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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[1]*Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil;* [2]*National Institute of Science and Technology for Structural Biology and Bioimaging, Rio de Janeiro, Brazil;* [3]*Department of Biophysics, Federal University of São Paulo, São Paulo, Brazil; and* [4]*Institute of Biomedical Sciences, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil*

Dias J, Ferrão FM, Axelband F, Carmona AK, Lara LS, Vieyra A. ANG-(3–4) inhibits renal Na^+-ATPase in hypertensive rats through a mechanism that involves dissociation of ANG II receptors, heterodimers, and PKA. *Am J Physiol Renal Physiol* 306: F855–F863, 2014. First published February 12, 2014; doi:10.1152/ajprenal.00488.2013.—The physiological roles of ANG-(3–4) (Val-Tyr), a potent ANG II-derived peptide, remain largely unknown. The present study *1)* investigates whether ANG-(3–4) modulates ouabain-resistant Na^+-ATPase resident in proximal tubule cells and *2)* verifies whether its possible action on pumping activity, considered the fine tuner of Na^+ reabsorption in this nephron segment, depends on blood pressure. ANG-(3–4) inhibited Na^+-ATPase activity in membranes of spontaneously hypertensive rats (SHR) at nanomolar concentrations, with no effect in Wistar-Kyoto (WKY) rats or on Na^+-K^+-ATPase. PD123319 (10^{-7} M) and $PKA_{(5–24)}$ (10^{-6} M), an AT_2 receptor (AT_2R) antagonist and a specific PKA inhibitor, respectively, abrogated this inhibition, indicating that AT_2R and PKA are central in this pathway. Despite the lack of effect of ANG-(3–4) when assayed alone in WKY rats, the peptide (10^{-8} M) completely blocked stimulation of Na^+-ATPase induced by 10^{-10} M ANG II in normotensive rats through a mechanism that also involves AT_2R and PKA. Tubular membranes from WKY rats had higher levels of AT_2R/AT_1R heterodimers, which remain associated in 10^{-10} M ANG II and dissociate to a very low dimerization state upon addition of 10^{-8} M ANG-(3–4). This lower level of heterodimers was that found in SHR, and heterodimers did not dissociate when the same concentration of ANG-(3–4) was present. Oral administration of ANG-(3–4) (50 mg/kg body mass) increased urinary Na^+ concentration and urinary Na^+ excretion with a simultaneous decrease in systolic arterial pressure in SHR, but not in WKY rats. Thus the influence of ANG-(3–4) on Na^+ transport and its hypotensive action depend on receptor association and on blood pressure.

ANG-(3–4); kidney proximal tubules; ouabain-resistant Na^+-ATPase; spontaneously hypertensive rats; ANG II receptors; heterodimerization

SPONTANEOUSLY HYPERTENSIVE rats (SHR) are a suitable model for the study of mechanisms underlying the genesis of essential hypertension. Despite the vast number of studies since the development of this animal line (27), several important issues remain poorly understood, including those linked to the role of the renin-angiotensin-system (RAS) and small ANG II-derived

peptides, such as ANG-(3–4), which are easily formed with the aid of a broad ensemble of peptidases (13) in body fluids (2) and kidneys (35). The kidneys, which are key organs for the onset and establishment of hypertension in SHR (11), have the ability to concentrate ANG-(3–4) (23), thereby making them a potential target for antihypertensive effects of the dipeptide, a general feature of all ANG II-derived small peptides.

The antihypertensive actions of ANG-(3–4), described over a decade ago (34), were primarily attributed to inhibition of the ANG I-converting enzyme (ACE) (15, 19). We have since demonstrated that ANG-(3–4) powerfully antagonizes the effects of ANG II upon the Ca^{2+}-ATPase resident in basolateral membranes of proximal tubule cells (4, 6). This pump is probably responsible for the fine control of cytosolic Ca^{2+} in proximal tubule cells (8) and consequently of fluid reabsorption across the proximal tubular epithelium, which is modulated by cytosolic Ca^{2+} fluctuations (9, 12). Thus we hypothesized that ANG-(3–4) could also interact with Na^+-transporting mechanisms, especially those mediated by Na^+-transporting ATPases.

Two ATPases are involved in the active reabsorption of Na^+ in proximal tubule cells: the classic Na^+-K^+-ATPase (14) and the ouabain-resistant Na^+-ATPase (28). The latter, recently cloned and purified from epithelial cells (31, 32), is an important target for a possible modulatory effect of ANG-(3–4) because *1)* it is considered to be the key pump for fine-tuning of Na^+ reabsorption in the renal proximal tubule (7); and *2)* it is upregulated in SHR (29), where blood pressure values are sensitive to ANG-(3–4) (34).

