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Ang-(3–4) suppresses inhibition of renal plasma membrane calcium pump by Ang II

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ABSTRACT

We previously demonstrated that Ang II inhibits the renal plasma membrane Ca^{2+}-ATPase. In the present work we have studied the effect of Ang II, at concentrations similar to those found in the renal interstitium, on the Ca^{2+}-ATPase from proximal tubule cells. High Ang II concentration (5×10^{-7} mol/L) led to the recovery of Ca^{2+}-ATPase activity previously inhibited by 50% at low Ang II concentration (10^{-10} mol/L). Reactivation occurred in parallel with: (i) formation of only two dead-end metabolites [Ang-(3–4) and Tyr] after incubation of isolated membranes with micromolar Ang II; and (ii) dissociation of constitutive AT_1R/AT_2R heterodimers, which are preserved with 10^{-10} mol/L Ang II. When the membranes were incubated with 10^{-14} mol/L Ang-(3–4), inhibition by 10^{-10} mol/L Ang II was no longer observed. The counteracting effect of Ang-(3–4) was abolished by PD123319, an antagonist of AT_2R, and mimicked by CGP42112A, an agonist of AT_2R. Ang-(1–7) is an intermediate in the formation of Ang-(3–4) via a pathway involving angiotensin-converting enzyme (ACE), and complete dipeptide breakdown to Tyr and Val is impaired by low Ang II. We conclude that Ang-(3–4) may be a physiological regulator of active Ca^{2+} fluxes in renal proximal cells by acting within the renin–angiotensin axis.

1. Introduction

Over the past few decades, our knowledge of the renin–angiotensin system (RAS) has advanced significantly. Classically, the RAS was viewed as a restraining cascade in which the only participants are hepatic angiotensinogen, pulmonary angiotensin-converting enzyme (ACE) and a single biologically active hormone, angiotensin II (Ang II; for a review see [1]), the last of which interacts with two well-characterized plasma membrane receptors, AT_1 and AT_2 (AT_1R and AT_2R) [2]. Recent advances in studies of the RAS have revealed a more complex system that includes novel bioactive Ang II-derived peptides, additional specific receptors, local and intracrine RAS, new angiotensin-forming enzymes and alternative pathways of Ang II generation [3–6]. RAS plays a pivotal role in the regulation of blood pressure and body fluid homeostasis where the kidney is a critical target organ. Ang II maintains body Na^+, fluid balance and blood pressure by its vasoconstrictor effects as well as by stimulation at low concentrations of the $(Na^+ + K^+)$-ATPase, Na^+/HCO_3^- co-transporter, Na^+/H^+ exchanger [7] and ouabain-insensitive Na^+-ATPase [8] in the renal proximal tubules, which otherwise result in AT_1R activation.

Besides its hypertensive effects, Ang II also promotes vasodilatation and natriuresis at high concentrations, a condition in which the peptide probably interacts with the AT_2 receptor [9–11]. Because of its intracellular and extracellular formation in kidney proximal tubules [12,13] and the uptake of circulating Ang II [14], Ang II levels in the renal interstitium [15–17] and the proximal tubule fluid [18] are greater than in circulating plasma. Therefore, kidneys are probably the organs where high Ang II concentration physiologically activates AT_2R. In addition, Ang II can be cleaved to produce active metabolites, in particular Ang III, Ang IV and Ang-(1–7), which have already been shown to interact with AT_2R, leading to actions antagonistic to those generated by Ang II [19–21]. These metabolites also stimulate AT_1R and some new receptors that seem to be more specific [4,22].

Most, if not all, Ang II actions involve Ca^{2+} as a second messenger [2,23]. In kidney proximal tubules, as in other cells, the plasma membrane Ca^{2+}-ATPase is essential for the fine-tuning of intracellular calcium concentration [24,25]. Thereafter it appears to be an excellent target for hormones and autacoids (such as Ang II and its metabolites), which modulate sodium and water transport through calcium-dependent pathways [7,26,27]. Our group has recently found that picomolar concentrations of Ang II inhibit the plasma membrane Ca^{2+}-ATPase resident in the basolateral membranes of kidney proximal tubules, an effect mediated by PLC- and PKC-stimulated pathways linked to AT_1 and AT_2 receptors [28].

The aim of the present work was to investigate whether Ang II at higher (nanomolar or even micromolar) concentrations, *i.e.* in the range found in interstitial fluid [15–17], could modulate *per se* or through small peptides derived from its proteolysis [29–36] the

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neighboring plasma membrane Ca^{2+}-ATPase. Several observations have associated the effects of these Ang II-derived peptides with inhibition of ACE [33,37–39] and also with blocking of Ca^{2+} channels [33,36,40], thus leading to a decrease in intracellular Ca^{2+} levels. Since the plasma membrane Ca^{2+}-ATPase is considered to be the enzymatic machinery responsible for fine-tuning the cytosolic Ca^{2+} concentration in many cell types [24,25], including kidney cells [41], we hypothesized that, besides the effects mentioned above, Ang II-derived peptides could influence ATP-dependent Ca^{2+} extrusion from kidney proximal cells in a way that overcomes the inhibition promoted by picomolar Ang II [28]. Among the different small peptides derived from Ang II, Ang-(3–4) seemed to have potentially greater physiological relevance once it was found to be a natural inhibitor of ACE [37] – a property confirmed in later experiments [32,38,39] – and it appears to accumulate in renal tissue in high amounts [42]. We also investigated whether Ang II could coexist with Ang II-derived small peptides in a physiological network that influences the basolateral membrane Ca^{2+} pump through interactions with angiotensin receptors.

2. Materials and methods

2.1. Animal care

Sheep kidneys were obtained from an authorized slaughterhouse, and the health status of the animals was guaranteed by veterinary controls. Each membrane preparation was obtained from 6 to 10 kidney pairs. The study was approved by the local ethics committee for animal care (Universidade Federal do Rio de Janeiro), which follows the recommendations of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

2.2. Materials

Buffers, bovine serum albumin, ouabain, protease inhibitors, Ang II, Ang-(1–7), CGP42112A and PD123319 were obtained from Sigma. Losartan was from Merck Sharp & Dohme. The Ang-(2–7), Ang-(3–7), Ang-(1–5), Ang-(1–4) and Ang-(3–4) peptides were synthesized by EZBiolab. Percoll and the molecular weight calibration kit for SDS electrophoresis were from GE Healthcare. The anti-AT_1 and anti-AT_2 antibodies were purchased from Santa Cruz Biotechnology. Distilled water, deionized using the Milli-Q system of resins (Millipore Corp.), was used to prepare all solutions. ^{32}Pi was obtained from the Brazilian Institute of Energy and Nuclear Research. [γ-^{32}P]ATP was prepared as *per* Maia et al. [43]. Acetonitrile and trifluoroacetic acid were purchased from TEDIA Co., Inc. All other reagents were of the highest purity available.

2.3. Preparation of kidney tubule basolateral membranes

Basolateral membranes from sheep kidney proximal tubule cells were isolated and purified using the Percoll gradient method [44] and stored in 250 mmol/L sucrose under liquid N_2 at a final protein concentration of ~20 mg/mL. This preparation contains 30–40% of unsealed membrane fragments [44] and therefore allows free access of both ATP and angiotensin peptides to their respective and oppositely oriented sites. Under liquid N_2, Ca^{2+}-ATPase activity is preserved for more than 3 months. Protein assays involved the Folin-phenol reagent [45], with bovine albumin as standard. The specific activity of the basolateral membrane marker (Na^++K^+)-ATPase was enriched 8-fold over the kidney cortex homogenate. Using specific markers [46], different basolateral-enriched preparations selected at random showed residual contamination from subcellular membrane markers to be minimal, as described elsewhere [47]. To avoid contamination with cytosolic components, the membranes obtained after separation of nuclei and cell debris [44] were washed twice with a buffered (10 mmol/L Hepes–TRIS, pH 7.6) sucrose (250 mmol/L) solution supplied with 2 mmol/L EDTA, 0,15 mg/mL trypsin inhibitor (type II-S soybean) and 1 mmol/L phenylmethanesulfonyl fluoride. Moreover, the band at density 1.037 g/mL of the Percoll gradient – the narrow band containing the basolateral membranes – was recovered after careful removal of the top layer where glyceraldehydes 3-phosphate dehydrogenase, the cytosolic marker, is found. This special care is necessary to ensure that the Ang II metabolizing enzymes are membrane-bound and not of cytosolic origin.

