Involvement of the $G_{i/o}$ /cGMP/PKG pathway in the AT_2 -mediated inhibition of outer cortex proximal tubule Na^+ -ATPase by Ang-(1–7)

Lucienne da Silva LARA, Fabíola CAVALCANTE, Flavia AXELBAND, Aloa Machado DE SOUZA, Anibal Gil LOPES and Celso CARUSO-NEVES¹

Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, CCS-bloco G, 21949-900, Rio de Janeiro, RJ, Brazil

The molecular mechanisms involved in the Ang-(1–7) [angiotensin-(1–7)] effect on sodium renal excretion remain to be determined. In a previous study, we showed that Ang-(1–7) has a biphasic effect on the proximal tubule Na^+ -ATPase activity, with the stimulatory effect mediated by the AT_1 receptor. In the present study, we investigated the molecular mechanisms involved in the inhibition of the Na^+ -ATPase by Ang-(1–7). All experiments were carried out in the presence of 0.1 nM losartan to block the AT_1 receptor-mediated stimulation. In this condition, Ang-(1–7) at 0.1 nM inhibited the Na^+ -ATPase activity of the proximal tubule by 54 %. This effect was reversed by 10 nM PD123319, a specific antagonist of the AT_2 receptor, and by 1 μ M GDP[β -S] (guanosine 5'-[β -thio]diphosphate), an inhibitor of G protein. Ang-(1–7) at 0.1 M induced [³⁵S]GTP[S] (guanosine 5'-[γ -³⁵S]thio]triphosphate) binding and 1 μ g/ml pertussis toxin, an

inhibitor of $G_{i/o}$ protein, reversed the Ang-(1–7) effect. Furthermore, it was observed that the inhibitory effect of Ang-(1–7) on the Na^+ -ATPase activity was completely reversed by 0.1 μ M LY83583, an inhibitor of guanylate cyclase, and by 2 μ M KT5823, a PKG (protein kinase G) inhibitor, and was mimicked by 10 nM d-cGMP (dibutyryl cGMP). Ang-(1–7) increased the PKG activity by 152 % and this effect was abolished by 10 nM PD123319 and 0.1 μ M LY83583. Taken together, these data indicate that Ang-(1–7) inhibits the proximal tubule Na^+ -ATPase by interaction with the AT_2 receptor that subsequently activates the $G_{i/o}$ protein/cGMP/PKG pathway.

Key words: angiotensin-(1–7), angiotensin receptors, Na^+ -ATPase, renal epithelium, cellular signalling, extracellular volume.

INTRODUCTION

The kidneys possess all of the compounds that are necessary for the synthesis of Ang-(1–7) [angiotensin-(1–7): Asp-Arg-Val-Tyr-Ile-His-Pro] and are the site of action for this peptide [1–3]. The physiological role of Ang-(1–7) is not completely determined, but it has been proposed that this peptide is involved in electrolyte balance as well as blood pressure regulation [1–4]. These effects are associated with the modulation of renal sodium excretion and, at least in part, are due to modulation of the transcellular sodium reabsorption [1,3,5–7].

Ang-(1–7) modulates sodium reabsorption by both the proximal and distal segments of the nephron [8]. In the proximal tubule, Ang-(1–7) has a biphasic effect: at lower concentrations (1 pM), the peptide increases fluid and bicarbonate reabsorption, whereas, at higher concentrations (10 nM), reabsorption decreases [9]. 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 [10,11]. These enzymes are located in basolateral membranes and they are involved in the genesis of the sodium electrochemical gradient [10]. In a previous study, we found a biphasic effect of Ang-(1–7) on proximal tubule Na^+ -ATPase with no change in the Na^+/K^+ -ATPase activity [5]. The stimulatory effect of Ang-(1–7) on proximal tubule Na^+ -ATPase is mediated by the AT_1 receptor [5], whereas the receptor and molecular mechanisms that are involved in the inhibitory effects are not known.

Besides the AT_1 receptor, an interaction with two different receptor(s) subtypes, AT_2 and AT_{1-7} , could mediate Ang-(1–7) effects. The heterogeneous distribution of these receptors along the nephron would determine the overall effect of Ang-(1–7) [12,13]. All three receptors have seven transmembrane domains and belong to the family of GPCRs (G-protein-coupled receptors) [14–16]. The AT_2 receptor shares 34 % amino acid sequence identity with the AT_1 receptor [16]. It is more expressed during foetal development and its level drops after birth [16]. Studies indicate that sodium depletion or glomerular injury can regulate the AT_2 receptor [13,17]. It may play an important role in counteracting the physiological and pathophysiological effects that are mediated by AT_1 receptor in adult animals [18–20]. The nature of signalling mechanisms coupled to the AT_2 receptor are still controversial. Although the AT_2 receptor is a seven-transmembrane-domain receptor, its coupling to heterotrimeric G-proteins remains uncertain [20]. The AT_{1-7} receptor was cloned, and the signalling pathways activated by this receptor are not well established [14]. Furthermore, little is known about the involvement of AT_2 and AT_{1-7} receptors on the modulation of the sodium transporters as well as the pathways involved in this process [3,19].