Our main hypothesis is that the Na^+-ATPase resident in proximal tubule cells of SHR could be responsive to ANG-(3–4) concentrations in the range reported in renal tissue (24). We also postulate that type 2 ANG II receptors (AT_2R) associated with the antihypertensive actions of ANG II (29, 36) could be central to the mechanism of the responses of Na^+-ATPase to ANG-(3–4).

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 N$_2$. 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 P$_i$ 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 MgCl$_2$, 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 MgCl$_2$, 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).
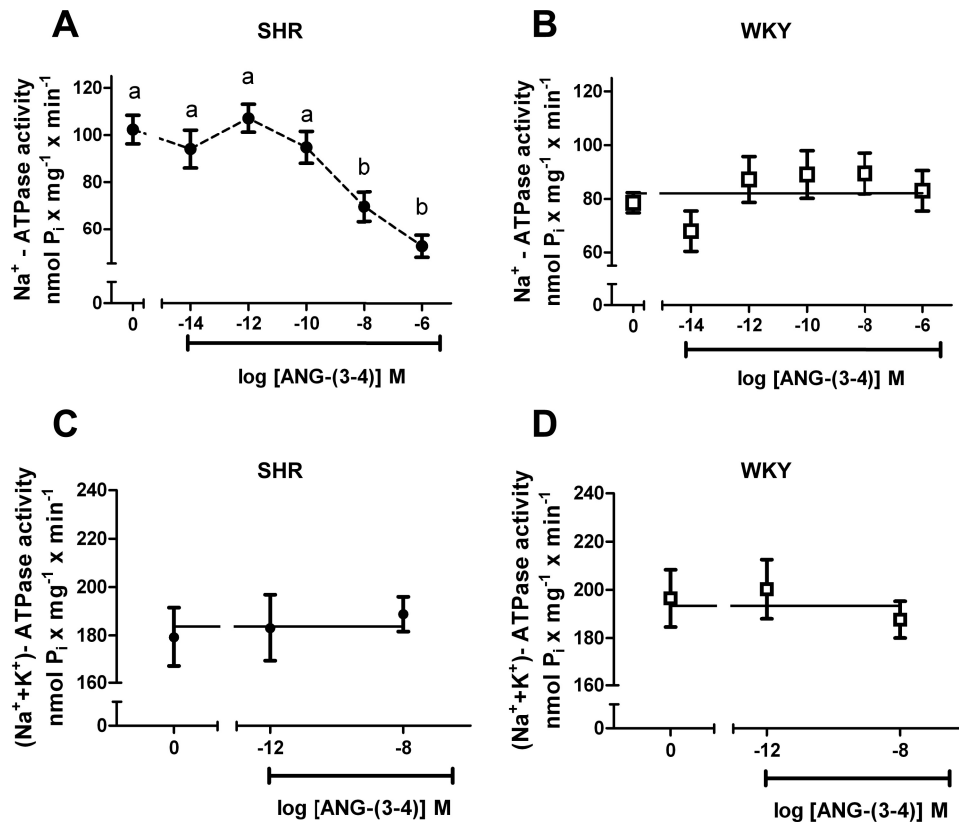

Fig. 1. ANG-(3–4) inhibits the ouabain-insensitive Na$^+$-ATPase in spontaneously hypertensive rats (SHR) but not in Wistar-Kyoto (WKY) rats. Values are means ± SE of 4–9 determinations in triplicate from different membrane preparations. *A*: concentration dependence of the inhibition by ANG-(3–4) on 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 AT$_1$/AT$_2$ receptor heterodimers (AT$_1$R/AT$_2$R) 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} M ANG II (Sigma-Aldrich, St. Louis, MO) or 10^{-8} 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-AT$_1$R 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 centrifuga-

