ANG-(3–4) inhibits renal Na\(^+\)-ATPase in hypertensive rats through a mechanism that involves dissociation of ANG II receptors, heterodimers, and PKA

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**Materials and Methods**

**Animals.** Eleven-week-old SHR (obtained from CEDEME/UNIFESP) and age-matched WKY rats (obtained from CEMIB/UNICAMP) were used. This age was selected because ANG-(3–4) has its maximum blood pressure-lowering effect in SHR at this age (34). All procedures were approved by the Committee for Experimental and Animal Ethics at the Federal University of Rio de Janeiro (protocol IBCCF106) and performed in accordance with its recom-
mendations. The animals were kept for at least 72 h in a room at 22 ± 3°C with a 12:12-h light-dark cycle with access to a standard rat chow and water ad libitum.

**Determination of arterial pressure and in vivo Na+ excretion.** After adaptation of the animals for 24 h in metabolic cages and, after an additional 24 h, urine samples were collected to measure Na+ concentration and volume. A single oral dose of 50 mg/kg ANG-(3–4) (EZBiolab, Carmel, IN) was given by gavage 3 h later. After a further 24 h, urine samples were collected again to measure the effect of ANG-(3–4) on Na+ excretion. Urinary Na+ was measured by the uranyl magnesium acetate precipitation method with a commercial kit (sodium rapid, Human, Wiesbaden, Germany).

The arterial pressure of the animals was measured by the noninvasive tail-cuff method (Letica 5002 storage pressure meter, Letica Scientific Instruments, Barcelona, Spain). Three independent measurements were taken after >10 min at 30–32°C. The rats were checked to ensure that they stopped moving before arterial pressures were read. After a 3-h recovery period, a single oral dose of 50 mg/kg ANG-(3–4) was given by gavage. Arterial pressure was monitored as described above at 3, 6, and 24 h after ANG-(3–4) administration.

**Isolation of proximal tubule cell membranes.** Proximal tubule cell membranes were isolated as previously described (40) from slices of the outer part of the kidney cortex (cortex corticis), where >90% of the cell population correspond to proximal tubules (41). The final membrane fraction was suspended in 250 mM sucrose (pH 7.4), and aliquots were stored in liquid N2. Total protein concentration was determined by the Folin method (18).

**Measurement of Na+-ATPase and Na+-K+-ATPase activity.** The activity of the two Na+ pumps was measured by quantification of Pi released during ATP hydrolysis, using a colorimetric method (38). Ouabain-resistant Na+-ATPase activity was determined by the difference between ATP hydrolysis in the absence and presence of its inhibitor, furosemide (both measurements were made in the presence of 1 mM ouabain). For quantification of Na+-ATPase activity, the reaction was started by adding the membrane preparation (final concentration 0.1 mg/ml), previously incubated for 10 min at 37°C with 1 mM ouabain to guarantee Na+-K+-ATPase inhibition, to the reaction media containing 20 mM HEPES-Tris (pH 7.0), 10 mM MgCl2, 120 mM NaCl, 5 mM ATP, and the specific inhibitors or peptides tested in each experimental set.

Na+-K+-ATPase activity was determined by the difference between ATP hydrolysis in the absence and presence of its specific inhibitor, ouabain. For quantification of Na+-K+-ATPase activity, the membrane preparation (final concentration 0.05 mg/ml) was added to the reaction mixture containing 50 mM Bis-Tris-propane (pH 7.4), 0.2 mM EDTA, 5 mM MgCl2, and 120 mM NaCl. After incubation for 10 min at 37°C, the reaction was started by adding a mix of ATP (5 mM) and KCl (24 mM).

