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Ouabain-insensitive Na^+-ATPase of proximal tubules is an effector for urodilatin and atrial natriuretic peptide

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Abstract

In the present paper we studied the effect of urodilatin and atrial natriuretic peptide (ANP) on the proximal tubule Na^+-ATPase and (Na^+K^+)ATPase activities. Urodilatin and ANP inhibit the Na^+-ATPase activity but not the (Na^+K^+)ATPase activity. Maximal effect was observed at a concentration of 10^{-11} M for both peptides. In this condition, the enzyme activity decreases from 10.8 ± 1.6 (control) to 5.7 ± 0.9 or 6.1 ± 0.7 nmol Pi mg^{-1} min^{-1} in the presence of urodilatin or ANP, respectively. This effect was completely reversed by 10^{-6} M LY83583, a guanylyl cyclase inhibitor, and mimicked by 10 nM cGMP. Furthermore, both ANP and urodilatin increase cGMP production by 33% and 49%, respectively. This is the first demonstration that it was shown that urodilatin and ANP directly modulate primary active sodium transport in the proximal tubule. The data obtained indicate that this effect is mediated by the activation of the NPR-A/guanylate cyclase/cGMP pathway.

Keywords: Na^+-ATPase; Urodilatin; ANP; Furosemide; Proximal tubule

1. Introduction

Modulation of Na^+ reabsorption in the kidney is critical for maintenance of the extracellular volume and for long-term regulation of blood pressure [1,2]. Several compounds are involved in this process such as urodilatin and atrial natriuretic peptide (ANP) [3,4]. ANP is a 28-amino-acid peptide described initially to be produced in the atrial cardiomyocytes [4,5] Actually, it is known that ANP can be produced in several tissues including renal tubules [6,7]. Urodilatin is a 32-amino-acid peptide similar to ANP, extended by four amino acids in the NH_2-terminal portion (ANP 95–126) [8–10]. This peptide is produced in renal distal segments where it may act as a paracrine factor modulating the function of the different segments of the nefron [6,9]. It has been shown that ANP and urodilatin increase renal sodium excretion being this effect correlated, in part, to the inhibition of tubular sodium transport [4,6].

Approximately 70% of the filtered sodium is reabsorbed in the proximal tubule and consequently small changes in this process could have major consequences for overall body sodium metabolism. The transcellular sodium reabsorption in the proximal tubule involves two primary active transporters: the ouabain-sensitive (Na^+K^+)ATPase and the ouabain-insensitive, furosemide-sensitive Na^+-ATPase [11,12]. These enzymes are located in basolateral membranes and they are involved in the genesis of the Na^+ electrochemical gradient [11]. The Na^+-ATPase is about 10 times less active than the (Na^+K^+)ATPase [12], which suggests that this enzyme may be involved in fine tuning, whereas the (Na^+K^+)ATPase is responsible for most of the Na^+ reabsorption in the proximal tubule.

Although urodilatin and ANP play an important role in the regulation of renal sodium excretion nothing is known about their mechanisms of action along the nephron. It was observed that ANP and urodilatin modulate renal active Na^+ transport [6,13], although it is not known if this effect, in proximal tubule, is due to direct modulation of primary active sodium transporters or not [6]. Therefore, the aim of this study was to examine the possible effects of urodilatin and ANP on the Na^+-ATPase and (Na^+K^+)ATPase activities of isolated basolateral membranes from proximal tubule.

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2. Materials and methods

2.1. Materials

ATP, ouabain, furosemide, EGTA, HEPES, sodium azide, ANP, C-type natriuretic peptide and brain natriuretic peptide were purchased from Sigma Chemical Co., St. Louis, MO, USA. Percoll was from Pharmacia Biotech, Uppsala, Sweden. All other reagents were of the highest purity available. [^{32}P]Pi 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 et al. [14].