2.4. Measurements of plasma membrane Ca^{2+}-ATPase activity

Except when otherwise noted, membranes (0.2 mg/mL, final protein concentration) were preincubated for 30 min at 37 °C with a solution containing 250 mmol/L sucrose, Ang II (concentrations as in Figs. 1, 4–6) and 1 mmol/L ouabain – the latter to guarantee complete inhibition of (Na^++K^+)-ATPase activity. The membrane suspension was then mixed with the basic reaction medium containing (in mmol/L) bis-TRIS-propane buffer 50 (pH 9.0), $MgCl_2$ 5, NaN_3 10, KCl 120, EGTA 0.2, and $CaCl_2$ 0.27 (20 µmol/L free Ca^{2+}). The total $CaCl_2$ needed for the desired free Ca^{2+} concentration was calculated as in Ref. [48]. Ca^{2+}-ATPase was calculated as the difference between the total activity and that determined in the presence of 2 mmol/L EGTA. In a series of experiments, different combinations of Ang II, Ang-(3–4), losartan or PD123319 (antagonists of AT_1R and AT_2R, respectively), CGP42112A (agonist of AT_2R) and L-NAME (an inhibitor of nitric oxide synthase; NOS) were assayed at the concentrations indicated in the corresponding figure legends or in the text (Figs. 4–6). Assays were started by adding [γ-^{32}P]ATP (5 mmol/L, ~1 Ci/mol) and continued at 37 °C for 20 min, and arrested by adding 4 vol activated charcoal in 0.1 mol/L HCL [49]. After centrifugation of the tubes at 18,000 ×*g* to sediment the charcoal, aliquots of the supernatant were counted in a liquid scintillation counter.

2.5. Analysis of angiotensin metabolites by high performance liquid chromatography (HPLC)

Ang II, Ang-(1–7), Ang-(1–5) or Ang-(3–4) samples (30 µmol/L) were incubated in water or 250 mmol/L sucrose (both conditions gave the same results) at 37 °C for the times indicated in the corresponding figure or figure legend. After addition of acetate buffer (5 mmol/L, pH 4.5), the tubes were centrifuged at 56,000 ×*g* for 30 min at 4 °C. The supernatants were concentrated to dryness in a speedvac instrument (Savant), resuspended in 0.1 mL 0.1% TFA (v/v) in deionized water (buffer A) and immediately injected into a C-18 reverse phase column (Rexcrom, 25 cm × 4.6 mm, Regis Technologies Inc.) coupled to a LC10AS-HPLC model (Shimadzu) through a 50 µL loop. Mobile-phase solvents were mixtures of buffer A (above) and 90% acetonitrile in 0.1%

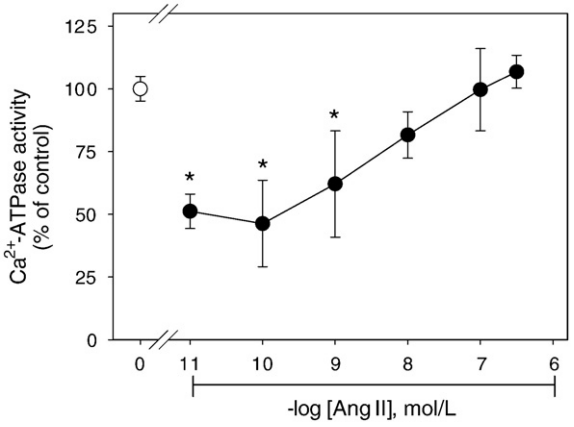

Fig. 1. Nanomolar concentrations of Ang II lead to complete recovery of the Ca^{2+}-ATPase activity inhibited at picomolar levels. Ca^{2+}-ATPase activity was measured in the presence of Ang II concentrations shown on the abscissa (●). Data points are means ± SE of at least four determinations in triplicate using different membrane preparations. The SE values were calculated from the absolute values of activities and converted to percentages. The absolute activity of the control was 12.8 ± 0.6 nmol $P_i \times mg^{-1} \times min^{-1}$ (○).

TFA (v/v) (buffer B) filtered through nitrocellulose filters (0.47 μm). To separate the metabolic products of Ang II, the column was isocratically eluted with 5% buffer B in A for the first 3 min, with a linear gradient of 5–100% of buffer B from 3 to 20 min, and finally with a linear gradient of 100–5% of buffer B from 20 to 24 min at a flow rate of 0.7 mL/min. Detection was at 214 nm. The column was pre-equilibrated with 5% buffer B for 40 min before use. Standard Ang II and Ang II-derived peptides were analyzed after incubation with membranes previously denaturated by adding acetate buffer at pH 4.5.

2.6. HPLC coupled to mass spectrometry (LC/ESI-MS)

LC/ESI-MS data were obtained on a Micromass instrument, model ZMD (Waters Corporation), coupled to a Waters Alliance model 2690 system using a Waters Nova-Pak C_{18} column (2.2×150 mm, 3.5 μm particle size, 60 Å pore size). For the LC step, the column was eluted with a linear gradient of acidified CH_3CN from 3% to 57% in 30 min at a flow rate of 0.4 mL/min. Absorbance was measured between 191 and 400 nm using a Waters photodiode array model 996. Mass measurements were performed in positive mode under the following conditions: mass range between 500 and 1500 m/z; nitrogen gas flow: 4.1 L/h; needle: 4 kV; cone voltage: 35 V; source heater: 140 °C; solvent heater: 400 °C.

2.7. Detection of AT_1/AT_2 receptor heterodimerization in basolateral membranes

Basolateral membranes (1 mg/mL) were initially incubated for 30 min at 37 °C (a) without Ang II, (b) with 10^{-10} mol/L Ang II or (c) with 10^{-6} mol/L Ang II, and then solubilized at room temperature in 0.01% (w/v) CHAPS for 30 min. Anti-AT_2R specific antibody (1:200) was mixed with protein A-agarose, gently stirred for 20 min and supplemented with an equal volume of bovine serum albumin solution (1 mg/mL) in 0.01% CHAPS. This mixture was then added to the solubilized membranes and left overnight at 4 °C under gentle agitation. The immunoprecipitates were separated from their supernatants by centrifugation at 1000 ×g for 4 min (4 °C), washed three times with Tris-buffered saline, mixed with Laemmli buffer and heated for 4 min (100 °C) to remove the antibodies and the protein A-agarose. After centrifugation at 16,100 ×g for 2 min (4 °C), aliquots of the receptor-containing supernatants were electrophoresed (10% SDS-PAGE), transferred to nitrocellulose membranes, and probed with anti-AT_1R or anti-AT_2R antibodies (1:500) as described in the corresponding figure legend (Fig. 7A,C). In a series of experiments, the supernatants recovered after overnight immunoprecipitation with the anti-AT_2R antibody were subjected to the procedure described above using anti-AT_1R antibody for both immunoprecipitation and probing (Fig. 7B).