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**Fig. 1.** ANG-(3–4) inhibits the ouabain-insensitive Na+-ATPase in spontaneously hypertensive rats (SHR) but not in Wistar-Kyoto (WKY) rats. Values are means ± SE of 4–9 determinations in triplicate from different membrane preparations. A: concentration dependence of the inhibition by ANG-(3–4) on proximal tubule Na+-ATPase from SHR in the range shown on the abscissa. Different lower-case letters above the symbols indicate statistical differences among the corresponding mean values [“a” symbols correspond to P values ranging from 0.427 to 0.638 with respect to the assay in the absence of ANG-(3–4); “b” symbols correspond to P = 0.005 and P < 0.001 for the conditions 10^-8 and 10^-6 M ANG-(3–4) compared with the control given no peptide]. Differences were assessed by 1-way ANOVA followed by a Newman-Keuls posttest. B: Na+-ATPase from WKY rats is insensitive to ANG-(3–4). A horizontal line (82.1 ± 3.1 nmol P_i·mg^-1·min^-1; mean ± SE) is adjusted to the experimental points. No statistical differences were found among mean values (P = 0.240 for 1-way ANOVA; posttest was not carried out). C and D: proximal tubule Na+-K+-ATPase is insensitive to ANG-(3–4) in both SHR and WKY rats. Horizontal lines are adjusted to the experimental points (183.7 ± 6.1 nmol P_i·mg^-1·min^-1 for SHR; 193.3 ± 6.1 nmol P_i·mg^-1·min^-1 for WKY rats). No statistical differences were found between the mean values of activities at different ANG-(3–4) concentrations with each strain (P = 0.843 for SHR and P = 0.730 for WKY rats; posttest was not carried out).
Detection of AT1/AT2 receptor heterodimers (AT1R/AT2R) by immunoprecipitation followed by Western blot analysis. Membranes (0.5 mg/ml) were initially incubated in sucrose (pH 7.4) for 20 min at 37°C with $10^{-10} \text{ M ANG II (Sigma-Aldrich, St. Louis, MO)}$ or $10^{-8} \text{ M ANG-(3–4)}$ and solubilized for 30 min at room temperature with 0.01% CHAPS (wt/vol). This mixture was diluted in RIPA buffer [50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% NP-40 (vol/vol), 0.25% sodium deoxycholate, and 1 mM EDTA], in a final volume of 1 ml and incubated with the monoclonal anti-AT1R antibody (TONI-1, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4°C. Subsequently, protein A/G-agarose (1:50, Santa Cruz Biotechnology) was added and left overnight at 4°C under gentle agitation. The immunoprecipitates were separated from supernatants by centrifugation at 1,000 g for 5 min (4°C) and washed three times with Tris-buffered saline. The resulting pellets were mixed with Laemmli buffer, heated for 4 min at 100°C, and centrifuged to remove protein A/G-agarose. Aliquots of the receptor-containing supernatants were electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-AT2R (C-18, Santa Cruz Biotechnology) antibody (1:500) to detect receptor heterodimers. Membranes were stripped for 30 min at 55°C with 0.2 M glycine (pH 2.2) and reprobed with anti-AT1R antibody (N-10, 1:500, Santa Cruz Biotechnology). The intensity of the bands at 45 kDa was measured by image-analysis software (Scion Image 4.0.3.2), and the amount of AT1R/AT2R heterodimers was determined by the ratio of AT2R to AT1R band intensities.

Fig. 2. Oral administration of ANG-(3–4) (50 mg/kg body wt) decreases systolic arterial pressure and increases urinary Na+ excretion in SHR (left) but not in WKY rats (right). Different lower-case letters above the symbols indicate statistical differences among the corresponding mean values. Systolic arterial pressure was monitored 3 h before (Pre) and 3, 6, and 24 h after ANG-(3–4) treatment to SHR (A) and WKY rats (E; n = 10 in all times; each value corresponds to 3 successive measurements in a period of 10–15 min). In A, $P < 0.001$ (3 h vs. Pre), $P < 0.001$ (6 h vs. Pre), $P = 0.100$ (24 h vs. Pre). Differences were assessed by 1-way ANOVA followed by a Newman-Keuls posttest. In E, $P = 0.060$ (1-way ANOVA without posttest). For urinary Na+ analysis, 24 h-urine samples were collected before (Pre) and after (Post) a single oral dose of ANG-(3–4). B and F: urinary volume (UV) corrected by the body weight in SHR (n = 10, before and after; $P = 0.489$) and WKY rats (n = 10; $P = 0.562$). C and G: urinary Na+ concentration ([Na+]u) in SHR (n = 10; $P = 0.033$) and WKY rats (n = 10; $P = 0.972$). D and H: urinary Na+ excretion in 24 h (UNaV) in SHR (n = 10; $P < 0.001$) and WKY rats (n = 10; $P = 0.523$). Differences were assessed by unpaired t-test within each strain.
Statistical analysis. Data were plotted and analyzed using GraphPad Prism 5.01. The results are expressed as means ± SE. Student’s t-test was used for comparison between two means, whereas three or more means were compared by one-way ANOVA, followed by a Newman-Keuls multiple comparison test. Values were considered significantly different at \( P < 0.05 \). Sigma Plot 12.5 software was used to calculate exact \( P \) values in multiple comparisons.