2.2. Preparation of isolated basolateral membranes from proximal tubule

Cortex homogenates and basolateral membranes were prepared from adult pig kidney. The kidneys were removed immediately after the animal's death and maintained in cold solution containing (mM): sucrose 250, HEPES–Tris (pH 7.6) 10, EDTA 2 and PMSF 1 [8–10]. Thin slices of the cortex (cortex-corticis) were removed 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 SCR20B centrifuge using an RP12-2 rotor at 4 °C. The supernatant was collected and stored at −4 °C. The fraction containing the basolateral membranes was isolated by the Percoll gradient method [12]. The membrane preparation was resuspended in 250 mM sucrose at a final concentration of 4–10 mg of protein ml^{-1} and stored at −4 °C. The specific activity of the basolateral-marker (Na^+K^+)ATPase was eightfold enriched when compared with that measured in the initial preparation of cortex homogenate. The same enrichment was observed to the Na^+-ATPase specific activity. On the other hand, the alkaline phosphatase and 5′-nucleotidase specific activities, markers of luminal membrane, were only 1.2- and 0.25-fold enriched, respectively. Residual contamination with other subcellular membrane markers was minimal. The succinate dehydrogenase specific activity (a marker for mitochondrial contamination), acid phosphatase specific activity (a marker for lysosomal membranes) and glucose-6-phosphatase specific activity (a marker for endoplasmic reticulum) were decreased by 95%, 90% and 94%, respectively [15,16].

2.3. Measurement of ATPase activity

The composition of the standard assay medium (0.1 ml) was: 10 mM $MgCl_2$; 5 mM [γ-^{32}P]ATP; 20 mM HEPES–Tris (pH = 7.0); 1 mM EGTA; and 90 mM NaCl; 10 mM Azida for the measurement of the Na^+-ATPase activity or 120 mM NaCl and 30 mM KCl for the measurement of the (Na^+K^+)ATPase activity, except as noted in figure legends.

ATPase activity was measured according to the method described by Grubmeyer and Penefsky [17]. The reaction was started by the addition of cortex homogenate or isolated basolateral membranes to a final protein concentration of 0.3–0.5 mg/ml, and stopped after 30 min by the addition of charcoal activated by HCl (0.1 N). The [^{32}P]Pi released was measured in an aliquot of the supernatant obtained after centrifugation of the charcoal suspension for 5 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 from the difference between the [^{32}P]Pi released in the absence and in the presence of 2 mM furosemide or in the presence and in the absence of NaCl, all conditions in the presence of 1 mM ouabain [12]. It was observed the same protein concentrations were determined by the Folin phenol method [18] using bovine serum albumin as standard. Each experiment was performed in at least four independent preparations of basolateral membranes or cortex homogenate.

2.4. cGMP measurement

The composition of the reaction medium was: 10 mM $MgCl_2$; 5 mM ATP; 20 mM HEPES–Tris (pH = 7.0); 10 mM Azide; 1 mM EGTA and 90 mM NaCl. The reaction was started by the addition of isolated basolateral membranes to a final protein concentration of 0.3 mg/ml, and stopped after 10 min by the addition of 0.9-ml ice-cold 6% trichloroacetic acid. cGMP was measured with a commercially available cGMP enzyme immunoassay (EIA) system (Amersham, Buckinghamshire, UK), following the protocol supplied with the kit.

2.5. Statistical analysis

Each experiment was performed in an independent preparation of basolateral membranes or cortex homogenate. The data were analyzed 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 in percentage of the control ± S.E. of the means.

3. Results and discussion

3.1. Effect of urodilatin and ANP on (Na^+K^+)ATPase and Na^+-ATPase activities

In order to avoid the effect of urodilatin and ANP on luminal sodium transporters, all experiments were performed using basolateral membranes isolated from pig kidney proximal tubules.