2.8. Statistical analysis

Ca^{2+}-ATPase activity data are expressed as means ± SE. Differences between mean values of activities in the absence or presence of different Ang II and Ang-(3–4) concentrations, losartan (AT_1R antagonist), PD123319 (AT_2R antagonist) and CGP42112A (AT_2R agonist) were assessed by ANOVA, followed by Student–Dunnet analysis (Figs. 1, 4–7). In Fig. 2, statistical differences between control and preincubation with 5×10^{-7} mol/L Ang II were assessed by Student's *t* test. Significance was set at $p<0.05$. HPLC chromatograms were repeated at least three times and the elution profiles of the different peptides were replicated consistently.

3. Results

3.1. Progressive recovery of Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activity inhibited by 10^{-11} to 10^{-10} mol/L Ang II was recovered progressively as the initial peptide concentrations were

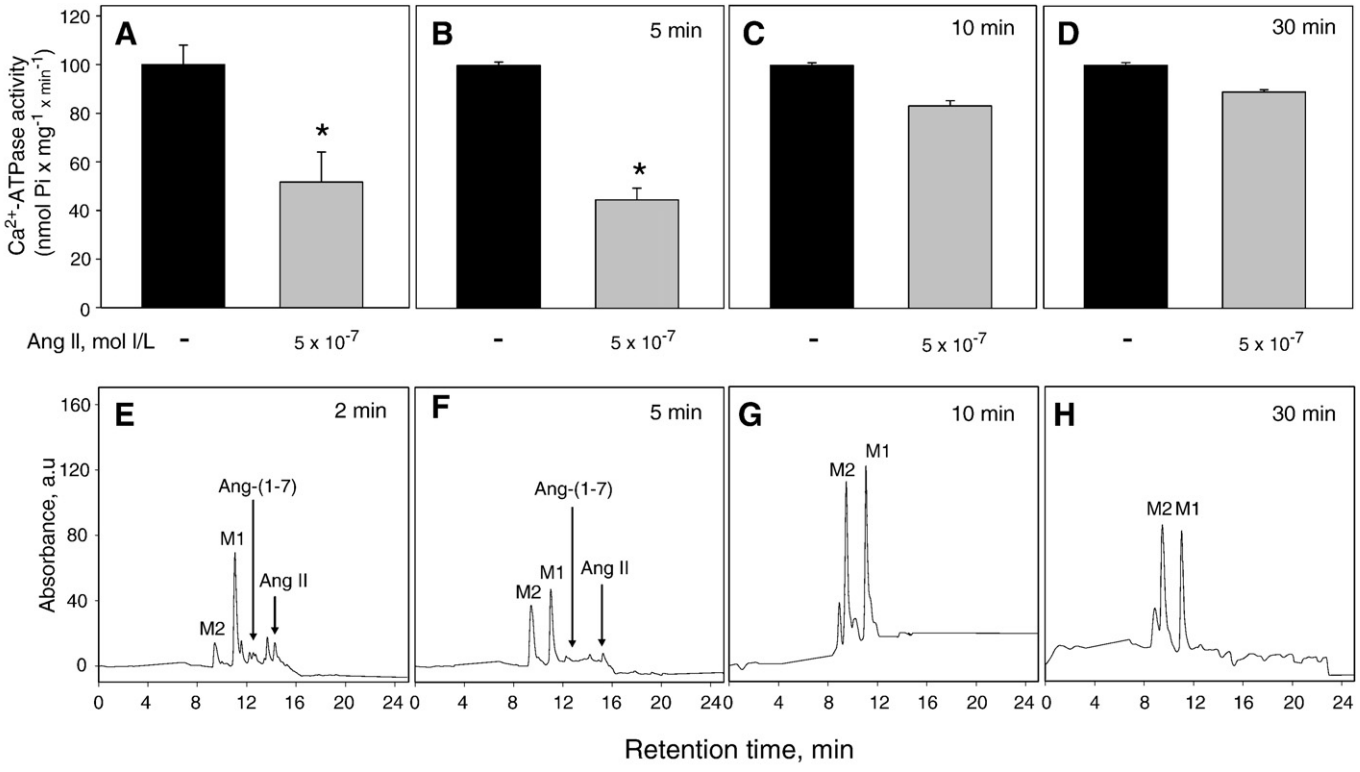

Fig. 2. Simultaneous recovery of Ca^{2+}-ATPase activity and formation of two end-metabolites derived from Ang II. The upper panels A, B, C and D show Ca^{2+}-ATPase activities at the indicated times and are expressed as percentages of the corresponding time-matched control assayed without Ang II. In these assays, Ang II (5×10^{-7} mol/L) was simultaneously added to the basolateral membranes with the ATP-containing reaction medium. *: Statistically different from the control without Ang II. The lower panels E, F, G and H are representative HPLC chromatograms showing the evolution of Ang II metabolism to two stable peaks (M1 and M2) after incubation with the membranes during the indicated times.

gradually raised to 5×10^{-7} mol/L, when a value identical to the control was attained (Fig. 1). At this concentration, the enzyme activity was the same as that measured in controls without Ang II ($p>0.05$). The reactivation might be due to desensitization of the Ang II receptors [50] or to the formation of inactive or active and counteracting metabolites. In the latter case, an active metabolite could be Ang-(1–7), a well known counter-regulator of the Ang II actions in kidney [3,51–54], or another downstream peptide formed in the physiological RAS cascade [29–36,39]. Ca^{2+}-ATPase measurements with Ang II concentrations higher than 1 μmol/L (assayed up to 10 μmol/L) showed activities identical to the control without Ang II (not shown). Interestingly, all the signaling components of the cascade that links Ang II to the Ca^{2+} pump are present in basolateral membranes from proximal tubule cells [28], as in the case of the ouabain-insensitive Na^{+}-ATPase [8,19,51].

3.2. Recovery of Ca^{2+}-ATPase activity is associated with parallel Ang II metabolism

The experiments depicted in Fig. 2 were designed to test the hypothesis that in the presence of high Ang II concentrations such as those found in kidney tissue [15–17], metabolites are formed in the presence of the basolateral membranes. In addition, they were set out to verify whether this possible process was associated with reactivation of the Ca^{2+} pump over time, as found in Fig. 1 after 30 min preincubation followed by 20 min reaction with ATP. For this reason, the experiments shown in Fig. 2 were carried out without preincubation of the peptide with the membranes, to allow Ca^{2+}-ATPase activity to be correlated with preservation (or otherwise) of the integrity of Ang II over time. Indeed, time-dependent proteolysis was clearly demonstrated and it appears to be the key process in Ca^{2+}-ATPase reactivation. The evolution of Ang II proteolysis with time shows rapid conversion (2 min) to several metabolites, including a transient Ang-(1–7) peak with gradual formation of two other products (M1 and M2) that are probably formed in sequential order (compare panels E and F). The retention time of synthetic Ang-(1–7) with this solvent system, measured in a separate experiment, was 13.0 min, and this value could identify the transient intermediate shown in the bottom panels of Fig. 2 (panels E and F). As incubation was prolonged, Ang-(1–7) could not be detected by this method, and the two peaks were initially stabilized in equivalent amounts (in terms of Tyr absorbance) between 10 and 30 min (panels G and H).

The progressive evolution of Ang II towards the two peaks is accompanied by complete reversion of the Ca^{2+}-ATPase inhibition seen at the outset (compare panels A and B with panels C and D). These experiments clearly confirm that isolated basolateral membranes from kidney proximal tubules have an enzymatic complex capable of transforming Ang II into two dominant metabolites with retention times of 11.9 min (M1) and 10.2 min (M2); and, as importantly, they demonstrate that this process has a significant effect on active Ca^{2+} transport across the tubular epithelium. The experiments of Fig. 2, however, do not clarify whether reactivation of the Ca^{2+} pump is due to almost complete consumption of Ang II or the formation of functionally active peptides that counteract the inhibitory influence of very low residual Ang II. In panels G and H, we cannot see whether a low residual Ang II is present, which might have an inhibitory influence on the Ca^{2+} pump that could be counteracted by the small metabolites. This point is addressed in the following section.