The aim of this work was to investigate the molecular mechanisms that are involved in the inhibition of the Na^+ -ATPase activity by Ang-(1–7) in isolated basolateral membranes from proximal tubules. In these studies, the AT_1 receptor-mediated stimulatory component was blocked by losartan. Our results show that Ang-(1–7) inhibits the Na^+ -ATPase activity through

Abbreviations used: Ang-(1–7), angiotensin-(1–7); d-cAMP, dibutyryl cAMP; d-cGMP, dibutyryl cGMP; GDP[β -S], guanosine 5'-[β -thio]diphosphate; GPCR, G-protein-coupled receptor; GTP[S], guanosine 5'-[γ -thio]triphosphate; NOS, nitric oxide synthetase; PACOCF₃, palmitoyl trifluoromethyl ketone; PKA, protein kinase A; PKAi, PKA inhibitor peptide; PKC, protein kinase C; PKG, protein kinase G; PLA₂, phospholipase A₂; PTX, pertussis toxin; TBST, Tris-buffered saline containing 0.05 % Tween 20; TCA, trichloroacetic acid.

¹ To whom correspondence should be addressed (email caruso@biof.ufrj.br).

activation of AT₂ receptor coupled to G_{ai/o} protein. Furthermore, activation of the AT₂ receptor by Ang-(1–7) leads to activation of the cGMP/PKG (protein kinase G) pathway. Because all experiments were carried out in isolated basolateral membranes, all components necessary for these effects must be associated with the plasma membrane. This suggests a mechanism for rapid regulation mediated by the AT₂ receptor. These results open several possibilities in understanding the role of the AT₂ receptor in renal sodium excretion and, consequently, the long-term regulation of extracellular volume and blood pressure.

EXPERIMENTAL

Materials

ATP, d-cAMP (dibutyryl-cAMP), d-cGMP (dibutyryl-cGMP), GTP[S] (guanosine 5'-[γ -thio]triphosphate), GDP[β -S] (guanosine 5'-[β -thio]diphosphate), PMA, PKAi [PKA (protein kinase A) inhibitor peptide], PTX (pertussis toxin), ouabain, EDTA, Hepes, Tris, Ang-(1–7), saralasin ([Sar¹,Ile⁸]angiotensin II), PD123319, Triton X-100, Protein A-agarose and histone were purchased from Sigma Chemical Co. The AT₁ receptor selective antagonist, losartan, was obtained from Medley S.A. [³⁵S]GTP[S] (guanosine 5'-[γ -³⁵S]thio]triphosphate) and Percoll were from Amersham Biosciences. Autoradiograph films (T-Mat) were from Eastman Kodak. KT5823, LY 83583, PACOCF₃ (palmitoyl trifluoromethyl ketone), genistein, calphostin C, polyclonal anti-PKG antibody and horseradish-peroxidase-conjugated sheep anti-rabbit antibody were purchased from Calbiochem. Antibody against AT₂ receptor was obtained from Santa Cruz Biotechnology. Peptide antagonist A779 {[D-Ala⁷]-Ang-(1–7)} and non-peptide agonist (AVE 0991) of the AT₁₋₇ receptor were kindly donated by Dr R. A. S. Santos (Departamento de Fisiologia e Biofísica, Universidade Federal de Minas Gerais, Belo Horizonte, BH, Brazil). All other reagents were of the highest purity available. [³²P]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 -³²P]ATP was prepared as described by Maia et al. [21].

Preparation of isolated basolateral membranes

The basolateral membranes of the outer cortex were prepared from adult pig kidneys. The kidneys were obtained from a commercial slaughterhouse immediately after the death of the animals and were maintained in a cold solution containing 250 mM sucrose, 10 mM Hepes/Tris, pH 7.6, 2 mM EDTA and 1 mM PMSF. Thin slices of outer cortex were removed using a scalpel. After dissection, slices were homogenized in the same cold solution with a Teflon/glass homogenizer. The homogenate was centrifuged at 1900 *g* for 10 min at 4°C in a SCR20B centrifuge using an RP12-2 rotor (Hitachi). The supernatant was collected and stored at 4°C. The fraction enriched in basolateral membranes was isolated by the Percoll gradient method [22]. The membrane preparation was resuspended in 250 mM sucrose at a final concentration of 5–10 mg of protein/ml and was stored at –20°C. The Na⁺/K⁺-ATPase activity, a marker for basolateral membranes, was 10–12-fold higher than the activity found in the cortex. The same enrichment was observed for the Na⁺-ATPase specific activity; both were determined as described in [11]. On the other hand, the alkaline phosphatase and 5'-nucleotidase specific activities, markers of the luminal membrane, were only 1.2- and 0.25-fold enriched respectively. Residual contamination with other subcellular membrane fractions was minimal. The specific activities of succinate dehydrogenase (a marker for mitochondrial