RESULTS

Inhibition of renal ouabain-resistant \( \text{Na}^+ \)-ATPase by \( \text{ANG}-(3–4) \) in SHR. \( \text{ANG}-(3–4) \) selectively inhibits in a dose-dependent manner \( \text{Na}^+ \)-ATPase activity resident in the plasma membranes from kidney proximal tubules cells of SHR rats (Fig. 1A) without influencing the WKY rats (Fig. 1B), indicating that the dipeptide actions on \( \text{Na}^+ \) transport are conditioned by alterations that contribute to the onset of hypertension. Selectivity is also seen in the molecular machinery involved in proximal \( \text{Na}^+ \) reabsorption. The \( \text{Na}^+-\text{K}^+-\text{ATPase} \) does not seem to be a target for \( \text{ANG}-(3–4) \), at least in isolated membranes from either SHR (Fig. 1C) or WKY rats (Fig. 1D).

A single oral dose of \( \text{ANG}-(3–4) \) stimulates \( \text{Na}^+ \) excretion in SHR. To test whether \( \text{ANG}-(3–4) \) has an effect on \( \text{Na}^+ \)-ATPase associated with modifications in \( \text{Na}^+ \) excretion by SHR, despite the absence of influence on \( \text{Na}^+-\text{K}^+-\text{ATPase} \) activity, the dipeptide was administered in a single oral dose of 50 mg/kg body wt. Three hours after \( \text{ANG}-(3–4) \) administration, SHR exhibited lower systolic arterial pressure (Fig. 2A), an antihypertensive effect that still persisted 6 h after \( \text{ANG}-(3–4) \). The pressure levels returned to the initial elevated values 1 day after administration. The dipeptide also increased the 24-h \( \text{Na}^+ \) excretion in SHR (U_{NaV}) due to increased urinary \( \text{Na}^+ \) concentration ([Na\textsubscript{ur}]\text{NaV}) without significantly augmenting urinary flow (UV) (Fig. 2, B–D). As in the case of \( \text{Na}^+ \)-ATPase activity, none of these parameters were modified after \( \text{ANG}-(3–4) \) treatment in WKY rats (Fig. 2, E–H).

AT\(_2\)R and PKA are components of a signaling pathway involved in inhibition of \( \text{Na}^+ \)-ATPase by \( \text{ANG}-(3–4) \) in SHR. We previously showed that AT\(_2\)R is the first step in a signaling cascade that ultimately has the basolateral plasma membrane \( \text{Ca}^{2+} \)-ATPase as a target for fentomolar \( \text{ANG}-(3–4) \) concentrations (4, 6). To establish whether this class of receptors participates in the same pathway that culminates in the inhibition of \( \text{Na}^+ \)-ATPase in SHR, the influence of the AT\(_2\)R antagonist PD123319 was examined. PD123319 (10\textsuperscript{-7} M) completely suppressed the inhibitory effect of 10\textsuperscript{-8} M \( \text{ANG}-(3–4) \), which is indicative of this participation (Fig. 3A). Conversely, involvement of AT\(_1\)R is unlikely because 10\textsuperscript{-7} M losartan had no effect upon the inhibition caused by \( \text{ANG}-(3–4) \) (Fig. 3B). Looking downstream for a key effector of a signal generated at the AT\(_2\)R level, full reversal of the inhibition due to 10\textsuperscript{-7} M PKA\textsubscript{5–24} peptide, the specific inhibitor of PKA, points to its involvement in the inhibition of \( \text{Na}^+ \)-ATPase from SHR (Fig. 3C).