3.3. Identification of the predominant final metabolites M1 and M2 by HPLC and detection of the lower residual Ang II by LC/ESI-MS

Looking at the possible Ang II metabolites that may be formed in kidney basolateral membranes by limited proteolysis *via* Ang-(1–7) or even through a parallel pathway with Ang III as an intermediate [3,29,39], we assayed different Ang-derived Tyr-containing peptides

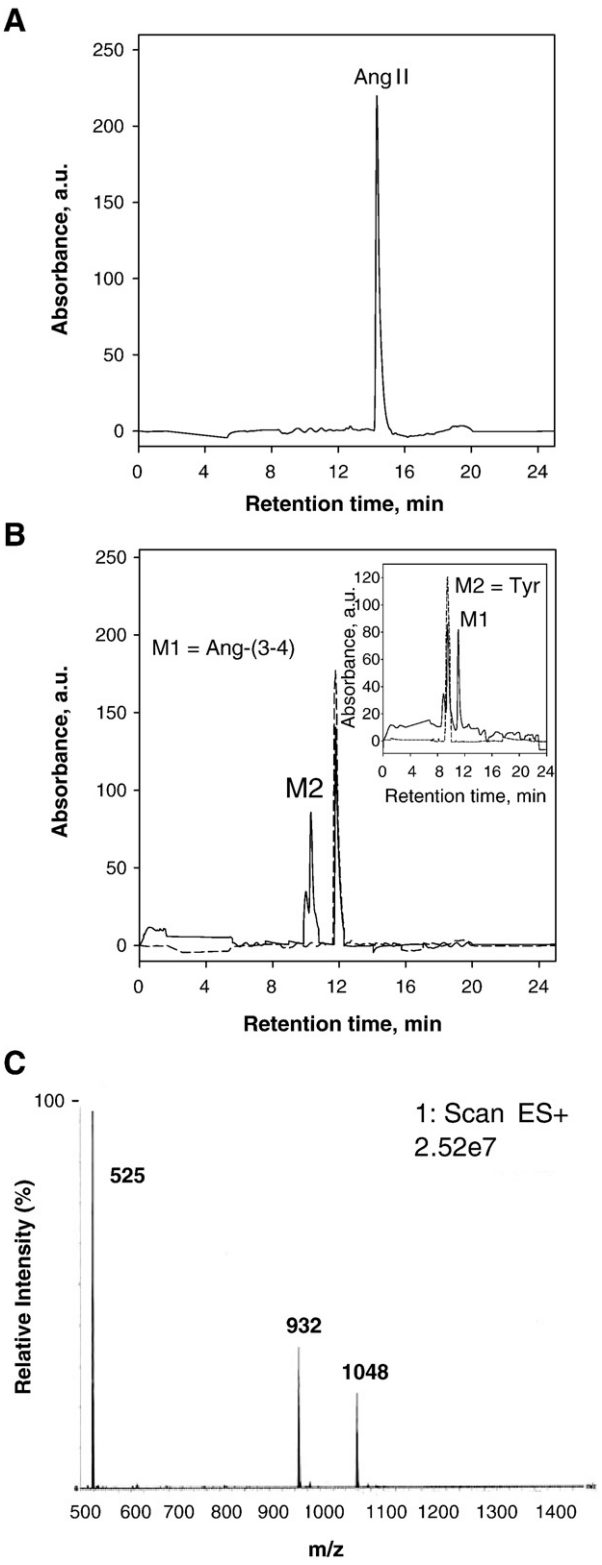

Fig. 3. Ang-(3–4) is the Ang II metabolite M1 shown in Fig. 2. Ang II was incubated for 30 min with the basolateral membranes. The resulting supernatant was analyzed by HPLC. (A) Initial Ang II peak, obtained with denatured membranes. (B) Peaks M1 and M2 from the chromatogram obtained after incubation of Ang II with the membranes for 30 min were mixed with the synthetic Ang-(3–4) standard (Val-Tyr) and injected into the HPLC apparatus. The inset to panel B shows that M1 corresponds to pure Tyr. Dashed lines represent the Ang-(3-4) or Tyr (inset) standards. (C) Residual Ang II and Ang III after 30 min of incubation with the membranes, detected by mass spectrometry. For details see Materials and methods section.

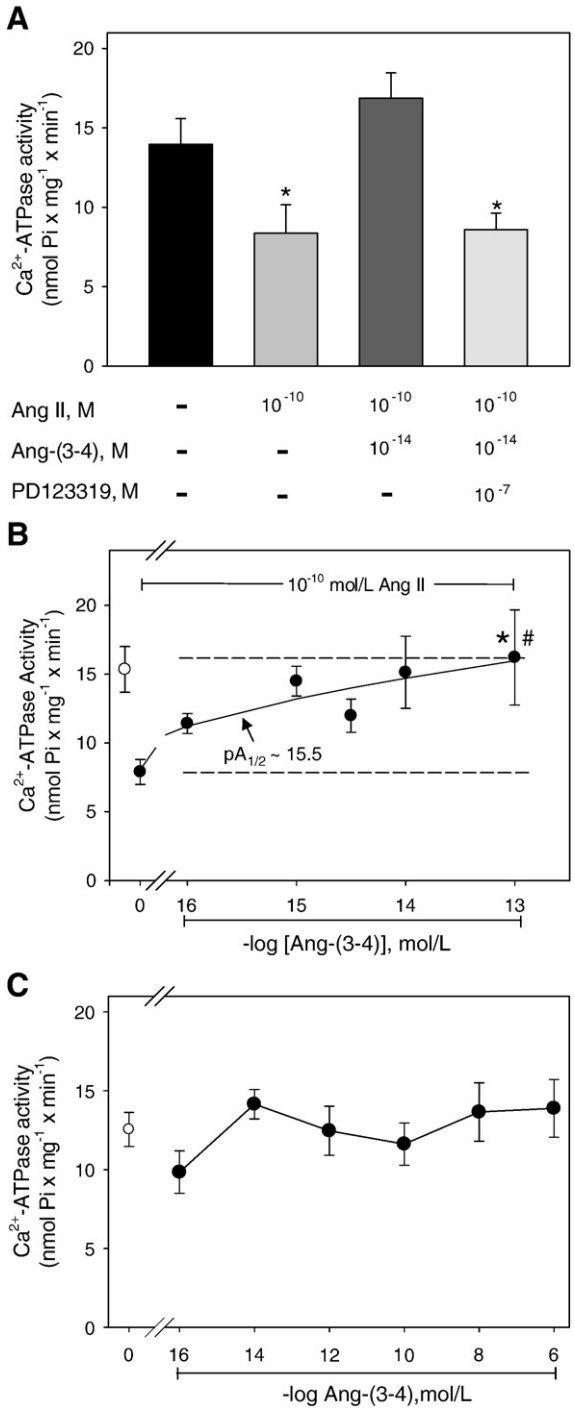

Fig. 4. Femtomolar Ang-(3–4) reactivates the Ang II-inhibited Ca^{2+}-ATPase after binding to AT_2 receptors. (A) Basolateral membranes preincubated or not with 10^{-10} mol/L Ang II, as shown, were assayed for Ca^{2+}-ATPase activity as described under Materials and methods in the presence or absence of Ang-(3–4) and the AT_2R antagonist PD123319 in the combinations and concentrations shown on the abscissa. (B) (●): Ca^{2+}-ATPase activity was measured with the combination of 10^{-10} mol/L Ang II and increasing concentrations of Ang-(3–4) as shown. $pA_{1/2}$ ~15.5 was calculated by hand (arrow). (○): control without peptide additions. C) (●): Ca^{2+}-ATPase activity measured in the presence of the Ang-(3–4) concentrations indicated on the abscissa with no Ang II. (○): control without peptide additions. Data bars and points indicate means ± SE of at least six determinations in triplicate using different membrane preparations. *: Statistically different from the control without additions.