contamination), acid phosphatase (a marker for lysosomal membranes) and glucose-6-phosphatase (a marker for endoplasmic reticulum) were decreased by 95, 90 and 94 % respectively, determined as described in [23,24].

Protein concentration was determined by the Folin phenol method [25] using BSA as a standard.

Measurement of the ATPase activity

The composition of the assay medium for the measurement of the Na⁺-ATPase activity (0.1 ml) was 4 mM MgCl₂, 4 mM ATP (specific activity of approx. 10⁴ Bq/nmol of ATP), 20 mM Hepes/Tris, pH 7.0, 90 mM NaCl, 0.1 nM losartan and 1 mM ouabain.

The ATPase activity was measured according to the method described by Grubmeyer and Penefsky [26]. The reaction was started by adding membranes to a final protein concentration of 0.3–0.5 mg/ml, and was stopped after 10 min by adding 0.1 M HCl-activated charcoal. The [³²P]P_i released was measured in an aliquot of 0.2 ml of the supernatant obtained after centrifugation of the charcoal suspension for 5 min at 1500 *g* in a clinical centrifuge. Spontaneous hydrolysis of [γ -³²P]ATP was measured simultaneously in tubes to which protein was added after the acid. The Na⁺-ATPase activity was calculated from the difference between the [³²P]P_i released in the absence and in the presence of 2 mM furosemide, both in the presence of 1 mM ouabain [11].

Protein kinase activity assay

The protein kinase activity of isolated basolateral membranes was measured by protein kinase inhibitor-sensitive incorporation of [³²P]P_i from [γ -³²P]ATP (7 μ Ci/ μ mol), using histone as substrate. The composition of the reaction medium was 4 mM MgCl₂, 20 mM Hepes/Tris pH 7.0, 1.5 mg/ml histone and 0.7 mg/ml protein. After 10 min, the reaction was stopped with 40 % TCA (trichloroacetic acid) and the sample was immediately placed on ice. An aliquot (0.1 ml) was filtered through a Millipore filter (0.45 μ m pore size) and was washed with ice-cold 20 % TCA solution and 0.1 M phosphate buffer (pH 7.0). The radioactivity was quantified by liquid-scintillation counting (Packard Tri-Carb 2100 TR). The specific PKG, PKC (protein kinase C) and PKA activities were calculated from the difference between the activity in the absence and in the presence of 2 μ M KT5823, 10 nM calphostin C or 10 nM PKAi respectively. cGMP, phorbol ester (PMA) and cAMP were used as activators of PKG, PKC and PKA respectively.

GTP[S] binding

The GTP[S] binding was measured according to the method described by Lazareno et al. [27]. The composition of the reaction medium (50 μ l) was 100 mM NaCl, 10 mM MgCl₂ and 20 mM Hepes/Tris, pH 7.0. Basolateral membranes (1 mg/ml) were incubated with 5 nM [³⁵S]GTP[S] and 0.1 nM Ang-(1–7). The assay solutions were incubated for 1 min at 37°C and the reaction was stopped by the addition of an ice-cold solution containing 100 mM NaCl, 10 mM MgCl₂ and 20 mM Hepes/Tris, pH 7.0. The samples were immediately filtered on borosilicate filters (MFS Advantec, GB140, 25 mm diameter). The radioactivity was quantified by liquid-scintillation counting. The basal binding was determined in the absence of the agonist, and non-specific binding was measured in the presence of 1 mM unlabelled GTP.

Immunoprecipitation

Basolateral membranes from proximal tubules (5 mg/ml) were initially incubated in the presence of 0.1 nM losartan (control) or in the presence of 0.1 nM losartan and 0.1 nM Ang-(1–7) for

10 min. The membranes were solubilized in buffer containing 0.01 % Triton X-100 for 30 min at room temperature (24 °C). Anti-(AT₂ receptor) polyclonal antibody (1:200 dilution) was added at 4 °C with constant stirring for 2 h. Protein A-agarose was added to the mixture for an additional 30 min under the same conditions (according to the manufacturer's instructions). The samples were centrifuged at 4500 *g* for 10 min. The supernatant was removed, and the immunoprecipitates were subjected to SDS/10 % PAGE followed by immunoblotting.