tion 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-AT$_2$R (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-AT$_1$R 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 AT$_1$R/AT$_2$R heterodimers was determined by the ratio of AT$_2$R to AT$_1$R band intensities.
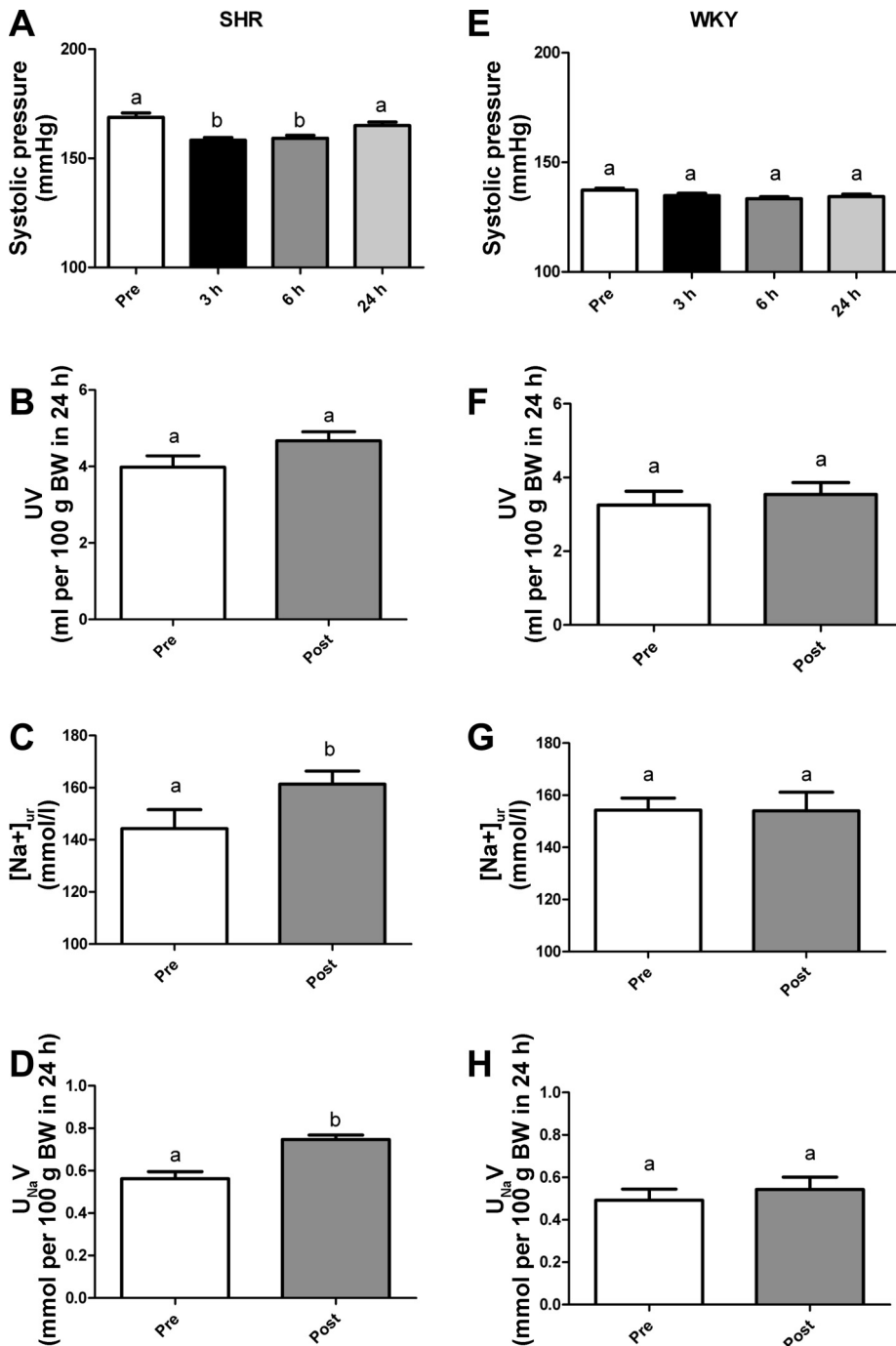

Fig. 2. Oral administration of ANG-(3–4) (50 mg/kg body wt) decreases systolic arterial pressure and increases urinary Na$^+$ excretion in SHR (*left*) but not in WKY rats (*right*). Different lower-case letters above the symbols indicate statistical differences among the corresponding mean values. Systolic 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$^+$]$_{ur}$) in SHR ($n = 10$; $P = 0.033$) and WKY rats ($n = 10$; $P = 0.972$). *D* and *H*: urinary Na$^+$ excretion in 24 h (U$_{Na}$V) 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 Na$^+$-ATPase by ANG-(3–4) in SHR. ANG-(3–4) selectively inhibits in a dose-dependent manner Na$^+$-ATPase activity resident in the plasma membranes from kidney proximal tubules cells of SHR rats (Fig. 1*A*) without influencing the WKY rats (Fig. 1*B*), indicating that the dipeptide actions on Na$^+$ transport are conditioned by alterations that contribute to the onset of hypertension. Selectivity is also seen in the molecular machinery involved in proximal Na$^+$ reabsorption. The Na$^+$-K$^+$-ATPase does not seem to be a target for ANG-(3–4), at least in isolated membranes from either SHR (Fig. 1*C*) or WKY rats (Fig. 1*D*).