by HPLC to compare their retention times with M1 and M2. Synthetic Ang-(2–7), Ang-(3–7), Ang-(1–5) and Ang-(1–4) gave retention times of 13.3, 13.1, 12.3 and 10.9 min; therefore, they were discarded as possible candidates for M1 and M2. Since Ang-(3–4) (Val-Tyr) can be formed in proximal tubules [31,34], the following experiments tested the hypothesis that one of the final metabolites shown in Fig. 2 could be this dipeptide. Samples of basolateral membranes were incubated with Ang II for 30 min and the supernatants containing the final peaks M1 and M2 were recovered, dried, mixed with the synthetic Ang-(3–4) standard (Val-Tyr) and then assayed by HPLC. Fig. 3B shows that a higher single peak at 11.9 min occurs when product M1 is mixed and eluted (compare continuous and dashed lines); in addition, the inset shows that the retention time of M2 matches that of tyrosine. Therefore, these experiments helped to identify the final proteolytic products as Ang-(3–4) and Tyr (Fig. 2). Although *uv–vis* HPLC detection did not identify Ang II after incubation of the peptide with the membranes for 30 min, mass spectrometric analysis of the sample showed a residual amount of about 98 pmol Ang II ($M + H^+ = 1048$ kDa), originating from 7.5 nmol Ang II (Fig. 3C). The peak at 932 kDa corresponds to Ang III and that at 525 kDa is a usual product of Ang II ionization in electron spray analysis. Small fragments, such as a dipeptide, are below the detection sensitivity of the method.

3.4. Ang-(3–4) counteracts the inhibition of the Ca^{2+} pump by Ang II through a mechanism sensitive to an AT_2R antagonist and mimicked by an AT_2R agonist

To investigate whether Ang-(3–4) actually counteracts the inhibition of renal Ca^{2+}-ATPase by picomolar Ang II concentrations, the activity of the pump was assayed in the presence of both peptides. Inhibition promoted by 10^{-10} mol/L Ang II was completely cancelled when an Ang-(3–4) concentration as low as 10^{-14} mol/L was present (Fig. 4A), with $pA_{1/2}$ ~ 15.5 (Fig. 4B), a value that clearly indicates the high potency of the dipeptide. The fact that PD123319 abolishes the recovery of Ca^{2+}-ATPase activity promoted by Ang-(3–4) can be considered evidence that the dipeptide effect involves the participation of AT_2R. Moreover, the AT_2R agonist CGP42112A (10^{-10} mol/L) also mimics the Ang-(3–4) effect in counteracting the inhibition by low Ang II (Fig. 5). Both Ang-(3–4) and CGP42112A *per se* have no effect on the enzyme activity (Figs. 4C and 5, respectively), an indication that their action is due to inactivation of the membrane-associated signaling cascade stimulated by Ang II [28]. When 10^{-6} mol/L Ang II is preincubated with the membranes for 30 min before adding ATP, enough time to form Ang-(3–4) (Figs. 2H and 3B),

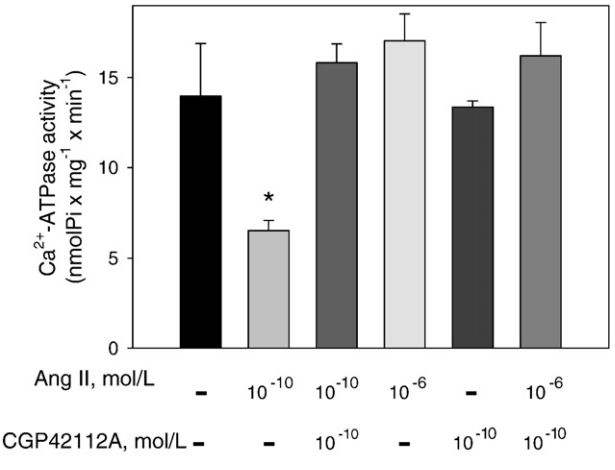

Fig. 5. Binding of the AT_2R agonist CGP42112A suppresses inhibition of Ca^{2+}-ATPase by 10^{-10} mol/L Ang II, with no effect in the presence of 10^{-6} mol/L Ang II. Basolateral membranes were preincubated with Ang II and then the samples were supplied or not with CGP42112A as shown, before addition of reaction medium to measure Ca^{2+}-activity. Data bars indicate means ± SE of at least six determinations in triplicate using different membrane preparations. *: Statistically different from the control without additions.

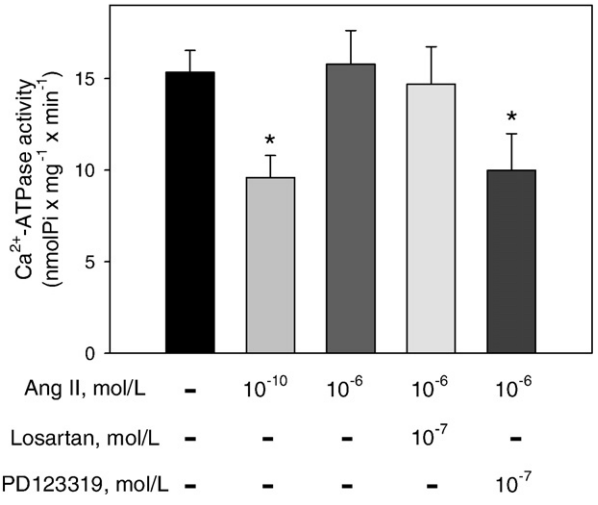

Fig. 6. PD123319 but not losartan blocks recovery of Ca^{2+}-ATPase activity in the presence of 10^{-6} mol/L Ang II. Basolateral membranes were assayed for Ca^{2+}-ATPase activity as described under Materials and methods in the presence of Ang II, losartan and PD123319 in the combinations and concentrations shown on the abscissa. Data bars indicate means ± SE of at least six determinations in triplicate using different membrane preparations. *: Statistically different from the control without additions.

which coexists with a very low residual Ang II (Fig. 3C), the reactivation of Ca^{2+}-ATPase is impaired by PD123319 but not by the AT_1R antagonist losartan (Fig. 6). This is further evidence that only AT_2R appears to be involved in the recovery of enzyme activity upon addition of Ang-(3–4).

3.5. Constitutive AT_1R/AT_2R heterodimers are maintained with picomolar Ang II and dissociate in the presence of micromolar Ang II concentrations

The observation that PD123319 cancels reactivation by Ang-(3–4) of the inhibited Ca^{2+}-ATPase appears, at a first sight, to contradict previous results [28]. Addition of 10^{-7} mol/L PD123319 in combination with 10^{-11} mol/L Ang II sufficed for full recovery of Ca^{2+}-ATPase activity [28], whereas 10^{-7} mol/L PD123319 prevents the reactivation promoted by Ang-(3–4) in the presence of 10^{-10} mol/L Ang II (Fig. 4). These apparently opposing groups of data led us to explore another hypothesis to explain the participation of Ang-(3–4) in a process that clearly involves AT_2R: the requirement of an AT_1/AT_2 heterodimer for inhibition of the Ca^{2+} pump by 10^{-10}—10^{-11} mol/L Ang II, and Ang-(3–4)-induced dissociation of the heterodimer to allow the Ca^{2+}-ATPase activity to recover. The experiments depicted in Fig. 7 were undertaken to show whether AT_1/AT_2 heterodimers are formed and dissociated under three experimental conditions: (a) without Ang II, (b) with only picomolar Ang II or (c) where residual Ang II – enough to