Immunoblotting

The presence in basolateral membranes of PKG and AT₂ receptor protein was determined by immunoblotting. Total basolateral membrane protein (70 µg) was resolved by SDS/10 % PAGE. The proteins were transferred on to PVDF membranes, which were then blocked with TBST (Tris-buffered saline containing 0.05 % Tween 20) plus 5 % (w/v) non-fat dried milk powder for 1 h at room temperature. The membranes were incubated for 1.5 h with the appropriate antibody [polyclonal anti-PKG antibody, diluted 1:1000, and anti-(AT₂ receptor), diluted 1:1000]. Following three consecutive washes with ice-cold TBST, the membranes were incubated with horseradish-peroxidase-conjugated anti-rabbit antibody (Amersham Biosciences) diluted 1:2000 in TBST plus 5 % (w/v) non-fat dried milk powder and then washed three more times with TBST. The signals were visualized using ECL[®]-plus (enhanced chemiluminescence) (Amersham Biosciences).

Data analysis

The means were compared by one-way ANOVA, taking into account the treatment of experimental groups. The magnitudes of differences were evaluated using the multiple comparative Bonferroni test. The data are presented as means ± S.E.M. The *n* values correspond to the results obtained from different basolateral membrane preparations.

RESULTS

Identification of the receptor involved in the inhibitory effect of the Ang-(1–7) upon Na⁺-ATPase activity

In a previous study, we showed that Ang-(1–7) has a biphasic effect on the proximal tubule Na⁺-ATPase activity, with the stimulatory effect mediated by the AT₁ receptor [5]. In the present study, we examined the possibility that the inhibitory component could be due to the action of Ang-(1–7) through another receptor(s). We measured the enzyme activity in the presence of 0.1 nM losartan to block the AT₁ receptor-mediated stimulation. Increasing Ang-(1–7) from 10 fM to 0.1 µM inhibited the Na⁺-ATPase activity in a dose-dependent manner, with the maximum effect observed at 0.1 nM (Figure 1A). Ang-(1–7) at 0.1 nM decreased the enzyme activity from 17.1 ± 2.7 (control) to 7.8 ± 1.4 nmol of P_i/min per mg. This inhibitory effect of Ang-(1–7) was completely abolished by 0.1 pM saralasin, a non-specific antagonist of AT receptors (Figure 1B). Saralasin alone (10 nM) did not modify the enzyme activity. These data suggest that Ang-(1–7) interaction with AT receptor inhibits the proximal tubule Na⁺-ATPase activity.

A new angiotensin receptor has been cloned: the AT₁₋₇ receptor [14]. This receptor is present in several tissues, including the kidneys, and binds Ang-(1–7) exclusively [14]. Furthermore, the availability of an AT₁₋₇ receptor-agonist (AVE0991), as well as a specific peptide antagonist, A779, makes it possible to investigate whether the effects of Ang-(1–7) are mediated by this re-

Figure 1 Ang-(1–7) inhibits Na⁺-ATPase activity

ATPase activity was measured as described in the Experimental section, always in the presence of 0.1 nM losartan. (A) Ang-(1–7) concentrations ranged from 10 fM to 0.1 µM (●). ○, Control in the absence of Ang-(1–7). (B) Effects of saralasin (Sar) at concentrations increasing from 1 fM to 10 nM. Saralasin at 10 nM did not modify the enzyme activity (results not shown). Ang-(1–7) at 0.1 nM was added where indicated (*n* = 18). Results are means ± S.E.M. *, *P* < 0.05 compared with the control.

ceptor [28,29]. Figure 2(A) shows the effect of AVE0991 on the Na⁺-ATPase activity in the presence of 0.1 nM losartan. Increasing concentrations of this agonist from 10 fM to 0.1 µM did not modify the enzyme activity. In addition, A779 did not abolish the Ang-(1–7) inhibitory effect on the Na⁺-ATPase activity (Figure 2B). A779 alone (0.1 µM) did not modify the enzyme activity (results not shown).

To determine the involvement of the AT₂ receptor in the inhibitory effect of Ang-(1–7) on the Na⁺-ATPase activity, we performed experiments in the presence of PD123319, a specific AT₂ receptor antagonist (Figure 3). Increasing PD123319 from 1 pM to 0.1 µM abolished the inhibition by Ang-(1–7), with complete reversal obtained at 0.1 nM. The presence of the AT₂ receptor was detected by immunoblotting. After the immunoprecipitation of the AT₂ receptor, immunoblotting for AT₂ receptor revealed a band at 50 kDa which corresponds to the correct molecular mass for AT₂ (Figure 3B). These data show that Ang-(1–7) inhibits the proximal tubule Na⁺-ATPase through the AT₂ receptor.