Stimulatory action of \( \text{ANG II} \) on \( \text{Na}^+ \)-ATPase in WKY, but not the inhibitory one in SHR, are modified by \( \text{ANG}-(3–4) \). \( \text{ANG II} \) is a physiological modulator of \( \text{Na}^+ \)-ATPase in WKY rats via AT\(_1\)R → PKC (30), \( \text{ANG II} \) depresses the activity of the pump in SHR rats via AT\(_2\)R (29), and \( \text{ANG}-(3–4) \) inhibits \( \text{Na}^+ \)-ATPase activity in SHR via AT\(_2\)R → PKA (Figs. 1A and 3, A, and C). Therefore, we assayed the two peptides in the combinations shown in Fig. 4 to investigate their possible

Fig. 3. AT\(_2\) receptor (AT\(_2\)R) and PKA participate in the signaling cascade that culminates in the inhibition of \( \text{Na}^+ \)-ATPase by \( \text{ANG}-(3–4) \). \( \text{Na}^+ \)-ATPase activity was assayed in the absence or presence of \( \text{ANG}-(3–4) \) in combination with PD123319 (A), PKA\textsubscript{5–24} peptide (B), or losartan (C), as shown on the respective abscissa. Values are means ± SE of 4–8 determinations in triplicate using different membrane preparations. Different lower-case letters above the bars indicate statistical difference among mean values. In A, \( P = 0.003 \) for \( \text{ANG}-(3–4) \) vs. control without additions, \( P = 0.767 \) for \( \text{ANG}-(3–4) \) plus PD123319 vs. control, and \( P = 0.785 \) for PD123319 alone vs. control. In B, \( P = 0.002 \) for \( \text{ANG}-(3–4) \) vs. control, \( P = 0.010 \) for \( \text{ANG}-(3–4) \) plus losartan vs. control, and \( P = 0.479 \) for losartan vs. control. In C, \( P = 0.010 \) for \( \text{ANG}-(3–4) \) vs. control without additions, \( P = 0.380 \) for \( \text{ANG}-(3–4) \) plus PKA\textsubscript{5–24} vs. control and \( P = 0.632 \) for PKA\textsubscript{5–24} vs. control.
interactions at the level of Na\textsuperscript{+}-ATPase from normotensive and hypertensive rats. The results show that only the stimulus of $10^{-10}$ M ANG II on Na\textsuperscript{+}-ATPase in WKY rats, possibly a prohypertensive action, is cancelled by $10^{-8}$ M ANG-(3–4) (Fig. 4A). Inhibition of the pump by $10^{-10}$ M ANG II in SHR was not modified by the dipeptide ($10^{-8}$ M), which gave similar inhibition alone (Fig. 4B).

AT\textsubscript{2}R and PKA participate in the counteracting action of ANG-(3–4) upon stimulation of Na\textsuperscript{+}-ATPase by ANG II. We investigated whether the same signaling route involved in the inhibition of Na\textsuperscript{+}-ATPase in SHR participates in the counteracting effect of ANG-(3–4) on the activation of Na\textsuperscript{+}-ATPase by ANG II in WKY rats. This hypothesis was confirmed, as shown by the results in Fig. 5. When $10^{-7}$ M PD123319 was added together with ANG-(3–4), the full stimulatory effect of ANG II is preserved (Fig. 5A), strong evidence of the participation of AT\textsubscript{2}R. When the inhibitor PKA\textsubscript{5–24} ($10^{-6}$ M) was present alone (Fig. 5B), Na\textsuperscript{+}-ATPase activity reached the levels found with ANG II, confirming that PKA activity plays a key downstream role in the mechanism involved in the regulation of the pump by ANG II/ANG-(3–4).