To determine the effect of urodilatin and ANP on the sodium pumps, we measured the (Na^+K^+)ATPase and Na^+-ATPase activities in the presence of different concentrations of both peptides (Fig. 1). The increase in concentrations of both peptides from 10^{-18} to 10^{-7} M inhibits the Na^+-ATPase activity in a dose-dependent manner being the maximum effect observed at 10^{-11} M (Fig. 1). In this condition, enzyme activity decreases from 10.8 ± 1.6 (control) to 5.7 ± 0.9 or 6.1 ± 0.7 nmol Pi mg^{-1} min^{-1} in the presence of urodilatin or ANP, respectively. Simultaneous addition of 10^{-11} M ANP and 10^{-11} M urodilatin does not have an additive effect on the Na^+-ATPase activity (data not shown).

On the other hand, in the same range of concentrations, urodilatin or ANP do not modulate the (Na^+K^+)ATPase activity in basolateral membranes isolated from proximal tubule (Fig. 1). The same lack of effect was observed in external cortex homogenates preparation and in the presence of subsaturating Na^+ concentrations (data not shown). These data are in agreement with the observation that ANP only inhibits the proximal tubule (Na^+K^+)ATPase activity after stimulation of the enzyme with norepinephrine or angiotensin II [19]. Taken together these data suggest that ANP and urodilatin may play a role in counteracting the activation of proximal tubule (Na^+K^+)ATPase by antinatriuretic compounds. However, the direct effect of both compounds cannot be ruled out since it was observed that the modulation of the (Na^+K^+)ATPase depends on cell integrity [19,20]. Further experiments are necessary to clarify this issue.

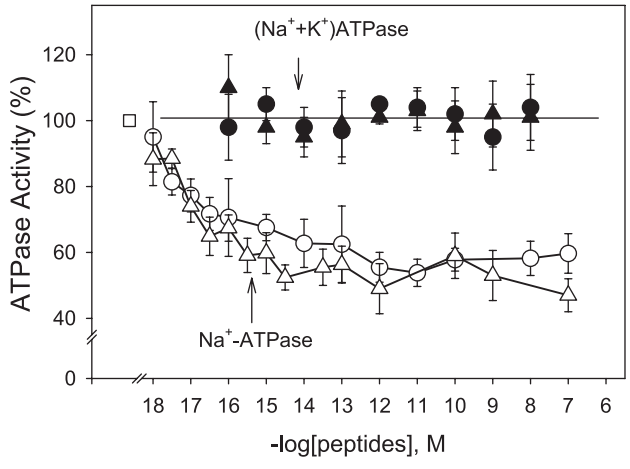

Fig. 1. Urodilatin (circles) and ANP (triangles) concentration dependence of Na^+-ATPase (open symbols) and (Na^+K^+)ATPase (closed symbols) activities in isolated basolateral membranes of renal proximal tubules. ATPase activities were measured as described in Materials and methods. The urodilatin and ANP concentrations were increased from 10^{-18} to 10^{-7} M. Results are expressed as percentage of the control, without peptides (square). The absolute values ± S.E. of the Na^+-ATPase and (Na^+K^+)ATPase activities were 10.8 ± 1.6 and 103.1 ± 7.6 nmol Pi mg^{-1} min^{-1}, respectively. *Statistically significant when compared to control ($P < 0.05$) ($n = 10$).