Involvement of G-protein in the inhibitory effect of the Ang-(1–7) upon Na⁺-ATPase activity

Although the AT₂ receptor belongs to the family of GPCRs, it can exert its effects through both G-protein-dependent and -independent pathways [30]. To test the involvement of a G-protein in the Ang-(1–7)-mediated inhibition of the Na⁺-ATPase activity, we assayed activity in the presence of GDP[β-S], a non-hydrolysable GDP analogue (Figure 4A). The simultaneous addition of 0.1 nM

Figure 2 Involvement of the AT₁₋₇ receptor in the modulation of Na⁺-ATPase activity by Ang-(1-7)

ATPase activity was measured as described in the Experimental section, always in the presence of 0.1 nM losartan. (A) Effects of AVE0991, agonist of the AT₁₋₇ receptor, on enzyme activity. The agonist concentration was increased from 10 fM to 0.1 μ M (●). ○, Control in the absence of AVE0991 ($n=9$). (B) Effects of A779, antagonist of the AT₁₋₇ receptor, at concentrations increasing from 10 pM to 0.1 μ M. A779 at 10 nM did not modify the enzyme activity (results not shown). Ang-(1-7) at 0.1 nM was added where indicated ($n=7$). Results are means $\pm$ S.E.M. *, $P < 0.05$ compared with the control.

Ang-(1-7) and GDP[β -S] (from 10 nM to 1 μ M) abolished the Ang-(1-7) effect. Complete reversal was obtained at 1 μ M GDP[β -S]. At this concentration, GDP[β -S] alone did not change the Na⁺-ATPase activity.

In addition, we measured the effect of 0.1 nM Ang-(1-7) on [³⁵S]GTP[S] incorporation in basolateral membranes. At this concentration, Ang-(1-7) increases the [³⁵S]GTP[S] binding by 40% (Figure 4B). This effect was reversed completely by 1 μ g/ml PTX. Under our experimental conditions, non-specific binding, defined as binding in the presence of 1 mM GTP, was less than 10% of the control. Furthermore, it was observed that 1 μ g/ml PTX also abolished the inhibitory effect of Ang-(1-7) on the Na⁺-ATPase activity (results not shown). These data suggest that a G_{i/o} protein mediates the effect of Ang-(1-7).

Involvement of the cGMP/PKG pathway in the inhibitory effect of Ang-(1-7) upon Na⁺-ATPase activity

The molecular mechanisms triggered by AT₂ receptor activation are not completely known. To identify the mechanism involved in the AT₂-mediated modulation of the outer cortex Na⁺-ATPase activity by Ang-(1-7), several pathways were studied. Figure 5(A) shows the effect of 50 nM PACOCF₃, a plasma membrane-associated PLA₂ (phospholipase A₂) inhibitor, and 1 mM genistein, a tyrosine kinase inhibitor, on the AT₂-mediated inhibition of the outer cortex Na⁺-ATPase activity by Ang-(1-7). Neither inhibitor

Figure 3 Effects of PD123319, an AT₂ receptor antagonist, on the inhibition of Na⁺-ATPase activity by Ang-(1-7)

(A) ATPase activity was measured as described in the Experimental section, always in the presence of 0.1 nM losartan. The effects of PD123319 concentrations increasing from 1 pM to 0.1 μ M are shown. PD123319 at 10 nM did not modify the enzyme activity (results not shown). Ang-(1-7) at 0.1 nM was added where indicated. Results are means $\pm$ S.E.M. ($n=8$). *, $P < 0.05$ compared with the control. (B) Immunoblot detection of the AT₂ receptor in the proximal tubule basolateral membranes. The AT₂ receptor was pulled down using an anti-(AT₂ receptor) polyclonal antibody. After immunoprecipitation, the basolateral membranes, supernatant and the immunoprecipitated proteins were separated by SDS/10% PAGE, then transferred on to a PVDF membrane and incubated with a primary anti-(AT₂ receptor) polyclonal antibody as described in the Experimental section. The blot is representative of three carried out with different preparations. IP, immunoprecipitation; WB, Western blotting; BLM, basolateral membranes without immunoprecipitation.

changed the effect of Ang-(1-7) on the enzyme activity. We also tested the effect of Ang-(1-7) on the PKA and PKC activities in the presence of 0.1 nM losartan (Figure 5B). Ang-(1-7) did not change either of the kinase activities. The addition of 1 μ M d-cAMP increased the PKA activity by 180%, while the addition of 1 pM phorbol ester (PMA), an activator of PKC, increased the PKC activity by 250%.

Taken together, these data rule out the involvement of tyrosine kinase, PLA₂, PKC and PKA in the AT₂-mediated inhibition of the outer cortex basolateral membrane Na⁺-ATPase by Ang-(1-7).

