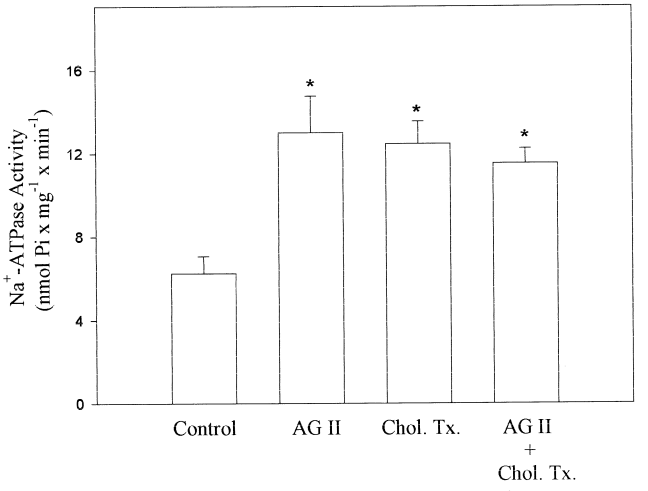


Fig. 9. Modulation of the effect of AG II on Na^+-ATPase activity in isolated basolateral membrane of renal proximal tubule by 10^{-9} M cholera toxin (Chol. Tx.). ATPase activity was measured as indicated in Section 2 ($n=5$). 10^{-8} M AG II was added where indicated. Results are expressed as absolute values of the enzyme activity. *Statistically significant when compared to control ($P<0.05$).

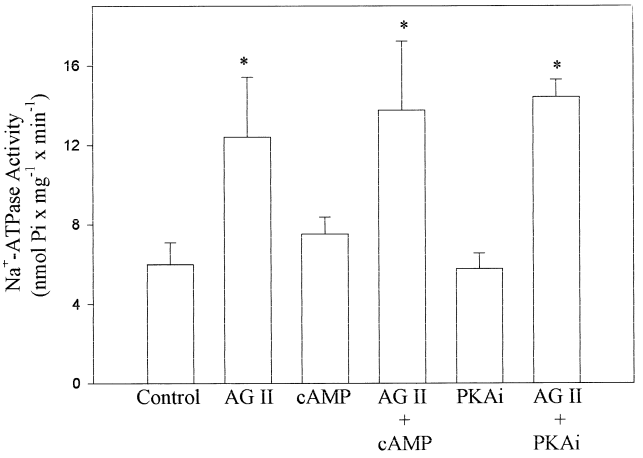


Fig. 10. Possible involvement of cAMP in the effect of AG II on the enzyme activity. Where indicated, 5 μM dibutyryl-cAMP (d-cAMP), 10 nM protein kinase A peptide inhibitor (PKAi) and 10^{-8} M AG II were added. ATPase activity was measured as indicated in Section 2 ($n=6$). 10^{-8} M AG II was added where indicated. Results are expressed as absolute values of the enzyme activity. *Statistically significant when compared to control ($P<0.05$).

pertussis toxin alone did not change the ATPase activity (4.64 ± 0.73 and 5.58 ± 0.65 nmol $^{32}P_i$ $mg^{-1}\,min^{-1}$, in the absence and in the presence of pertussis toxin, respectively).

Fig. 9 illustrates results related to the interactions between cholera toxin, a classical stimulatory G-protein (G_s) activator, and AG II. In the presence of 10^{-9} M cholera toxin the Na^+-ATPase activity increased from 6.24 ± 0.83 nmol $^{32}P_i$ $min^{-1}\,mg^{-1}$ (control) to 12.47 ± 1.07 nmol $^{32}P_i$ $min^{-1}\,mg^{-1}$. Similarly, the simultaneous addition of 10^{-9} M cholera toxin and 10^{-8} M AG II raised the Na^+-ATPase activity to 11.53 ± 0.72 nmol $^{32}P_i$ $min^{-1}\,mg^{-1}$. These results demonstrate the lack of additivity of the AG II and cholera toxin stimulatory effects. Based on these data, it is possible to conclude that AG II activates the Na^+-ATPase activity via a pertussis toxin-insensitive and cholera toxin-sensitive G-protein.

3.4. Possible involvement of cAMP on the effect of AG II on the enzyme activity

It has been described that intracellular cAMP levels modulate the effect of AGII in proximal tubule cells [28]. To determine the role of cAMP on the effect of AG II on the Na^+-ATPase activity present in the basolateral membrane of the proximal tubule experiments were performed in the presence of dibutyryl-cAMP (d-cAMP) and protein kinase A inhibitor peptide (PKAi), as shown in Fig. 10. Neither 5 μM d-cAMP nor 10 nM PKAi changed the Na^+-ATPase activity either in the presence or in the absence of 10^{-8} M AG II. These data indicate that the effect of AG II on the Na^+-ATPase activity is not mediated by cAMP.