A single oral dose of ANG-(3–4) stimulates Na$^+$ excretion in SHR. To test whether ANG-(3–4) has an effect on Na$^+$-ATPase associated with modifications in Na$^+$ excretion by SHR, despite the absence of influence on Na$^+$-K$^+$-ATPase activity, the dipeptide was administered in a single oral dose of 50 mg/kg body wt. Three hours after ANG-(3–4) administration, SHR exhibited lower systolic arterial pressure (Fig. 2*A*), an antihypertensive effect that still persisted 6 h after ANG-(3–4). The pressure levels returned to the initial elevated values 1 day after administration. The dipeptide also increased the 24-h Na$^+$ excretion in SHR (U$_{Na}$V) due to increased urinary Na$^+$ concentration ([Na$^+$]$_{ur}$) without significantly augmenting urinary flow (UV) (Fig. 2, *B–D*). As in the case of Na$^+$-ATPase activity, none of these parameters were modified after 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 Na$^+$-ATPase by 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 Ca^{2+}-ATPase from kidney proximal cells as a target for femtomolar ANG-(3–4) concentrations (4, 6). To establish whether this class of receptors participates in the same pathway that culminates in the inhibition of Na$^+$-ATPase in SHR, the influence of the AT$_2$R antagonist PD123319 was examined. PD123319 (10^{-7} M) completely suppressed the inhibitory effect of 10^{-8} M ANG-(3–4), which is indicative of this participation (Fig. 3*A*). Conversely, involvement of AT$_1$R is unlikely because 10^{-7} M losartan had no effect upon the inhibition caused by ANG-(3–4) (Fig. 3*B*). Looking downstream for a key effector of a signal generated at the AT$_2$R level, full reversal of the inhibition due to 10^{-6} M PKA$_{(5–24)}$ peptide, the specific inhibitor of PKA, points to its involvement in the inhibition of Na$^+$-ATPase from SHR (Fig. 3*C*).

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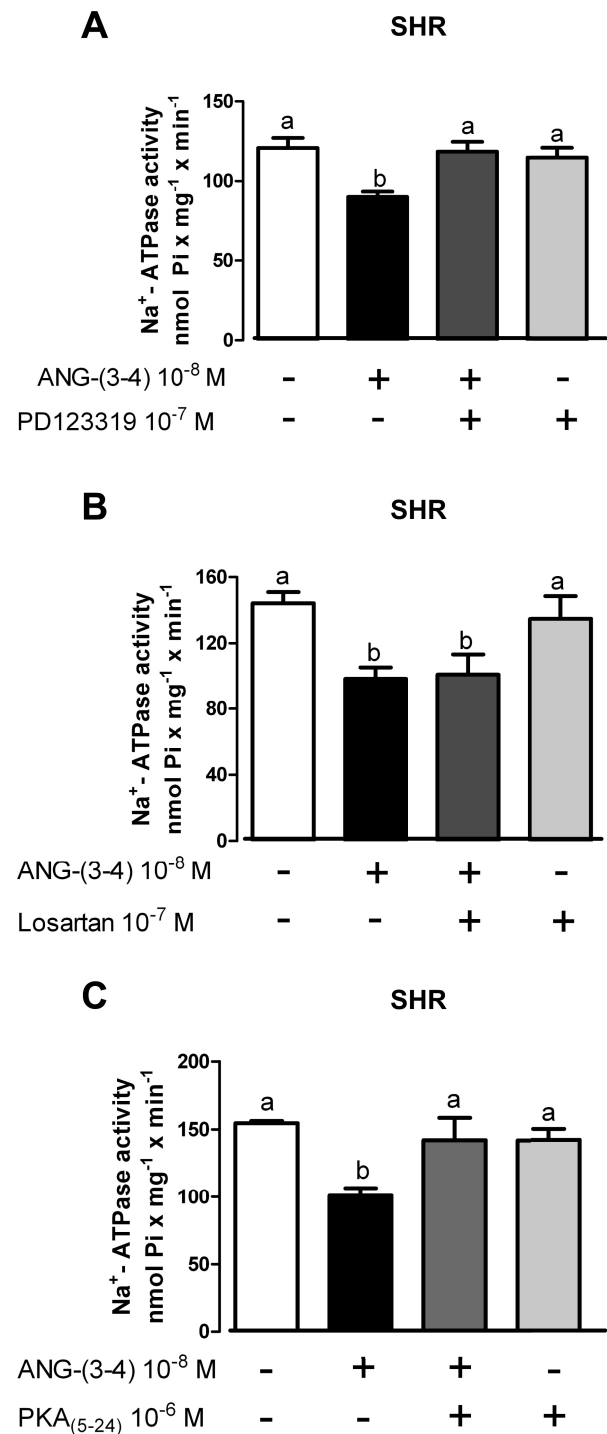
A   **SHR**