AT₂ receptor activation may either decrease or increase cGMP levels in different cell types [31,32]. Figure 6(A) shows that 0.1 μ M LY83583, a specific guanylate cyclase inhibitor, completely reversed the inhibitory effect of Ang-(1-7), whereas 10 nM d-cGMP inhibited the enzyme activity to 60%. This effect is similar, but not additive, to that of 0.1 nM Ang-(1-7).

Because an increase in cGMP activates PKG [31], we evaluated the effect of KT5823, a PKG inhibitor, on the inhibition of the Na⁺-ATPase activity by Ang-(1-7). KT5823 at 2 μ M blocked the peptide effect (Figure 6A, rightmost bar), but alone did not modify the enzyme activity (results not shown). The presence of PKG was detected by immunoblotting. A polypeptide with molecular mass between 75 and 105 kDa, which corresponds to the expected molecular mass for previously characterized PKG, was immunodetected by a polyclonal anti-PKG antibody (Figure 6B) [33].

Figure 4 Involvement of G-protein in the inhibition of Na⁺-ATPase activity by Ang-(1–7)

(A) Effect of GDP[β-S] on Ang-(1–7)-inhibited enzyme activity. ATPase activity was measured as described in the Experimental section, always in the presence of 0.1 nM losartan. GDP[β-S] concentration increased from 10 nM to 1 μM. GDP[β-S] at 1 μM did not modify the enzyme activity (results not shown). Ang-(1–7) at 0.1 nM was added where indicated ($n = 5$). (B) Binding of [³⁵S]GTP[S] by basolateral membranes induced by Ang-(1–7). Binding of [³⁵S]GTP[S] was measured as described in the Experimental section, always in the presence of 0.1 nM losartan. Ang-(1–7) at 0.1 nM and 1 μg/mL PTX were added where indicated ($n = 4$). Results are means $\pm$ S.E.M. *, $P < 0.05$ compared with the control.

In the next group of experiments, we measured PKG activity. Ang-(1–7) at 0.1 nM stimulated PKG activity by 152 %, an effect similar, but not additive, to the d-cGMP effect. Both 10 nM PD123319 and the guanylate cyclase inhibitor LY83583 at 0.1 μM reversed the effect of Ang-(1–7) (Figure 7). LY83583 alone (0.1 μM) decreased the basal PKG activity by 70 %. These data indicate that inhibition of Na⁺-ATPase by Ang-(1–7) is mediated by peptide interaction with AT₂ receptors that subsequently leads to the activation of PKG.

DISCUSSION

Ang-(1–7) is involved in electrolyte balance as well as blood pressure regulation [1,3,4], although little is known about the receptors and signalling pathways involved. In the present work, it was observed that an inhibitory effect of Ang-(1–7) on the proximal tubule Na⁺-ATPase activity is mediated by the AT₂ receptor and involves the activation of a G_{i/o}-dependent cGMP/PKG pathway. Moreover, the data in the present paper and another from our group [12] show for the first time the direct modulation of the sodium pump by the AT₂ receptor. This observation opens several possible routes to understanding the role of the AT₂ receptor in renal

Figure 5 AT₂ receptor-mediated Ang-(1–7) effects on Na⁺-ATPase activity does not involve PLA₂, tyrosine kinase, PKC or PKA

(A) Effects of PACOCF₃, a plasma membrane-associated PLA₂ inhibitor, and genistein, a tyrosine kinase inhibitor, on the inhibition of Na⁺-ATPase activity by 0.1 nM Ang-(1–7). ATPase activity was measured as described in the Experimental section, always in the presence of 0.1 nM losartan. PACOCF₃ and genistein did not modify the enzyme activity (results not shown). The compounds were added where indicated. Results are means $\pm$ S.E.M. ($n = 4$). (B) PKA (closed bars) and PKC (open bars) activities were measured as described in the Experimental section, in the presence of 0.1 nM losartan. Ang-(1–7) at 0.1 nM, d-cAMP at 1 μM or PMA at 1 pM was added where indicated. Results are expressed as percentages of the control ($n = 4$). *, $P < 0.05$ compared with the control.

sodium excretion and, consequently, the long-term regulation of extracellular volume and blood pressure.

Effects of Ang-(1–7) are mediated by angiotensin receptors such as AT₁, AT₂ and AT₁₋₇ [1–3,14,18]. The following data indicate that the inhibitory effect of Ang-(1–7) on the proximal Na⁺-ATPase activity is mediated by the AT₂ receptor: (i) this effect is completely reversed by 0.1 nM PD123319, a specific antagonist of the AT₂ receptor; (ii) A779, a specific antagonist of the AT₁₋₇ receptor, did not change the effect of Ang-(1–7); and (iii) all experiments were performed in the presence of losartan, a specific antagonist of the AT₁ receptor. Different groups have shown that the AT₂ receptor is expressed in the adult kidney, with the proximal tubule being one site of expression [34,35]. Furthermore, it localizes to the luminal membrane of this segment [35]. In the present study, we have shown the presence of the AT₂ receptor in basolateral membranes, suggesting another important site for AT₂ receptor-mediated regulation of sodium reabsorption in the proximal tubule.