4. Discussion

In the present work, we studied the regulation of the Na^+-ATPase and Na^+,K^+-ATPase activities of the proximal tubules basolateral membrane from pig kidney by AG II. It was observed that AG II has a selective effect on the Na^+-ATPase activity but does not regulate, at least directly, the Na^+,K^+-ATPase activity. Proverbio et al. [30] described two forms of Na^+-stimulated ATPase activity in aged microsomal fractions from guinea-pig kidney cortex. One is the well known ouabain-sensitive Na^+,K^+-ATPase, the other is the ouabain-insensitive, Na^+-stimulated ATPase activity, which is sensitive to ethacrynic acid and furosemide. Proverbio and Del Castillo [31] showed, in basolateral membranes from proximal tubular cells of guinea-pigs, the presence of an ouabain-insensitive Na^+-stimulated ATPase activity. The expression of this enzyme was demonstrated in different cells of several species [15,17,18]. Using the same preparation, we had previously observed that an increase in Na^+ concentration, in the presence of 1 mM ouabain, raised the ATPase activity inhibited by 2 mM furosemide [32]. This ATPase activity, that is 10 times lower than the Na^+,K^+-ATPase activity, did not change with the addition of K^+ to a final concentration of 30 mM. These data indicate that the basolateral membrane of proximal tubule cells from pig kidney expresses both Na^+-ATPase and Na^+,K^+-ATPase activities. Furthermore, we also observed that AG II increases, in a similar way, both the Na^+ stimulated and the furosemide-sensitive ATPase activity, indicating that the effect of AG II is specific for the ouabain-insensitive Na^+-ATPase activity (data not shown).

It is well characterized that some of the physiological effects of the renin–angiotensin system are due,

in part, to the direct modulation of renal transporters by AG II [1]. It has been described that low concentrations of AG II (10^{-12} to 10^{-10} M) increase sodium and water reabsorption in the proximal tubules [3,33–35]. However, the mechanism involved in this effect is still unclear. Our data demonstrate that AG II, in a concentration as low as 10^{-10} M, increases the Na^+-ATPase activity in both preparations by 82% (Fig. 1). Considering that AG II plays a central role in extracellular volume regulation through its action in sodium reabsorption, we propose that direct modulation of the Na^+-ATPase might be a mechanism by which the hormone exerts this function. This hypothesis is in agreement with Munday and coworkers [19] who suggest that AG II increases sodium extrusion in rat kidney cortex slices through the activation of a ouabain-insensitive Na^+ pump.

The presence of AG II receptors in the cortical nephron segments, mainly in the convoluted proximal tubules, was demonstrated in rat kidney by autoradiographic studies using I^{125}-AG II [36]. In the proximal tubule the AG II receptors are uniformly distributed in both luminal and basolateral membranes [9,37]. Burns et al. [9] showed that over 80% of the AG II receptors found in the basolateral membrane of proximal tubules from rat and rabbit kidneys are of the AT_1 type. The stimulatory effect of AG II on the Na^+-ATPase activity is mediated by receptor binding, since it was observed that saralasin completely abolished this AG II action (Fig. 2). The AT_1 receptor has disulfide bonds linking its four extracellular domains, making it susceptible to the reducing power of thiols, such as dithiothreitol (DTT) [8]. We suggest that AG II regulates the Na^+-ATPase activity through AT_1 receptors because the experiments performed in the presence of DTT failed to describe the AG II stimulatory action on the ATPase activity (Fig. 3). The confirmation of the receptor type mediating AG II function, as well as the comprehension of its biological actions, can only be achieved with the use of selective non-peptide antagonists such as losartan and PD123319 [38]. AT_1 receptors are sensitive to nano- and micromolar concentrations, whereas AT_2 receptors are sensitive to PD123319, but only to millimolar concentrations of losartan [39]. As indicated in Fig. 4, the AG II stimulating effect on the Na^+-ATPase activity is abolished by losartan in a nanomolar range. Besides, PD123319 did not revert the effect of AG II on the enzyme activity (Fig. 5). These data confirm our hypothesis that the effect of AG II on the Na^+-ATPase activity of the proximal tubule is mediated by AT_1 subtype receptors. The involvement of AT_1 receptors on the effect of AG II in different transporters of the proximal tubule has been described in other papers [40,41]. The increase in sodium reabsorption in proximal tubule has been associated to the modulation of three different transporters: 1) the basolateral Na^+/HCO_3^- [42]; 2) the basolateral Na^+,K^+-ATPase [43]; 3) the luminal Na^+/H^+ exchanger [41]. These data indicate that AG II modulates the Na^+ transport in both luminal and basolateral membranes. Aperia et al. (1994) [43] observed that AG II, in concentrations as low as 10^{-11} M, increased the Na^+,K^+-ATPase activity in the presence of subsaturating concentrations of Na^+ but had no effect in the presence of saturating Na^+ concentrations. In our experiments, we observed that AG II did not change the Na^+,K^+-ATPase activity (Fig. 1). This apparent contradiction could be due to the use of saturating Na^+ concentrations in our experiments. The possible effect of AG II observed in this paper can not be attributed to the modulation of the Na^+/H^+ exchanger or the Na^+/HCO_3^- transporter since: (1) The preparation used was enriched in basolateral membrane, and the Na^+/H^+ exchanger, involved in sodium reabsorption, is located in the luminal membrane; and (2) furosemide does not inhibit these transporters [14]. Taken together these data suggest that the effect of AG II on Na^+ reabsorption in the proximal tubule involves several transporters, including the ouabain-insensitive Na^+-ATPase. It is plausible to postulate that modulation of the Na^+,K^+-ATPase activity could be correlated to the gross Na^+ reabsorption, while modulation of the ouabain-insensitive Na^+-ATPase would represent the fine adjustment of Na^+ reabsorption.