ANG-(3-4) 10^{-8} M   −   +   +   −
PD123319 10^{-7} M   −   −   +   +

B   **SHR**

ANG-(3-4) 10^{-8} M   −   +   +   −
Losartan 10^{-7} M   −   −   +   +

C   **SHR**

ANG-(3-4) 10^{-8} M   −   +   +   −
PKA$_{(5-24)}$ 10^{-6} M   −   −   +   +

Fig. 3. AT$_2$ receptor (AT$_2$R) and PKA participate in the signaling cascade that culminates in the inhibition of Na$^+$-ATPase by ANG-(3–4). Na$^+$-ATPase activity was assayed in the absence or presence of ANG-(3–4) in combination with PD123319 (*A*), PKA$_{(5–24)}$ 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 ANG-(3–4) vs. control without additions, $P = 0.767$ for ANG-(3–4) plus PD123319 vs. control, and $P = 0.785$ for PD123319 alone vs. control. In *B*, $P = 0.002$ for ANG-(3–4) vs. control, $P = 0.010$ for ANG-(3–4) plus losartan vs. control, and $P = 0.479$ for losartan vs. control. In *C*, $P = 0.010$ for ANG-(3–4) vs. control without additions, $P = 0.380$ for ANG-(3–4) plus PKA$_{(5–24)}$ vs. control and $P = 0.632$ for PKA$_{(5–24)}$ vs. control.
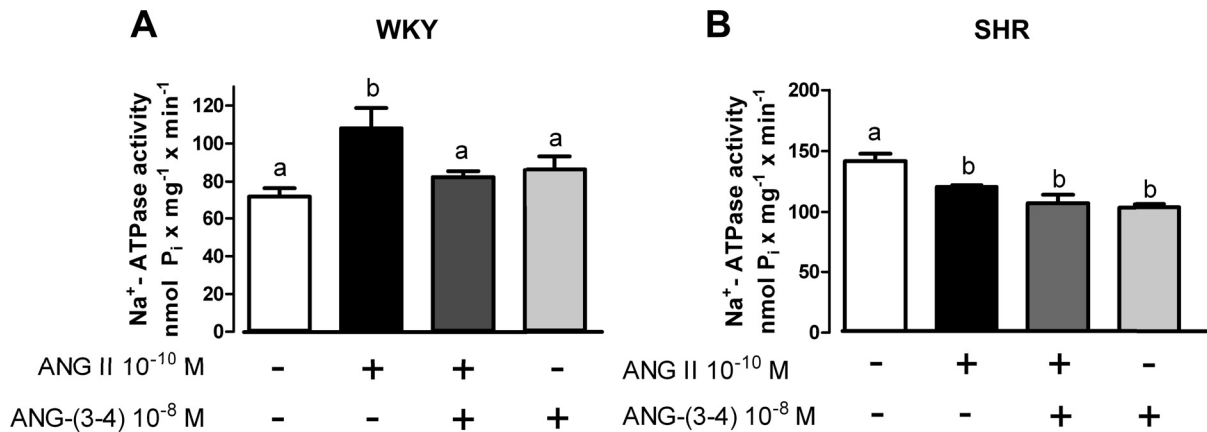
Fig. 4. ANG-(3–4) suppresses the stimulation by ANG II of Na$^+$-ATPase activity in WKY rats, whereas it does not modify the inhibition seen in SHR. ANG II and ANG-(3–4) were assayed in the combinations shown on the abscissa. Values are means ± SE of 4–5 determinations in triplicate using different membrane 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} 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.

interactions at the level of Na$^+$-ATPase from normotensive and hypertensive rats. The results show that only the stimulus of 10^{-10} M ANG II on Na$^+$-ATPase in WKY rats, possibly a prohypertensive action, is cancelled by 10^{-8} M ANG-(3–4) (Fig. 4*A*). 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. 4*B*).