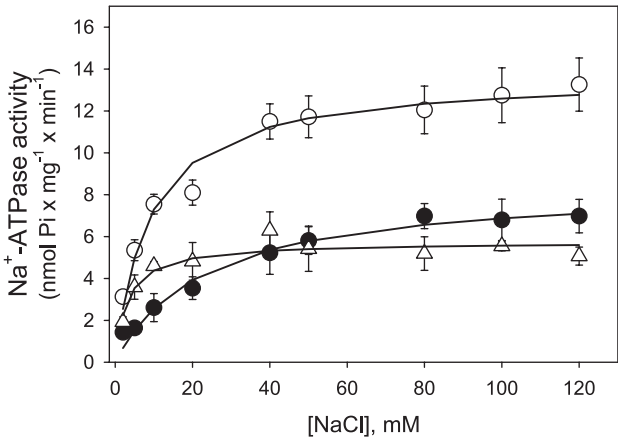

Fig. 2. Na^+ concentration dependence of Na^+-ATPase activity in isolated basolateral membranes of renal proximal tubule with or without peptides (open circles). When indicated, 10^{-11} M urodilatin (closed circles) or 10^{-11} M ANP (open triangles) was added. The Na^+-stimulated ATPase activity was calculated as the difference between the ATPase activity in the presence and absence of Na^+, both in the presence of ouabain 2 mM (see Materials and methods). Na^+ concentration was increased from 2 to 120 mM. The kinetic parameters were calculated by the following equation: $v = V_{max}+[S]/K_m[S]$. Results are expressed as mean ± S.E. ($n = 5$).

Fig. 2 shows the effect of 10^{-11} M ANP or 10^{-11} M urodilatin on the Na^+ concentration dependence of the Na^+-ATPase activity in the presence of 1.0 mM ouabain. The Na^+ concentration that promotes half maximal stimulation ($K_{0.5}$) was 9.6 ± 1.5 mM and the maximal rate (V_{max}) was 13.5 ± 1.1 nmol Pi mg^{-1} min^{-1}. Urodilatin decreases both $K_{0.5}$ and V_{max} to 3.1 ± 0.8 mM and 5.7 ± 0.2 nmol Pi mg^{-1} min^{-1}, respectively. ANP decreases V_{max} to 8.4 ± 0.4 nmol Pi mg^{-1} min^{-1} and increases $K_{0.5}$ to 22.9 ± 3.9 mM.

3.2. Possible involvement of guanylyl cyclase/cGMP on inhibition of Na^+-ATPase activity by urodilatin and ANP

The effect of ANP and urodilatin on renal sodium excretion is mediated by receptors containing an intrinsic guanylyl cyclase activity [21]. The binding of these peptides to their receptor increases guanylyl cyclase activity leading to a rise in cGMP concentration. In the next experimental group, we verified the effect of both ANP and urodilatin on the cGMP level (Fig. 3). The addition of 10^{-11} M ANP or 10^{-11} M urodilatin increases the cGMP level by 33% and 49%, respectively. Furthermore, 10^{-6} M LY83583, a guanylyl cyclase inhibitor, completely reverses the inhibitory effect of ANP and urodilatin on the Na^+-ATPase activity (Fig. 4). These data agree with the observation that ANP stimulates cGMP synthesis in rat proximal tubule [22].

Fig. 5 shows the effect of dibutyryl-cGMP (d-cGMP) on the Na^+-ATPase activity of basolateral membrane. An increase in d-cGMP concentrations from 1 to 10 nM inhibits the Na^+-ATPase activity in a dose-dependent manner, being the maximal effect at 10 nM. In this condition, the enzyme

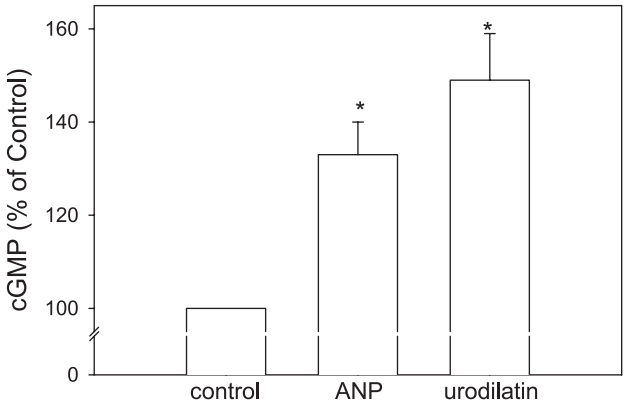

Fig. 3. Changes in cGMP induced by ANP (10^{-11} M) and urodilatin (10^{-11} M). cGMP was measured as described in Materials and methods. Results are expressed as percentage of the control. In the control condition the cGMP level is 6.7 pmol mg^{-1}. *Statistically significant when compared to control ($P<0.05$) ($n=3$).

activity was decreased in 58% (Fig. 5A). This effect is similar and not additive to urodilatin or ANP (Fig. 5B).