ANG-(3–4) does not induce dissociation of AT\textsubscript{1}R/AT\textsubscript{2}R dimers in proximal tubule membranes in SHR, a physiological outcome encountered in WKY rats. We had earlier postulated that dissociation of AT\textsubscript{1}R/AT\textsubscript{2}R dimers is an important feature in the signaling mechanism associated with the recovery by ANG-(3–4) in basolateral plasma membrane Ca\textsuperscript{2+}-ATPase activity that is inhibited by picomolar ANG II concentrations (4, 6). ANG-(3–4) at $10^{-8}$ M dissociated 50% of the AT\textsubscript{1}R/AT\textsubscript{2}R dimers detected in the proximal tubule membranes of WKY rats, whereas $10^{-10}$ M ANG II had no effect (Fig. 6). The SHR present an AT\textsubscript{1}R/AT\textsubscript{2}R dimer population that is $I$.

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### Fig. 4. ANG-(3–4) suppresses the stimulation by ANG II of Na\textsuperscript{+}-ATPase activity in WKY rats, whereas it does not modify the inhibition seen in SHR. ANG II and ANG-(3–4) were assayed in the combinations shown on the abscissa. Values are means ± SE of 4–5 determinations in triplicate using different membrane preparations. A: WKY rats; B: SHR. Different lower-case letters above the bars indicate statistical differences among mean values. In A, $P = 0.009$ for $10^{-10}$ M ANG II vs. control with no peptides, $P = 0.491$ for $10^{-10}$ M ANG II plus ANG-(3–4) vs. control, and $P = 0.301$ for $10^{-8}$ M ANG-(3–4) vs. control. In B, values with “b” correspond to $P = 0.008$ for $10^{-10}$ M ANG II vs. control with no peptides, $P < 0.001$ for $10^{-10}$ M ANG II plus $10^{-8}$ M ANG-(3–4) vs. control, and $P < 0.001$ for $10^{-8}$ M ANG-(3–4) vs. control.

### Fig. 5. AT\textsubscript{2}R and PKA participate in the counteracting effect of ANG-(3–4) upon the activation of Na\textsuperscript{+}-ATPase by ANG II in normotensive rats. Values are means ± SE of 4–5 determinations in triplicate using different membrane preparations. Assays were carried out in the presence of ANG II and ANG-(3–4) with PD123319 (A) or PKA\textsubscript{5–24}, peptide (B). Different lower-case letters above the bars indicate statistical differences among mean values. In A, $P = 0.047$ for $10^{-10}$ M ANG II vs. control without additions, $P = 0.032$ for $10^{-10}$ M ANG II together with $10^{-8}$ M ANG-(3–4) plus $10^{-7}$ M PD123319 vs. control, $P = 0.302$ for $10^{-10}$ M ANG II plus $10^{-8}$ M ANG-(3–4) vs. control, and $P = 0.506$ for PD123319 alone vs. control. In B, $P = 0.013$ for $10^{-10}$ M ANG II vs. control without additions, $P = 0.245$ for $10^{-10}$ M ANG II plus $10^{-8}$ M ANG-(3–4) vs. control, $P = 0.047$ for $10^{-10}$ M ANG II together with ANG-(3–4) plus $10^{-6}$ M PKA\textsubscript{5–24}, and $P = 0.032$ for PKA\textsubscript{5–24} alone vs. control.
Fig. 6. ANG-(3–4) induces dissociation of AT₁R/AT₂R dimers in WKY rats but not in SHR. Proximal tubule cell membranes from WKY rats and SHR were incubated with ANG II and ANG-(3–4), in the combinations shown on the abscissa, before receptor immunoprecipitation (lanes 1–3 were incubated with ANG II and ANG-(3–4), in the combinations shown on the abscissa). A: representative immunodetection of AT₁R using a goat polyclonal antibody after receptor immunoprecipitation using a mouse monoclonal antibody against AT₁R. B: representative immunodetection of AT₂R in the same nitrocellulose membrane using a rabbit polyclonal antibody. C: densitometric quantification of AT₁R and AT₂R immunosignals from 5 experiments using different membrane preparations. Bars represent AT₂R-to-AT₁R density ratios. Different lowercase letters above the bars indicate statistical differences between mean values. For WKY rats, \( P = 0.799 \) for 10\(^{-10}\) M ANG II vs. control with no peptides and \( P = 0.037 \) for 10\(^{-8}\) M ANG-(3–4) vs. control. For SHR, \( P = 0.969 \) for 10\(^{-10}\) M ANG II vs. control with no peptides and \( P = 0.950 \) for 10\(^{-8}\) M ANG-(3–4) vs. control. Comparison between WKY rats and SHR with no peptides (first columns in each panel): \( P = 0.031 \). The negative control is shown in lane 4 (A and B). Immunoprecipitation was carried out in WKY membranes in the absence of the AT₂R monoclonal antibody.

smaller than in WKY, 2) similar to that in WKY rats after the ANG-(3–4)-induced dissociation, and 3) completely insensitive to ANG-(3–4).