Even though AT₂ levels are very low in contrast with the highly expressed AT₁ receptor, it has been proposed that this receptor

Figure 6 Involvement of the cGMP/PKG pathway in mediating the inhibitory effect of Ang-(1-7) on the Na⁺-ATPase activity

(A) Effect of LY83583, d-cGMP and KT5823 on the inhibition of the Na⁺-ATPase activity by 0.1 nM Ang-(1-7). ATPase activity was measured as described in the Experimental section, always in the presence of 0.1 nM losartan. LY83583 at 0.1 μM and KT5823 at 2 μM did not modify the enzyme activity (results not shown). Ang-(1-7) at 0.1 nM, d-cGMP at 10 nM, LY83583 at 0.1 μM or KT5823 at 2 μM were added where indicated. Results are means ± S.E.M. (*n* = 7). *, *P* < 0.05 compared with the control. (B) Immunoblot detection of PKG in the proximal tubule basolateral membranes. Basolateral membrane proteins were separated by SDS/7.5% PAGE, then transferred on to a PVDF membrane and incubated with a primary anti-PKG monoclonal antibody as described in the Experimental section. The blot is representative of four carried out with different preparations. MW, molecular mass.

has an important physiological and pathophysiological role in the modulation of renal sodium excretion [36–39]. Results obtained from AT₂ receptor-deficient mice indicated that this receptor induces natriuresis and diuresis [38]. De Gasparo et al. [20] observed that the activation of the AT₂ receptor in luminal membranes of proximal tubules leads to inhibition of sodium reabsorption, but the transporters involved were not determined. It has been demonstrated that the ouabain-insensitive Na⁺-ATPase is a primary active transporter target for compounds that are involved in the regulation of renal sodium excretion, such as the peptides of the renin-angiotensin system [5,6,12]. The Na⁺-ATPase is approx. 10-fold less active than the Na⁺/K⁺-ATPase [10], which suggests that this enzyme may be involved in fine tuning, whereas the Na⁺/K⁺-ATPase is responsible for most of the sodium reabsorption in the proximal tubule. The data obtained in the present study, together with observations reported by our group in previous papers [5,6] indicate that the Na⁺-ATPase is involved, at least in part, in the effect of Ang-(1-7) on the proximal tubule sodium reabsorption. In addition, because AT₁ receptor-mediated Ang-(1-7) stimulates sodium reabsorption, we postulate that the interaction of Ang-(1-7) with both the AT₁ and AT₂ receptors may permit fine-tuning of sodium reabsorption in the proximal tubule.

The molecular cloning of the AT₂ receptor revealed that this receptor belongs to the GPCR family [16]. However, the bio-

Figure 7 Effects of Ang-(1-7) on PKG activity

PKG activity was measured as described in the Experimental section, in the presence of 0.1 nM losartan. Ang-(1-7) at 0.1 nM, d-cGMP at 10 nM, LY83583 at 0.1 μM or PD123319 at 10 nM were added where indicated. Results are expressed as percentages of the control. *, *P* < 0.05 compared with the control (*n* = 9).

chemical association of the AT₂ receptor with this class of proteins is a subject of debate. Some authors have demonstrated in different tissues, such as renal tissue, that AT₂ receptor binding is not susceptible to GTP analogues, suggesting a role for G-protein-independent pathways [17,30]. In the kidney, AT₂ activation elicits both G-protein-independent and -dependent pathways [12,35], and little is known about the G-protein isoform that is activated. However, previous work has shown that a G_s protein is involved in the AT₂ receptor response in isolated basolateral membranes from the inner cortex [12]. Our present data indicate that the AT₂ receptor-mediated Ang-(1-7) effect on the proximal tubule Na⁺-ATPase of the outer cortex involves the activation of a G_{i/o} protein because: (i) GDP[β-S], a non-hydrolysable GDP analogue that inhibits G-proteins, abolished the peptide effect; (ii) the peptide treatment increases the [³⁵S]GTP[S] incorporation in a PTX-sensitive manner, and (iii) PTX abolished the inhibitory effect of Ang-(1-7) on the enzyme activity. These data represent the first demonstration that the proximal tubule basolateral membrane AT₂ activation involves a G_{i/o} protein, and thus reveal a new molecular mechanism that is involved in the AT₂ receptor-induced renal response.