The AT_1 receptor mediated effect of AG II has been described to be coupled to a G-protein [8,27]. In this work, we used the non-hydrolysable GTP analogue, GTPγS, and a non-hydrolysable GDP analogue, GDPβS to characterize G-protein involvement. In this context, GTPγS enhanced Na^+-ATPase activity in a dose-dependent manner, compatible with AG II mechanism (Fig. 6). On the other

hand, GDPβS reverted the AG II stimulatory action on the enzyme activity (Fig. 7). It was recently reviewed by Douglas and Hopfer [28] that some of the G-proteins can be identified due to their sensitivity to toxins as well as by their specific interactions with effectors. The members of the stimulatory G-protein catalytic subunits ($G\alpha_s$) are well described as adenylyl cyclase activators, and therefore enhance the cAMP intracellular levels. In addition, they are activated by cholera toxin. On the other hand, the inhibitory G-protein catalytic subunits ($G\alpha_i$), which promote a reduction on cAMP intracellular levels through the inactivation of adenylyl cyclase, are inhibited by pertussis toxin. Our results demonstrate that the G-protein activated by AG II, involved in the stimulation of Na^+-ATPase in proximal tubules basolateral membrane, is insensitive to pertussis toxin (Fig. 8). In fact, we further documented that AG II acts through a cholera toxin sensitive G-protein, which in turn stimulates cellular messengers that enhance the activity of the enzyme studied (Fig. 9).

In this paper we also investigate the possible involvement of cAMP on effect of AG II on the Na^+-ATPase activity. It has been observed that the stimulation of the Na^+/H^+ exchanger present in the luminal membrane of the proximal tubule cells is mediated a the decrease in the cAMP intracellular level [7]. The activation of Na^+-ATPase by AG II is not mediated by a decrease in cAMP level since this effect is not reversed by pertussis toxin or by d-cAMP (Figs. 8 and 10). On the other hand, the observation that cholera toxin mimicked the effect of AG II on the Na^+-ATPase activity could suggest that the activation of the adenylyl cyclase (Fig. 9) and consequent increase in cAMP level would be the signaling pathway. This hypothesis can be ruled out since protein kinase A inhibitor peptide did not change the effect of AG II (Fig. 10). The elements involved in cellular signaling remain to be elucidated.

The physiological role of the furosemide-sensitive Na^+-ATPase is still unclear. Initially, it was proposed that the Na^+-ATPase could be involved in cell volume regulation [14–16]. Recently, we observed that adenosine and bradykinin, natriuretic compounds, inhibit the Na^+-ATPase activity but do not change the Na^+,K^+-ATPase activity in isolated basolateral membrane of the proximal tubule and cortex homogenate from pig kidney [16,17]. The data presented in this paper reveals that AG II has also a selective effect on the Na^+-ATPase activity. These observations suggest that the primary active transport target involved in the regulation of sodium reabsorption in the proximal tubule by natriuretic and antinatriuretic compounds is the ouabain-insensitive Na^+-ATPase and not the ouabain-sensitive Na^+,K^+- ATPase.