AT$_2$R and PKA participate in the counteracting action of ANG-(3–4) upon stimulation of Na$^+$-ATPase by ANG II. We investigated whether the same signaling route involved in the inhibition of Na$^+$-ATPase in SHR participates in the counteracting effect of ANG-(3–4) on the activation of Na$^+$-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. 5*A*), strong evidence of the partici-

pation of AT$_2$R. When the inhibitor PKA$_{(5–24)}$ (10^{-6} M) was present alone (Fig. 5*B*), Na$^+$-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$_1$R/AT$_2$R dimers in proximal tubule membranes in SHR, a physiological outcome encountered in WKY rats. We had earlier postulated that dissociation of AT$_1$R/AT$_2$R dimers is an important feature in the signaling mechanism associated with the recovery by ANG-(3–4) in basolateral plasma membrane Ca^{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$_1$R/ AT$_2$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$_1$R/AT$_2$R dimer population that is *1*)
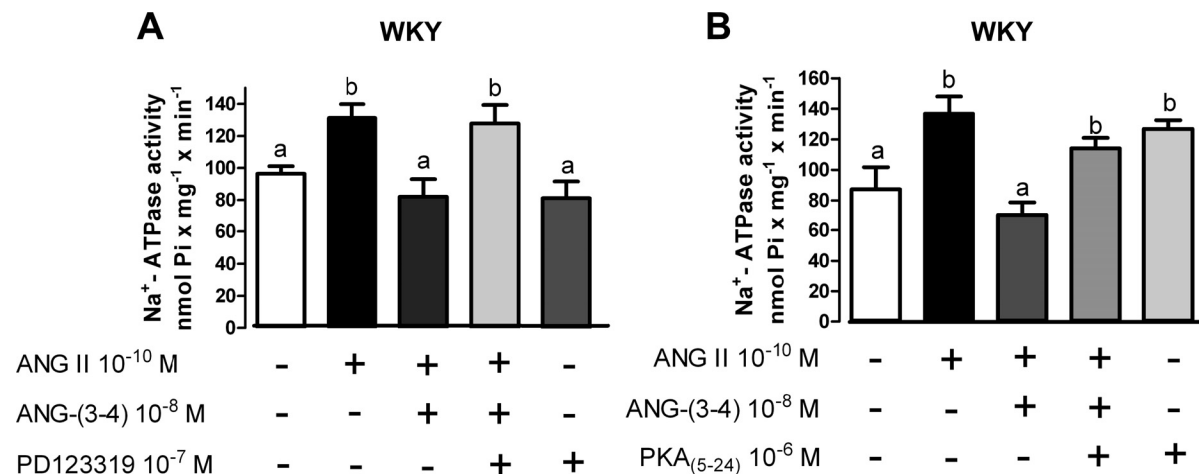