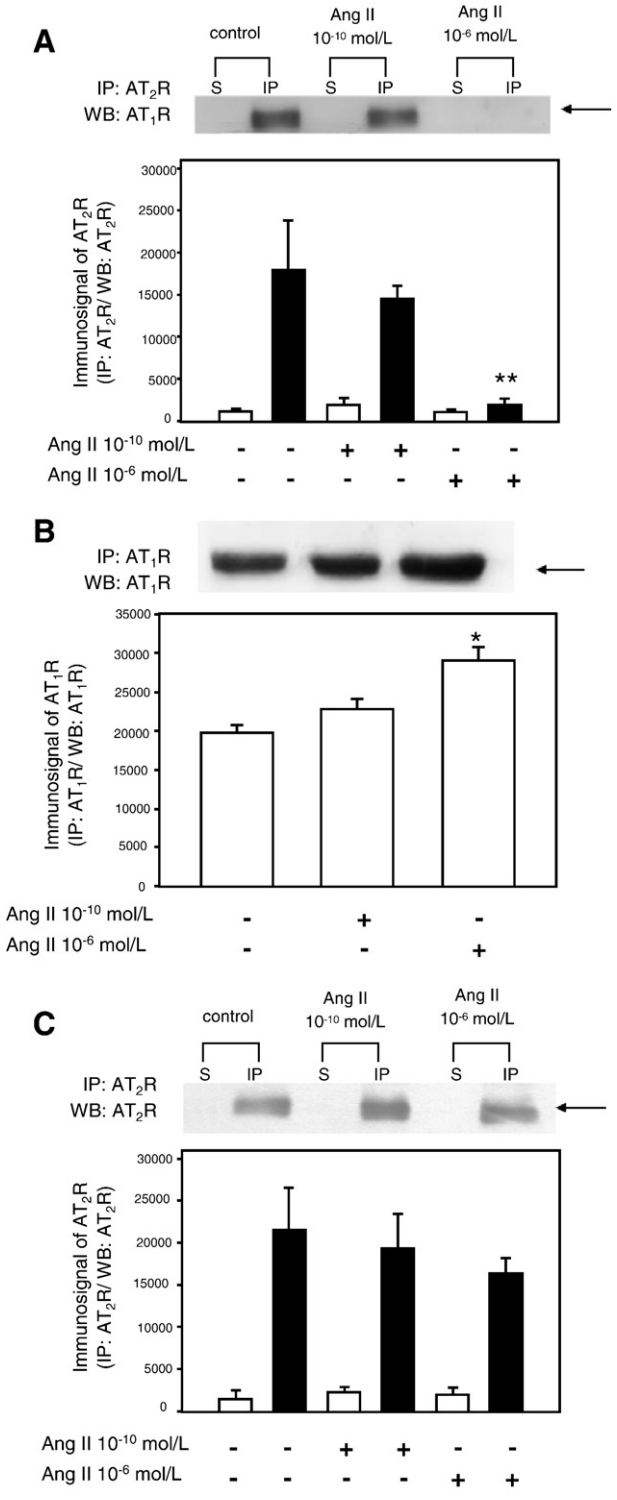

Fig. 7. High (10^{-6} mol/L) Ang II promotes dissociation of AT_1R/AT_2R heterodimers in basolateral membranes. *Lower panels.* (A) Immunoprecipation with anti-AT_2R antibody and probing with anti-AT_1R antibody (IP:AT_2R / WB:AT_1R) were assayed after preincubation of the membranes in the presence of the Ang II concentrations shown on the abscissa. Empty and filled bars: Immunodetection of AT_1R in the supernatants and pellets, respectively, after SDS-PAGE. (B) Immunoprecipitation with anti-AT_1R antibody and also probing with anti-AT_1R antibody (IP:AT_1R / WB:AT_1R) of the supernatant recovered after immunoprecipitation with anti-AT_2R antibody in the absence or presence of the Ang II concentrations shown on the abscissa. (C) Immunoprecipation with anti-AT_2R antibody and probing with anti-AT_2R antibody (IP:AT_2R / WB:AT_2R) were assayed after preincubation of the membranes in the presence of the Ang II concentrations shown on the abscissa. Empty and filled bars: Immunodetection of AT_2R in the supernatants and pellets, respectively, after SDS-PAGE. For details see Materials and methods section. **: Statistically different from the other immunoprecipitates (without additions or with 10^{-10} mol/L Ang II), $p<0.001$; *: Statistically different from the control without additions, $p<0.05$. *Upper panels.* Representative immunodetections of angiotensin receptors in supernatants (S) and pellets (IP) after AT_2R immunoprecipitation in the absence or presence of the Ang II concentrations indicated on the abscissa. Probing was carried out with anti-AT_1 (A) or anti-AT_2 antibodies (C). (B) Representative immunodetection of AT_1R in the supernatant recovered after immunoprecipitation with anti-AT_2R antibody in the absence or presence of the Ang II concentrations shown on the abscissa. Arrows in the upper panels indicate a molecular mass of 45 kDa determined with the use of a molecular weight calibration kit for SDS electrophoresis, visualized in the nitrocellulose membranes after staining with ponceau red.

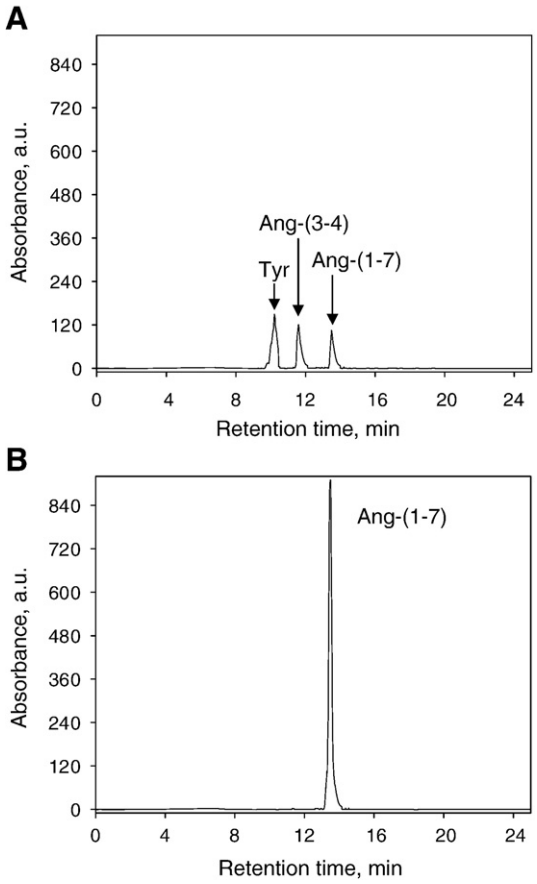

Fig. 8. ACE participates in the conversion of Ang-(1–7) into Ang-(3–4). Ang-(1–7) was incubated for 30 min with the basolateral membranes in the absence (A) or presence (B) of 150 μmol/L captopril (B) and the resulting supernatants were analyzed by HPLC. For details see Materials and methods section.

promote inhibition when assayed alone – and Ang-(3–4) are present after preincubation of 10^{-6} mol/L Ang II with the membranes for 30 min before the immunoassay. Fig. 7A shows that (i) there is a non-stimulated population of preexisting AT_1/AT_2 heterodimers in the membranes, (ii) these heterodimers are preserved with 10^{-10} mol/L Ang II, a condition in which the Ca^{2+}-ATPase is inhibited, and (iii) they dissociate when the membrane suspension is supplied with an initial 10^{-6} mol/L Ang II concentration (which results in residual Ang II plus Ang-(3–4) at the moment of anti-AT_2R antibody addition), a condition in which Ca^{2+}-ATPase activity is recovered.