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References

[1] P.J. Harris, L.G. Navar, Tubular transport responses to angiotensin, Am. J. Physiol. 248 (1985) F621–F630.
[2] D.W. Ploth, L.G. Navar, Intrarenal effects of the renin–angiotensin system, Fed. Proc. 38 (1979) 2280–2285.
[3] T. Wang, Y.L. Chan, Mechanism of angiotensin II action on proximal tubule transport, J. Pharmacol. Exp. Ther. 252 (1990) 689–695.
[4] G. Capasso, R. Unwin, F. Ciani, N.G. De Santo, G. De Tommaso, F. Russo, G. Giebisch, Bicarbonate transport along the loop of Henle. II. Effects of acid-base, dietary and neurohumoral determinants, J. Clin. Invest. 94 (1994) 830–838.
[5] D.Z. Levine, M. Incivitti, S. Buckman, V. Harrison, In vivo modulation of rat distal tubule net HCO_3^- flux by VIP, isoproterenol angiotensin II and ADH, Am. J. Physiol. 266 (1994) F878–F887.
[6] T. Wang, G. Giebisch, Effects of angiotensin II on electrolyte transport in the early and late distal tubules in rat kidney, Am. J. Physiol. 271 (1996) F143–F149.
[7] F.Y. Liu, M.G. Cogan, Angiotensin II stimulates early proximal bicarbonate absorption in the rat by decreasing cyclic adenosine monophosphate, J. Clin. Invest. 84 (1989) 83–91.
[8] T. Inagami, R.C. Harris, Molecular insights into angiotensin II receptor subtypes, NIPS 8 (1993) 215–218.
[9] K.D. Burns, T. Inagami, R.C. Harris, Cloning of a rabbit cortex AT_1 angiotensin II receptor that is present in prox-