The physiological actions of natriuretic peptides are mediated by specific receptors: NPR-A, NPR-B and NPR-C [21]. NPR-A and NPR-B present a guanylate cyclase activity in their domains while NPR-C lacks this domain [6,21]. It was observed that ANP and urodilatin bind preferentially to NPR-A receptor and this receptor is expressed in different nephron segments including proximal tubule [7]. Our results show that ANP and urodilatin have the same potency and affinity on the inhibition of Na^+-ATPase activity and stimulation of cGMP production. We then postulated that urodilatin and ANP modulate the

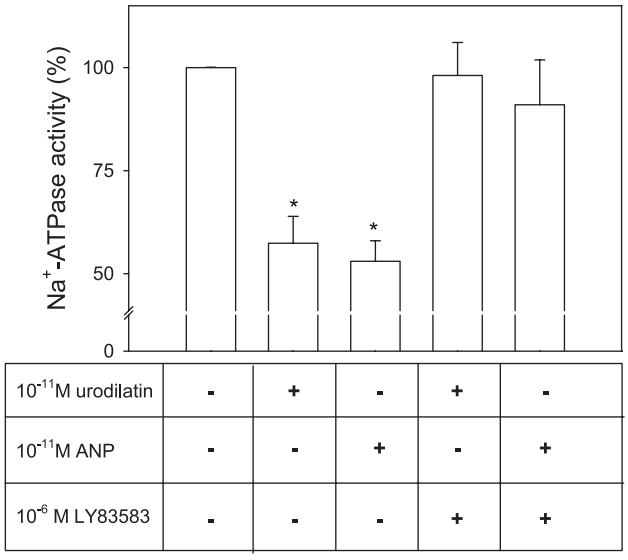

Fig. 4. Reversal of the effect of urodilatin and ANP on Na^+-ATPase activity by LY83583, a guanylyl cyclase inhibitor. ATPase activity was measured as described in Materials and methods. Results are expressed as percentage of the control. *Statistically significant when compared to control ($P<0.05$) ($n=6$).