Since no AT_1R-related immunosignal appeared in the supernatant in the 10^{-6} mol/L Ang II condition, probably because its amount was low, an attempt was made to circumvent this difficulty as follows. Using the same Ang II concentrations, the supernatant obtained after immunoprecipitation with anti-AT_2R antibody was re-immunoprecipitated and probed against AT_1R. Fig. 7B shows a substantial amount of this class of receptors, which clearly corresponds to a pool in the monomeric state that is significantly higher with 10^{-6} mol/L Ang II owing to the contribution of that dissociated from the dimers. In a control experiment, it can be seen that different Ang II levels have no effect on the AT_2R distribution, as demonstrated in the experiments where both immunoprecipitation and probing were carried out with anti-AT_2R antibody (Fig. 7C).

3.6. Ang-(3–4)-induced reactivation of Ca^{2+}-ATPase inhibited by 10^{-10} mol/L Ang II is not associated with an AT_2R/NOS coupled pathway

Several studies have shown that NO participates in signaling cascades coupled to AT_2R (for recent and early reviews see [35,55]). In view of the results in Figs. 4–6, reactivation assays were conducted in the presence of L-NAME, an inhibitor of NOS. In this series of experiments, reactivation of the Ca^{2+}-ATPase inhibited by 10^{-10} mol/L Ang II (7.76 ± 2.25 nmol $Pi \times mg^{-1} \times min^{-1}$) to reach the control value (14.15 ± 1.78 nmol $Pi \times mg^{-1} \times min^{-1}$, no additions), remained unchanged both in the presence of 10^{-10} mol/L Ang II in combination with 10^{-14} mol/L Ang-(3–4) plus 10^{-4} mol/L L-NAME (15.28 ± 1.12 nmol $Pi \times mg^{-1} \times min^{-1}$) and in the presence of 10^{-6} mol/L Ang II plus 10^{-4} mol/L L-NAME (12.80 ± 2.95 nmol $Pi \times mg^{-1} \times min^{-1}$) ($n = 5$; $p > 0.05$ when differences were analyzed between control and L-NAME conditions).

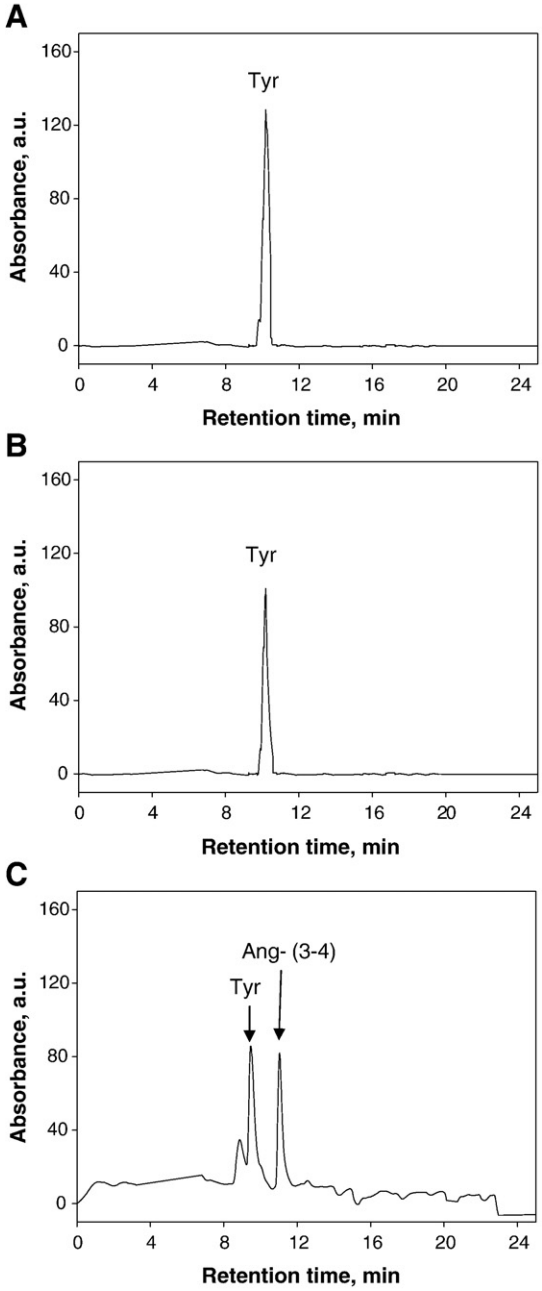

Fig. 9. Ang-(3–4) is hydrolyzed to Val and Tyr if the starting peptides are Ang-(1–5) or Ang-(1–4) but exhibits high stability in the presence of residual Ang II. Basolateral membranes were incubated in the presence of Ang-(1–5) (A), Ang-(1–4) (B) (in both cases for 30 min) or Ang II (C) (for 2 h) and the resulting supernatants were analyzed by HPLC. For details see Materials and methods section.

3.7. Enzymatic pathway from Ang II towards Ang-(3–4) in the basolateral membranes

Fig. 2 (E and F) shows a small transient peak of Ang-(1–7), which is generated during consumption of the starting Ang II, evidence that the former is an intermediate precursor of Ang-(3–4). In Fig. 8A, incubation of Ang-(1–7) with membranes gives Ang-(3–4) plus Tyr, and 150 μmol/L captopril, an inhibitor of the angiotensin-converting enzyme (ACE), blocks metabolization of Ang-(1–7) (Fig. 8B). Since ACE converts Ang-(1–7) into Ang-(1–5) [31], the following experiments were carried out by incubating Ang-(1–5) or Ang-(1–4) with the membranes (Fig. 9A,B). Unexpectedly, only Tyr was recovered after 30 min. Since the two final peaks, Ang-(3–4) and Tyr, shown in 2H and 3B remain stable after incubation of Ang II for 2 h (9C), it was concluded that the residual Ang II in Fig. 3C might block the final hydrolysis of Ang-(3–4) to Tyr, thus contributing to the stability of the dipeptide.

4. Discussion

We previously demonstrated that the Ca^{2+}-ATPase resident in the basolateral membranes of kidney proximal tubule cells is inhibited up to 50% in the presence of Ang II in the range 10^{-12} to 10^{-10} mol/L [28]. This inhibition (Fig. 1) is progressively suppressed as long as the Ang II level increases. A biphasic influence of Ang II on fluid reabsorption in intact proximal tubules has been also described, and these opposite effects have been ascribed to fluctuations in cytosolic Ca^{2+} in response of varying Ang II concentrations over a broad range [7,27]. Since the plasma membrane Ca^{2+} pump is considered the cell mechanism responsible for fine-tuning cytosolic Ca^{2+} [24,25,41], the complex regulation of Ca^{2+}-modulated fluid reabsorption is probably achieved, at least in part, by the observed biphasic effects on Ca^{2+}-ATPase.

Fig. 2 shows that recovery of Ca^{2+}-ATPase activity is a result of Ang-(3–4) formation, with Ang-(1–7) as an intermediate (Fig. 8A,B) that subsequently produces Ang-(1–5) and Ang-(1–4) in an ACE-dependent route [31,34]. Ang III is also formed as a result of Ang II hydrolysis catalyzed by an aminopeptidase A (Fig. 3C) [3,29]. A branch in which the sequential steps are Ang IV (formed from Ang III as the result of N-mediated aminopeptidase catalysis) and Ang-(3–7) (arising from the action of a carboxypeptidase on Ang IV) could culminate in Ang-(3–4) if an ectopeptidase was present [30]. Ang-(1–7) was also metabolized to Tyr and Val-Tyr by brush border membranes [31], whereas others [34] have demonstrated metabolism of both Ang I and Ang II by intact and disrupted proximal tubule cells, with Ang-(1–7) as an intermediate in a process that culminates in several small peptides including Ang-(3–4). These final metabolites could physiologically antagonize several Ang II effects through a possible inhibitory feedback within the same RAS cascade, as demonstrated in our case with the plasma membrane Ca^{2+}-ATPase.