imal tubule epithelium, Am. J. Physiol. 264 (1993) F645–F654.
[10] G.P. Brown, J.G. Douglas, Angiotensin II binding sites in rat and primate isolated renal tubular basolateral membranes, Endocrinology 112 (1983) 2007–2014.
[11] G.P. Brown, J.G. Douglas, Angiotensin II binding sites on isolated rat renal brush border membranes, Endocrinology 111 (1982) 1830–1836.
[12] J.F. Riordan, Angiotensin II, biosynthesis, molecular recognition, and signal transduction, Cell. Mol. Neurobiol. 15, (6) (1995) 637–651.
[13] G. Whittembury, F. Proverbio, Two modes of Na extrusion in cells from guinea-pig kidney slices, Pflügers Arch. 316 (1970) 1–25.
[14] F. Proverbio, R. Marín, T. Proverbio, The 'second' sodium pump and cell volume, Curr. Membr. Transp. 34 (1989) 105–119.
[15] R. Moretti, M. Martín, T. Proverbio, F. Proverbio, R. Marín, Ouabain-insensitive Na^+-ATPase activity in homogenates from different animal tissues, Comp. Biochem. Physiol. 98B (1991) 623–626.
[16] I.R. Arenstein, C. Caruso-Neves, L.F. Onuchic, A.G. Lopes, Mechanisms of cell volume regulation in the proximal segment of the malpighian tubule of *Rhodnius neglectus*, J. Membr. Biol. 146 (1995) 47–57.
[17] C. Caruso-Neves, L.G. Francisco-Pedro, L.P. Souza, C. Chagas, A.G. Lopes, Effect of adenosine on the ouabain-insensitive Na^+-ATPase activity from basolateral membrane of the proximal tubule, Biochim. Biophys. Acta 1329 (1997) 336–344.
[18] C. Caruso-Neves, A.S.E. Siqueira, G. Iso-Cohen, A.G. Lopes, Bradykinin modulates the ouabain-insensitive Na^+-ATPase activity from basolateral membrane of the proximal tubule, Biochim. Biophys. Acta (1998) submitted.
[19] K.A. Munday, B.J. Parsons, J.A. Poat, The effect os angiotensin on cation transport by rat kidney cortex slices, J. Physiol. 215 (1971) 269–282.
[20] J.C.C. Maia, S.L. Gomes, M.H. Juliani, in: C.M. Morel (Ed.), Genes of Antigenes of Parasites, A Laboratory Manual, Ed. Fundação Oswaldo Cruz, Rio de Janeiro, 1993, pp. 146–157.
[21] M.S. Grassl, P.S. Aronson, Na^+/HCO_3^- co-transport in basolateral membrane vesicles isolated from rabbit renal cortex, J. Biol. Chem. 261 (1986) 8778–8783.
[22] C. Grubmeyer, H.S. Penefsky, The presence of two hydrolytic sites on beef heart mitochondrial adenosine triphosphate, J. Biol. Chem. 256 (1981) 3718–3727.
[23] J.R. Del Castillo, R. Marín, T. Proverbio, F. Proverbio, Partial characterization of the ouabain-insensitive, Na^+-stimulated ATPase activity of kidney basal-lateral plasma membranes, Biochim. Biophys. Acta 692 (1982) 61–68.
[24] F. Proverbio, R. Marín, T. Proverbio, Na^+-ATPase is a different entity from the Na^+,K^+-ATPase in rat kidney basolateral plasma membranes, Biochim. Biophys. Acta 858 (1986) 202–205.
[25] P.L. Jørgensen, J.C. Skou, Purification and characterization of Na^+,K^+-ATPase, Biochim. Biophys. Acta 233 (1971) 366–380.
[26] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[27] J.G. Douglas, Angiotensin receptor subtypes of the kidney cortex, Am. J. Physiol. 253 (1987) F1–F7.
[28] J.G. Douglas, U. Hopfer, Novel aspect of angiotensin receptors and signal transduction in the kidney, Annu. Rev. Physiol. 56 (1994) 649–669.
[29] J.R. Raymond, Multiple mechanisms of receptor-G protein signaling specificity, Am. J. Physiol. 38, (2) (1995) F141–F158.
[30] F. Proverbio, M. Condrescu-Guidi, G. Wittembury, Ouabain-insensitive Na^+ stimulation of an Mg^{2+}-dependent ATPase in kidney tissue, Biochim. Biophys. Acta 394 (1975) 281–292.
[31] F. Proverbio, J.R. Del Castillo, Na^+-stimulated ATPase activities in kidney basal-lateral plasma membranes, Biochim. Biophys Acta 646 (1981) 99–108.
[32] C. Caruso-Neves, S. Coelho-Souza, G. Goes, A.G. Lopes, Modulation of the ouabain-insensitive Na^+-ATPase activity of the proximal tubule by pH, in preparation.
[33] K. Steven, Effect of peritubular infusion of angiotensin II on rat proximal nephron function, Kidney Int. 6 (1974) 73–80.
[34] P.J. Harris, J.A. Young, Dose-dependent stimulation and inhibition of proximal tubular sodium reabsorption by angiotensin II in the rat kidney, Pflügers Arch. 367 (1977) 295–297.
[35] V.L. Schuster, J.P. Kokko, J.R. Jacobson, Angiotensin II directly stimulates sodium transport in rabbit proximal convoluted tubules, J. Clin. Invest. 73 (1984) 507–515.
[36] H.M. Cox, K.A. Munday, J.A. Poat, Location of [^{125}I]-angiotensin II receptors on rat kidney cortex epithelial cells, Br. J. Pharmacol. 82 (1984) 891–895.
[37] S.K. Mujais, S. Kauffman, A.I. Katz, Angiotensin II binding sites in individual nephrons of the rat nephron, J. Clin. Invest. 77 (1993) 315–318.
[38] J.V. Duncia, D.J. Carini, A.T. Chiu, A.L. Johnson, W.A. Price, P.C. Wong, R.R. Wexler, P.B.M.W.M. Timmermans, The discovery of DuP 753, a potent, orally active nonpeptide angiotensin II receptor antagonist, Med. Res. Rev. 12 (1992) 149–191.
[39] F.M. Bumpus, K.J. Catt, A.T. Chiu, M. Degasparo, T. Goodfriend, A. Husain, M.J. Peach, D.G. Taylor, P.B.M.W.M. Timmermans, Nomenclature for angiotensin receptors: a report of the nomenclature committee of the council for high blood pressure research, Hypertens. Dallas 17 (1992) 720–721.
[40] J. Poggiolo, G. Lazar, P. Houillier, J.P. Gardin, J.M. Achard, M. Paillard, Effects of angiotensin II and non peptide receptor antagonists on transduction pathway in rat proximal tubules, Am. J. Physiol. 263 (1992) C750–C758.
[41] A. Cano, R.T. Miller, R.J. Alpern, P.A. Preisig, Angiotensin II stimulation of Na-H antiporter activity is cAMP inde-

pendent in OKP cells, Am. J. Physiol. 266 (1994) C1603–C1608.

[42] J. Geibel, G. Giebisch, W.F. Boron, Angiotensin II stimulates both Na^+-H^+ exchange and Na^+/HCO_3^- cotransport in the rabbit proximal tubule, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 7917–7920.

[43] A. Aperia, U. Holtbäck, M.L. Syrén, L.B. Svensson, J. Fryckstedt, P. Greengard, Activations of renal Na^+, K^+-ATPase: a final common pathway for regulation of natriuresis, FASEB J. 8 (1994) 436–439.