**DISCUSSION**

Besides the classic view of the RAS being restricted to ANG I and ANG II, experimental evidence over the last three decades indicates the existence of a great constellation of small ANG II-derived peptides in the kidney (35) and in other organs (13), but their physiological role remains relatively obscure. Among these peptides, the smaller is ANG-(3–4), whose antihypertensive properties were first described by Saito et al. (34), has received little attention despite its well-documented action on the cardiovascular system as a whole (19, 15, 24, 37). The circulating levels of this dipptide are up to three times lower in hypertensive human subjects compared with normotensive controls, and their plasma levels correlate inversely with systolic arterial pressure (20), indicating that endogenously formed ANG-(3–4) is important in regulating arterial blood pressure. This dipptide also has vasodilating (37, 39), anti-proliferative (24), and antioxidant (39) effects.

Interactions between ANG-(3–4) and RAS seem to occur beyond those at the ANG II receptor level. Matsui et al. (25) demonstrated that ANG-(3–4) and captopril compete for the same absorption site in the jejunal membranes, with a subsequent decrease in the plasma levels of the antihypertensive drug and that combined oral administration of both compounds to SHR attenuates their respective blood pressure-lowering effect. These observations could mean that ANG-(3–4) interacts with ANG II, forming enzymatic pathways by binding at the catalytic site of the ACE (15, 19).

We have now demonstrated a specific dose-dependent inhibitory influence of ANG-(3–4) on Na\(^+\)-ATPase activity in the proximal tubule cells of SHR, but not in WKY rats. Inhibition occurs only in hypertensive rats that have hyperactive AT₁R-linked signaling pathways in their proximal tubules and a constitutively high Na\(^+\)-ATPase activity (29), which raised the hypothesis that ANG-(3–4) acts through the AT₂R receptors. The signaling pathways associated with this class of receptors can elicit counteracting responses to those originating in AT₁R (26), although they can act through specific mechanisms and pathways unrelated to those of AT₁R (33). Since the AT₂R antagonist PD123319 or the PKA inhibitor PKA (5–24) completely cancelled the effect of ANG-(3–4) constitutes strong evidence for a signaling network that, starting with AT₂R being bound by the peptide, activates a cAMP-mediated downstream pathway that culminates in the downregulation of Na\(^+\)-ATPase in the basolateral membranes of SHR. Thus the Na\(^+\) flux mediated by this pump is depressed and can be seen as an antihypertensive action at renal level.

This view is supported by fact that the in vitro inhibitory effect of ANG-(3–4) on Na\(^+\)-ATPase matches those obtained in vivo regarding the influence of the peptide increasing the total Na\(^+\) excretion in 24 h and transiently lowering systolic blood pressure. Taking these data as a whole demonstrates that changes in Na\(^+\)-ATPase in hypertensive rats, without changes in Na\(^+\)-K\(^+\)-ATPase, result in parallel changes in overall renal Na\(^+\) transport and, consequently, in a global antihypertensive action. The selective influence on Na\(^+\)-ATPase can explain the lack of effect on urinary flux with a simultaneous rise in urinary Na\(^+\) concentration and total Na\(^+\) excretion over 24 h after a single dose of ANG-(3–4). Since this pump is considered the fine tuner of proximal Na\(^+\) reabsorption, whereas the Na\(^+\)-K\(^+\)-ATPase is responsible for the bulk reabsorption (7), the natriuretic effect of ANG-(3–4) seems to arise from a delicate and specific impact of great physiological significance upon only one Na\(^+\) pump, without affecting water balance.