Fig. 5. AT$_2$R and PKA participate in the signaling cascade associated with the counteracting effect of ANG-(3–4) upon the stimulus of Na$^+$-ATPase by ANG II in normotensive rats. Values are means ± SE of 4–5 determinations in triplicate using different membrane preparations. Assays were carried out in the presence of ANG II and ANG-(3–4) with PD123319 (*A*) or PKA$_{(5–24)}$ 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$_{(5–24)}$, and $P = 0.032$ for PKA$_{(5–24)}$ alone vs. control.
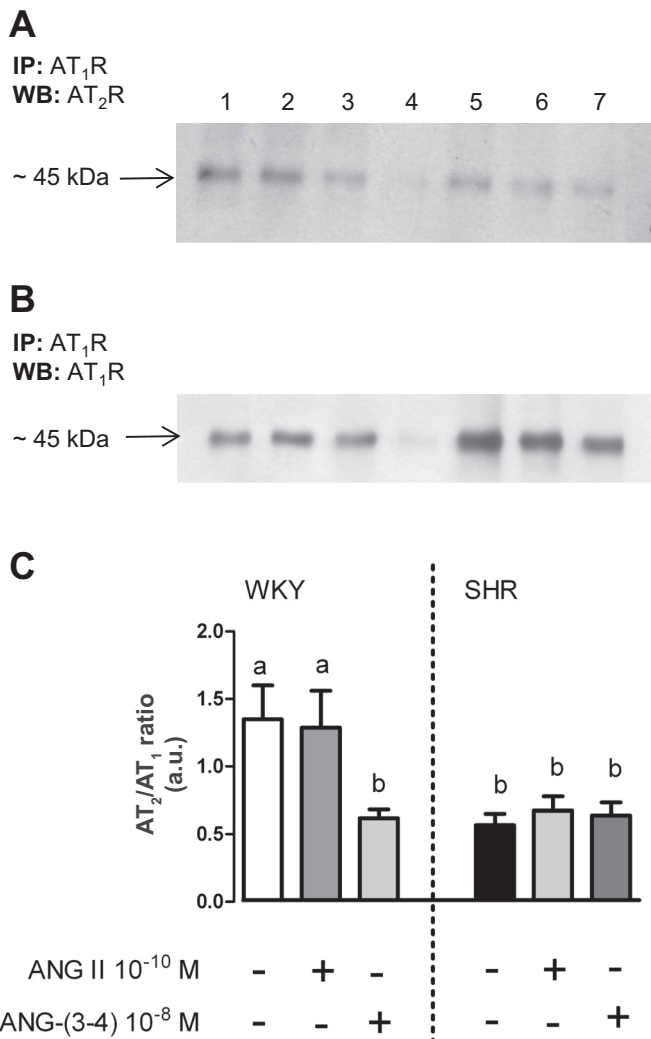
Fig. 6. ANG-(3–4) induces dissociation of AT$_1$R/AT$_2$R dimers in WKY rats but not in SHR. Proximal tubule cell membranes from WKY rats and SHR were incubated with ANG II and ANG-(3–4), in the combinations shown on the abscissa, before receptor immunoprecipitation (*lanes 1–3, 5–7*). *A*: representative immunodetection of AT$_2$R using a goat polyclonal antibody after immunoprecipitation using a mouse monoclonal antibody against AT$_1$R. *B*: representative immunodetection of AT$_1$R in the same nitrocellulose membrane using a rabbit polyclonal antibody. *C*: densitometric quantification of AT$_2$R and AT$_1$R immunosignals from 5 experiments using different membrane preparations. Bars represent AT$_2$R-to-AT$_1$R 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 AT$_1$R 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 dipeptide 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 dipeptide also has vasodilating (37, 39), antiproliferative (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 AT$_1$R-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 AT$_2$R receptors. The signaling pathways associated with this class of receptors can elicit counteracting responses to those originating in AT$_1$R (26), although they can act through specific mechanisms and pathways unrelated to those of AT$_1$R (33). Since the AT$_2$R 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 AT$_2$R 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. AT$_2$R 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 PKA$_{(5-24)}$ (10^{-6} M) returned Na$^+$-ATPase activity to the stimulated values obtained with ANG II alone.

The activity of Ca^{2+} 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) → AT$_2$R → PKA cascade is an extremely potent reactivator in the femtomolar range (6) of basolateral plasma membrane Ca^{2+}-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 AT$_1$R/AT$_2$R 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
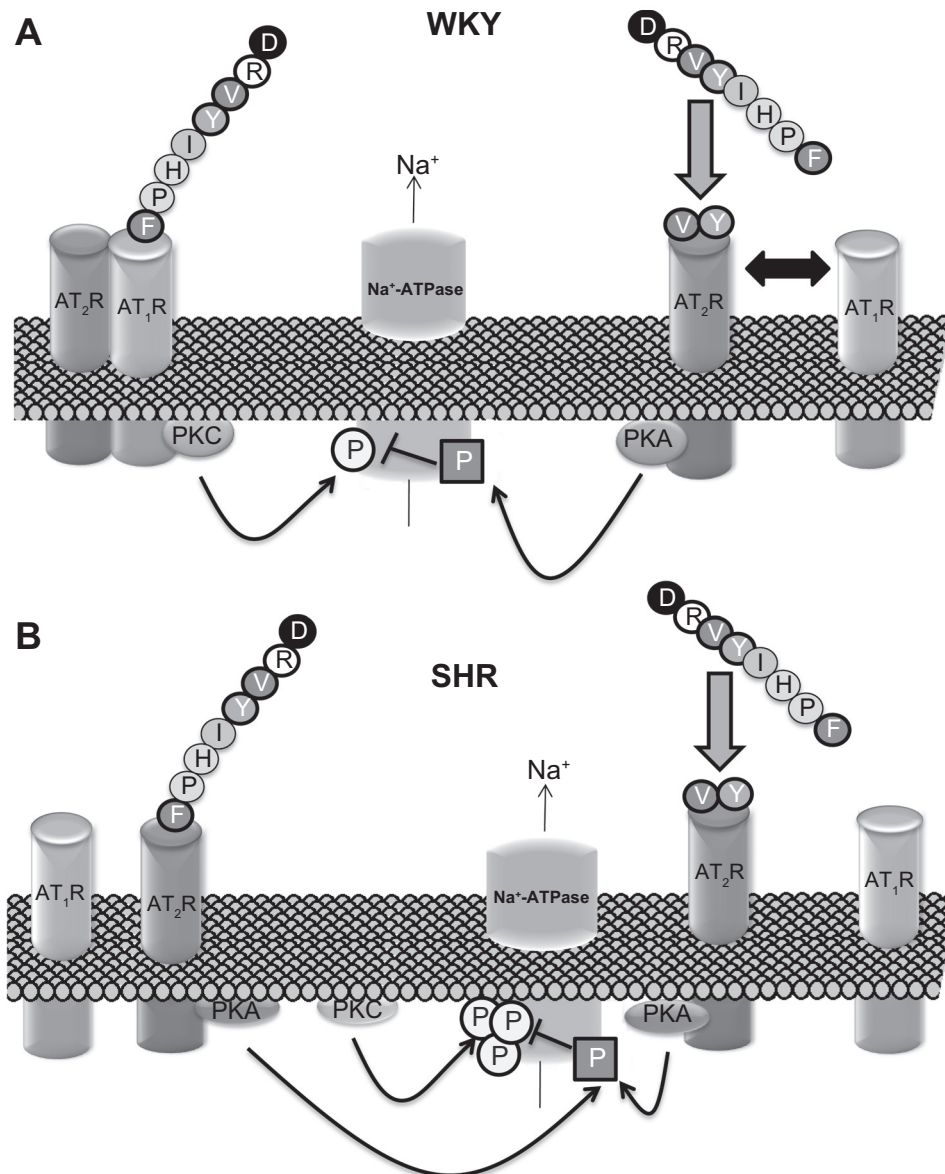