It should be emphasized that the products Ang-(3–4) and Tyr (Fig. 3A,B) coexist with a residual amount of Ang II (Fig. 3C), which on its own could suffice to inhibit the renal plasma membrane Ca^{2+}-ATPase. This gives strong support to the view that the pump reactivation observed in Fig. 2 is due to counteraction of the influence of the metabolites — as long as they are formed — rather than to recovery of the activity as a result of Ang II consumption. In addition, the observations that Ang-(3–4) is cleaved completely to Tyr when it is formed from Ang-(1–5) or Ang-(1–4), but not when the starting material is Ang II or Ang-(1–7) (compare Fig. 9A,B with Fig. 3), suggest that a regulatory mechanism that preserves Ang-(3–4) could be present *in vivo*. It may be that Ang III, another side-product of Ang II metabolism in basolateral membranes (Fig. 3C), also impairs the final cleavage of Ang-(3–4) to Tyr and Val as Ang II actually does. In this regard, Allred et al. [31] proposed that Ang II may have a protective effect on Ang-(1–7) by inhibiting its metabolism to Ang-(1–4) in brush border membranes. In the case of active Ca^{2+} transport, preservation of Ang-(3–4) could be viewed as a safeguard against abnormally high intracellular Ca^{2+} levels resulting from inhibition of Ca^{2+}-ATPase by Ang II. It may therefore be that a balance between Ang II and Ang-(3–4) is physiologically more important than the absolute Ang II levels, at least in kidney tissue.

The high potency of Ang-(3–4) in reactivating the Ca^{2+}-ATPase inhibited by picomolar Ang II ($pA_{1/2}$ ~15.5; Fig. 4A,B) strongly suggests that this end-point metabolite could be one of the most important modulators of the intrarenal RAS system and, in the case of transepithelial ion movements, of ATP-dependent Ca^{2+} fluxes. The view that renal active transport of Ca^{2+} — an essential second messenger in living systems [24] — could be one of the main targets for Ang-(3–4) is also supported by the observations that the dipeptide accumulates in high amounts in renal tissue and that renal ACE rather than plasma ACE is preferentially inhibited [42]. Thus, antagonizing the Ca^{2+}-mediated stimulatory influence of Ang II in the reabsorption of fluid in proximal tubules [7,8] could be the main mechanism by which Ang-(3–4) contributes to decreasing the volume of the extracellular fluid and, therefore, the arterial pressure. The huge difference between the $pA_{1/2}$ for the reactivation of plasma membrane Ca^{2+}-ATPase in renal tissue (~15.5; Fig. 4B) and the $pI_{1/2}$ demonstrated for the inhibition of blood serum ACE *in vitro* (~4.9; [39]) suggests that, in mammals, different pools of Ang-(3–4) might act in different ways depending on the body compartment.

The fact that PD123319 – but not losartan – abolishes the recovery of Ca^{2+}-ATPase activity promoted by Ang-(3–4) or high Ang II (Figs. 4A and 6) can be considered evidence that the dipeptide effect is mediated in some way by AT_2R. The participation of AT_2R receptors in suppressing inhibition of the Ca^{2+} pump by 10^{-10} mol/L Ang II is confirmed by the experiments of Fig. 5, showing that the AT_2R agonist CGP42112A also cancels the enzyme inhibition promoted by physiological Ang II concentrations. Moreover, the lack of receptor agonist effect in the presence of 10^{-6} mol/L Ang II – at which full recovery of Ca^{2+}-ATPase is observed and a significant amount of Ang-(3–4) is formed – indicates that they act in the same manner. Altogether, these observations add support to the idea that one important role of this type of receptor is to mediate the effects of Ang II-derived peptides [19–21,34], together with those elicited by Ang II itself, to bring about a delicate intracascade balance with AT_1R-mediated responses. From the data shown in Fig. 4A, it can be concluded that, besides Ang III [35] or Ang-(1–7) [30,56], Ang-(3–4) can bind to AT_2R in the femtomolar concentration range.

The influence of Ang-(3–4) on intracellular Ca^{2+} levels has been ascribed, in vascular smooth muscle cells, to inhibition of Ang II-induced Ca^{2+} influx by direct interaction with L-type Ca^{2+} channels rather than by an antagonistic effect on Ang II receptors [40]. It may be that such small peptides could accommodate sufficiently to block a channel. Moreover, it is also conceivable that owing to its small size, Ang-(3–4) may bind with very high affinity to the AT_2R component of an AT_1R/AT_2R dimer, thus overcoming the steric hindrance that longer peptides would have. Fig. 7A shows that, under conditions in which residual Ang II and Ang-(3–4) coexist, there is complete dissociation of the constitutive AT_1R/AT_2R heterodimers detected in the basolateral membranes of proximal tubule cells. Thus, the main mechanism by which Ang-(3–4) influences the renal plasma membrane Ca^{2+}-ATPase could be to induce dissociation of the AT_1R/AT_2R heterodimers, whereas their preservation with 10^{-10} mol/L Ang II (Fig. 7A) appears to be essential for the losartan- and PD123319-sensitive inhibition of the Ca^{2+} pump [28]. The existence of heterodimers with AT_1R is well documented and this structural arrangement has been implicated either in the antagonist effect of AT_2R [57] or in the potentiation of Ang II effects when heterodimerization with bradykinin B_2 receptors occurs [58,59]. AT_1R/AT_2R heterodimerization has also been proposed in a study on the role of AT_2R in coronary flow and left ventricular systolic pressure regulation [60]. In that study it was demonstrated that AT_2R-mediated effects occurs only if AT_1R ($AT_{1A}R$) are activated. In the case of kidney basolateral membranes the need of this interaction could explain the

lack of Ang-(3–4) effect if Ang II is not present and, therefore, AT_1R is not activated (Fig. 4C). Moreover, from the data of Figs. 3C, 4A,B and 7 it could be also hypothesized that, in the intact cell, AT_1R/AT_2R heterodimers might dissociate because of the internalization of AT_1R upon binding of Ang-(3–4) to AT_2R. Triggering of a signaling cascade after binding of Ang-(3–4) to AT_2R (and dimer dissociation) also seems possible, since PD123319 blocks recovery of the Ca^{2+}-ATPase activity (Fig. 4A), and CGP42112A (the AT_2R agonist) mimics the effect of the dipeptide (Fig. 5). The intermediates and the mechanisms of this pathway remain to be elucidated.

Finally, it can be argued that, as Ang-(3–4) consists of only two amino acids, it is likely to be an easy target for peptidases *in vivo*. However, Ang-(3–4) appears to have unexpected stability. The end-peak of Ang-(3–4) formed after a 30 min-incubation of Ang II with renal membranes (Fig. 2G, H) remained unmodified when the incubation was prolonged to 2 h (Fig. 9C). Evidence for its stability *in vivo* is the observation that it has a high mean residence time in several tissues (notably kidney) and a long elimination half-time after a single oral administration [32,42]. Moreover, in a recent paper, Pentzien and Meisel [39] have demonstrated the stability of Ang-(3–4) when it is incubated with human blood serum. The presence of Ang II could be another important factor in stabilizing the peptide, because Ang-(3–4) is completely cleaved to Val and Tyr when the proteolytic process is started with Ang-(1–5) or Ang-(1–4) instead of Ang II (Fig. 9).

In conclusion, the present study provides evidence that Ang-(3–4) is a potent reactivator of the kidney proximal tubule plasma membrane Ca^{2+}-ATPase inhibited by Ang II. Since in the proximal nephron segment about two thirds of the glomerular ultrafiltrate is reabsorbed and this process is highly sensitive to Ca^{2+} [2,23], Ang-(3–4) emerges as an important regulator of body fluid compartments.

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