The selective influence on renal Na\(^+\)-ATPase of SHR, as well as the earlier observations that ANG-(3–4) decreases
arterial blood pressure in hypertensive (15), but not in normotensive humans (21), gives experimental support to the idea presented above that ANG-(3–4) acts on proximal Na⁺ transport under conditions that culminate in well-established hypertension. However, the physiological role of ANG-(3–4) is clearly not restricted to hypertensive animals. ANG II is a key physiological activator of renal Na⁺-ATPase (7, 30). Since 10⁻⁸ M ANG-(3–4) blocks stimulation of Na⁺-ATPase in WKY rats due to 10⁻¹⁰ M ANG II, it is now clear that interacting regulatory actions between the two peptides seems to have important physiological significance. This adds support to the view that the effect of ANG-(3–4) is only significant when Na⁺-ATPase is activated, in either physiological or pathological conditions. This rationale has additional support; ANG-(3–4) is ineffective in modulating the inhibitory effect of 10⁻¹⁰ M ANG II on the constitutively hyperactive Na⁺-ATPase of SHR. AT₂R and the regulatory phosphorylation mediated by PKA also seems to play a role in the counteracting effect of ANG-(3–4) in normotensive rats. In the presence of ANG-(3–4), PD123319 (10⁻⁷ M), or PKA₁(5–24) (10⁻⁶ M) returned Na⁺-ATPase activity to the stimulated values obtained with ANG II alone.

The activity of Ca²⁺ ions plays a key role in the regulation of transport of fluid across the proximal tubular epithelium in a pathway involving PKC (9, 10). The ANG-(3–4) → AT₂R → PKA cascade is an extremely potent reactivator in the femtomolar range (6) of basolateral plasma membrane Ca²⁺-ATPase inhibited by 10⁻¹⁰ M ANG II (4), which occurs via a PKC-mediated pathway (3). Previous observations seen in the context of those described herein lead us to postulate a regulatory network in proximal tubule cells that involves antagonistic and interacting effects of ANG II/ANG-(3–4), and AT₁R/AT₂R and PKC/PKA as being involved in the regulation of Na⁺ reabsorption and, ultimately, in the physiological regulation of blood pressure. However, these signaling molecules and effectors certainly are involved with other player mechanisms not covered in this report. In phosphoproteomic analysis of ANG II-mediated responses in proximal tubule cells, several PKC

![Fig. 7. Proposed model for the Na⁺-ATPase modulation by ANG II and ANG-(3–4) in WKY rats (A) and SHR (B). In normotensive WKY animals, the basal activity of Na⁺-ATPase (central transmembrane cylinder) is determined by the balance between activating phosphorylations mediated by PKC (30) and inhibitory phosphorylation mediated by PKA (Fig. 5B). ANG II activates PKC after interaction with AT₁R/AT₂R heterodimers, as previously demonstrated for renal Ca²⁺-ATPase (3, 4). ANG-(3–4) promotes dissociation of the heterodimers (Fig. 6), binds to AT₂R (Fig. 5A), and favors PKA activation (Fig. 5B), thus blocking the activation of the pump by ANG II (Fig. 4A). In SHR, a higher basal PKC activity (16) leads to an imbalance between regulatory phosphorylations, and consequently to hyperactivation of the Na⁺ pump. The hypertensive animals have a smaller number of heterodimers (Fig. 6), thus favoring the interactions of ANG II with monomeric AT₁R, leading to activation of PKA and inhibition of the Na⁺ pump, the same and nonadditive effect observed with ANG-(3–4) (Fig. 4B). Small circles, amino acids in ANG II and ANG-(3–4) sequences; large circles in the intracellular aspect of the Na⁺-ATPase molecule, stimulatory phosphorylations of Na⁺-ATPase mediated by PKC; squares, inhibitory phosphorylations mediated by PKA. Large vertical arrows indicate conversion of ANG II to ANG-(3–4) in basolateral membranes of proximal tubule cells (4); thin black arrows from the kinases indicate stimulus of the corresponding activating (PKC) or inhibitory (PKA) phosphorylations of Na⁺-ATPase; truncated arrows indicate blockade of PKC-mediated activation of the pump upon activation of PKA. Horizontal double-headed black arrow indicates dissociation induced by ANG-(3–4).]
isoforms, cAMP-responsive proteins, and MAPK and ERK1/2 become phosphorylated, depending on whether pressor or nonpressor doses of ANG II are used (17). The constitutive ratio of PKC/PKA activity in proximal tubules could also play an important role in the physiological action of ANG-(3–4) in modulating Na⁺-ATPase in normotensive and hypertensive rats. This ratio is higher in SHR than in WKY rats (16); for this reason, only the latter animals respond to PKA/AT1R heterodimers (4) alone by augmentation of Na⁺-ATPase levels to those found with ANG II alone. When the AT₁R → PKC pathway is overactive, as in SHR, the effect of PKA/AT1R heterodimers is no longer apparent.