Despite the proposal that the AT₂ receptor modulates renal sodium excretion [17–19], the pathways involved in the regulation of renal function and sodium excretion remain obscure. In general, the activation of the G_{i/o} protein is associated with decreasing cAMP levels [30]. It is well known that the decrease in cAMP levels leads to the inhibition of PKA activity. In the present study, we show that Ang-(1-7), in the presence of 0.1 nM losartan, did not change the outer cortex PKA activity, ruling out the involvement of this pathway in the AT₂-mediated effect of Ang-(1-7) on the outer cortex Na⁺-ATPase. On the other hand, in a previous study, we showed that Ang-(1-7), through the AT₂ receptor, inhibits the Na⁺-ATPase activity of the inner cortex by activation of the G_s/cAMP/PKA pathway [12]. The involvement of this pathway in the AT₂ receptor-mediated inhibition of the outer cortex Na⁺-ATPase by Ang-(1-7) could also be ruled out because we have shown that its activation stimulates the enzyme activity instead of inhibiting it [40]. Furthermore, as discussed above,

Ang-(1–7) did not change the PKA activity through the AT₂ receptor.

Studies using the microdialysis technique have reported that activation of the AT₂ receptor stimulates renal production of cGMP in response to sodium depletion and that this effect is mediated by NO production [31]. However, the effect of AT₂-mediated cGMP production on renal sodium transporters has not yet been demonstrated. In the present study, we show that the inhibitory effect of Ang-(1–7) on Na⁺-ATPase activity in the outer cortex proximal tubule is mediated by activation of the cGMP/PKG pathway. This result is in agreement with the observation that G_{1α}-coupled AT₂ receptor activation does not modify the intracellular cAMP levels, but modulates the cGMP levels according to the cell type [30]. The data obtained in the present study and those from previous work [30] show that the signalling pathways that are involved in AT₂-mediated inhibition of Na⁺-ATPase of inner and outer cortex basolateral membranes by Ang-(1–7) are different. So it is plausible to postulate that the pathways coupled to the AT₂ receptor depend on the cell type and even the localization of these cells in the kidney. This hypothesis is supported by the observation that several pathways have been described to be coupled to the AT₂ receptor in the same or in different cell types [18,32].

Some groups have investigated the link between the activation of G_{1α}-protein and guanylate cyclase activity [31,41]. In cardiac myocytes, the activation of G_{1α}-coupled AT₂ receptors increases cGMP levels through serine/threonine phosphatase or tyrosine phosphatase stimulation [31,41]. On the other hand, NOS (nitric oxide synthetase) is a substrate for kinases, including PKC, which is activated by G_{1α}-coupled receptors [42]. This raises another possibility in which G_{1α}-coupled AT₂ receptor stimulates PKC and leads to the increase in NOS activity, followed by an increase in guanylate cyclase activity. Our preliminary data indicate that the inhibitory effect of Ang-(1–7) on the Na⁺-ATPase is completely abolished by NOS inhibitors. However, we showed that Ang-(1–7) did not change PKC activity through the AT₂ receptor. Further experiments will be necessary to clarify this issue.

All of the results of the present study were obtained using basolateral membranes isolated from proximal tubule cells. Several components of different cell signalling pathways are present in the basolateral membrane, including G-proteins, phospholipases and protein kinases [5,6,12]. In general, it is accepted that the activation of the kinases involves the redistribution of enzymes from cytosol to membrane. However, Chakravarthy et al. [43] showed that membranes of different cells have a significant amount of constitutive PKC that can be stimulated by signals that produce diacylglycerol. In previous studies, we showed that the isolated basolateral membranes of renal proximal tubules contain a constitutive PKC and PKA that, when activated by phorbol ester or cAMP respectively, phosphorylate other proteins located in this membrane [40,44]. Recently, we showed that urodilatin and ANP (atrial natriuretic peptide) increase cGMP levels which lead to the inhibition of the basolateral membrane Na⁺-ATPase, indicating the presence of guanylate cyclase in this membrane [45]. This result agrees with the observation that guanylate cyclase is present in the plasma membranes of different cell types [46]. In the present study, we have shown that isolated basolateral membranes of outer renal proximal tubules contain a constitutive PKG that is stimulated by cGMP. The presence of complete signalling complexes in plasma membrane, from receptors to all intermediary elements, to final membrane targets, illustrates an important means by which fine and fast modulation of renal sodium reabsorption by proximal tubule cells during acute sodium intake changes can occur. This hypothesis is in agreement with the proposal that cellular signalling could occur in distinct components or even in microdomains of the plasma membrane such as

caveolae. Such compartmentalization is usually associated with epithelial cell types, such as kidney cells [47].

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