Fig. 7. Proposed model for the Na$^+$-ATPase modulation by ANG II and ANG-(3–4) in WKY rats (*A*) and SHR (*B*). In normotensive WKY animals, the basal activity of Na$^+$-ATPase (central transmembrane cylinder) is determined by the balance between activating phosphorylation mediated by PKC (30) and inhibitory phosphorylation mediated by PKA (Fig. 5*B*). ANG II activates PKC after interaction with AT$_1$R/AT$_2$R heterodimers, as previously demonstrated for renal Ca^{2+}-ATPase (3, 4). ANG-(3–4) promotes dissociation of the heterodimers (Fig. 6), binds to AT$_2$R (Fig. 5*A*), and favors PKA activation (Fig. 5*B*), thus blocking the activation of the pump by ANG II (Fig. 4*A*). 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 AT$_2$R, leading to activation of PKA and inhibition of the Na$^+$ pump, the same and nonadditive effect observed with ANG-(3–4) (Fig. 4*B*). 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 in WKY represents AT$_1$R/AT$_2$R 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$_{(5–24)}$ alone by augmentation of Na$^+$-ATPase levels to those found with ANG II alone. When the AT$_1$R → PKC pathway is overactive, as in SHR, the effect of PKA$_{(5–24)}$ alone is no longer apparent.

The formation of AT$_2$R/AT$_1$R heterodimers and its potential physiological relevance were reported over a decade ago (1), the association being considered proof of the antagonistic effects of AT$_2$R on the AT$_1$R 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 AT$_1$R and AT$_2$R 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 AT$_1$R/AT$_2$R heterodimers compared with normotensive WKY rats, which might be related to the absolute expression of AT$_1$R and AT$_2$R. In a similar cell membrane fraction of the proximal tubules, this AT$_2$R 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 AT$_2$R, a higher proportion of total AT$_1$R should be found in the dimeric state. In SHR, the lower AT$_2$R content leaves a greater amount of total AT$_1$R 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 AT$_2$R/AT$_1$R heterodimers (4). These heterodimers are required for the inhibition of the plasma membrane Ca^{2+}-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 AT$_2$R/AT$_1$R are required for the physiological stimulation of Na$^+$-ATPase in normotensive rats, and *2*) lower levels of AT$_2$R/AT$_1$R 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 AT$_1$R/AT$_2$R- 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).

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: J.D., F.M.F., F.A., A.K.C., L.S.L., and A.V. provided conception and design of research; J.D., F.M.F., and F.A. performed experiments; J.D., F.M.F., F.A., A.K.C., L.S.L., and A.V. analyzed data; J.D. and A.V. interpreted results of experiments; J.D. prepared figures; J.D. and A.V. drafted manuscript; J.D., F.M.F., F.A., A.K.C., L.S.L., and A.V. edited and revised manuscript; J.D., F.M.F., F.A., A.K.C., L.S.L., and A.V. approved final version of manuscript.

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