The formation of AT₂R/AT₁R heterodimers and its potential physiological relevance were reported over a decade ago (1), the association being considered proof of the antagonistic effects of AT₁R on the AT₂R functions. However, mutual signaling between the two classes of receptors seems to be more complex in the membranes of proximal tubule cells. The supramolecular organization of AT₁R and AT₂R in proximal tubule cell membranes as well as the relative amounts of monomers are important features in both the constitutive status of the tensional levels in SHR and WKY rats and in the responses to ANG-(3–4) and ANG II. SHR membranes have fewer AT₁R/AT₂R heterodimers compared with normotensive WKY rats, which might be related to the absolute expression of AT₁R and AT₂R. In a similar cell membrane fraction of the proximal tubules, this AT₁R expression is significantly lower in SHR compared with WKY (16). This could explain the higher proportional amount of heterodimers in WKY; since these animals have more AT₂R, a higher proportion of total AT₁R should be found in the dimeric state. In SHR, the lower AT₂R content leaves a greater amount of total AT₁R immunoprecipitated in the monomeric state.

We previously demonstrated that micromolar ANG II concentrations, a condition in which substantial amounts of ANG-(3–4) are formed, promote dissociation of AT₁R/AT₂R heterodimers (4). These heterodimers are required for the inhibition of the plasma membrane Ca²⁺-ATPase by nanomolar ANG II (3). We have now directly shown that 10⁻⁸ M ANG-(3–4) reduces heterodimerization in tubule membranes of WKY rats to the levels constitutively found in SHR. This allows us to postulate that 1) higher levels of AT₂R/AT₁R are required for the physiological stimulation of Na⁺-ATPase in normotensive rats, and 2) lower levels of AT₂R/AT₁R heterodimers are a prerequisite for the action of ANG-(3–4) on Na⁺-ATPase. The peptide could also, and at the same time, control the equilibrium between monomeric and dimeric states of both classes of ANG II receptors.

Finally, the possible physiological relevance of ANG-(3–4) in the regulation of renal functions, especially of those related to Na⁺ transport, has been highlighted by its peculiar metabolism and turnover. ANG-(3–4) is formed in the basolateral membranes from ANG II, ANG III, and ANG-(1–7) from limited proteolysis mediated by a wide ensemble of peptidases (5). Moreover, the basal concentrations of the dipeptide in renal tissue (~7 pmol/g, i.e., ~10⁻⁸ M) (24) are those that were effective in this study, with the kidney having the ability to concentrate ANG-(3–4) (24). Since the systemic antihypertensive actions of exogenous ANG-(3–4) remain after the rapid return of its plasma levels to normal values (23), it is likely that depressor mechanisms take place at a tissue level. For its role in blood pressure regulation, the kidney is a privileged candidate. Interestingly, physiological ANG II (detected by mass spectrometry) is required in vitro to avoid complete degradation of ANG-(3–4) to Val and Tyr (4), further evidence of an interplay between the pathways of ANG II and ANG-(3–4) metabolism in the kidney cortex (5). The proposed interactions between ANG II and ANG-(3–4) in the basolateral membranes at the level of AT₁R/AT₂R and PKC/PKA-mediated signaling cascades in WKY rats and SHR that emerge from our data are given in Fig. 7 (for a detailed description, see the corresponding legend).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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