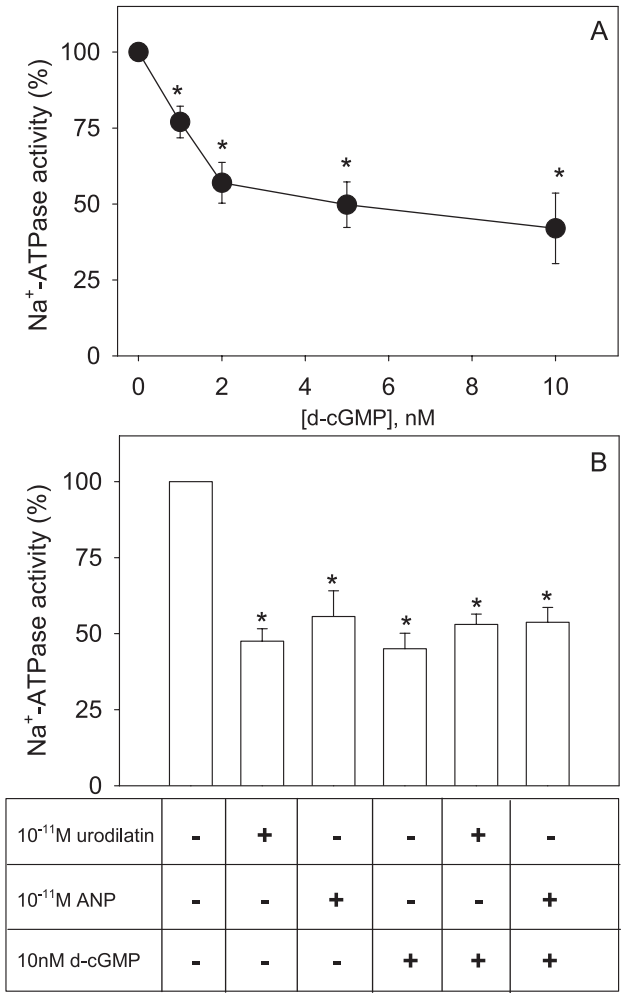

Fig. 5. Effect of dibutyryl-cGMP (d-cGMP) on Na^+-ATPase activity in the absence (A) or in the presence of 10^{-11} M urodilatin or 10^{-11} M ANP (B). In panel (A) d-cGMP concentration was increased from 1 to 10 nM. ATPase activity was measured as described in Materials and methods. Results are expressed as percentage of the control. *Statistically significant when compared to control ($P<0.05$) ($n=7$).

proximal tubule Na^+-ATPase through the activation of NPR-A/guanylate cyclase/cGMP pathway. This hypothesis agrees with the observation that the effect of ANP and urodilatin on renal sodium excretion is mediated by NPR-A receptors [6].

Since the effects of ANP and urodilatin are completely abolished by guanylate cyclase inhibitor, the possibility that both peptides bind directly to the enzyme could be ruled out. If the effect of cGMP on the enzyme activity is due to a direct effect on the enzyme, such as observed in some channels [23], or mediated by other protein such as PKG [6], it was not determined yet. Further experiments are necessary to clarify this issue.

In general the effects of ANP and urodilatin on the renal sodium excretion are associated to changes in the sodium reabsorption in the distal nephron segments [6]. It was observed that ANP modulates the proximal tubule sodium reabsorption, although little is known about the effects of

urodilatin in proximal tubule [6,10,25]. In the present paper we showed that urodilatin inhibits the proximal tubule Na^+-ATPase which is compatible with its effect on the renal sodium excretion. Taken together these data indicate that the proximal tubule Na^+-ATPase is a target to the action of urodilatin during the regulation of renal sodium excretion. Furthermore, the observation that urodilatin and ANP have the same effect on the proximal tubule Na^+-ATPase favors the hypothesis that urodilatin reproduces the mechanisms of sodium transport regulation triggered by ANP [6].

It has been proposed that urodilatin is produced in distal tubule and secreted into tubular lumen [6]. Our data show that urodilatin is able to modulate the proximal tubule Na^+-ATPase through the interaction of basolateral receptors, suggesting that there may be a mechanism of secretion into the interstitial space. In this way, the urodilatin produced could act as a paracrine compound modulating the sodium transport in adjacent segments such as proximal tubule. This hypothesis is supported by the following observations: (1) systemic or intrarenal administration of urodilatin increases the fractional renal sodium excretion [24]; (2) it has been shown that urodilatin could bind to the same ANP receptors [6]. Furthermore, it was observed the presence of ANP receptors in the luminal and basolateral side of mouse proximal tubules [25].

The Na^+-ATPase was initially described by Proverbio et al. [26,27] in aged microsomal fractions from guinea-pig kidney cortex. They described two forms of Na^+-stimulated ATPase activity: (a) the classical ouabain-sensitive (Na^+K^+)ATPase, and (b) the ouabain-insensitive Na^+-ATPase, which was sensitive to ethacrynic acid and furosemide. It was observed that this enzyme is located in basolateral membranes of the proximal tubule and it is also expressed in different cells of several species [11,12,28]. In the present paper, we observed that urodilatin and ANP selectively inhibit the Na^+-ATPase of proximal tubule, which is compatible with their effect on renal sodium excretion. Similar results were observed in our laboratory using angiotensins, kinins and adenosine [29–32]. Taken together these data support the hypothesis that the ouabain-insensitive Na^+-ATPase is a primary active transport target for compounds involved in the fine regulation of Na^+ reabsorption in proximal tubule.

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