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A scrutiny of the biochemical pathways from Ang II to Ang-(3–4) in renal basolateral membranes

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ABSTRACT

In a previous paper we demonstrated that Ang-(3–4) counteracts inhibition of the Ca^{2+}-ATPase by Ang II in the basolateral membranes of kidney proximal tubules cells (BLM). We have now investigated the enzymatic routs by which Ang II is converted to Ang-(3–4). Membrane-bound angiotensin converting enzyme, aminopeptidases and neprilysin were identified using fluorescent substrates. HPLC showed that Plummer's inhibitor but not Z–pro–prolinal blocks Ang II metabolism, suggesting that carboxypeptidase N catalyzes the conversion Ang II→ Ang-(1–7). Different combinations of bestatin, thiorphan, Plummer's inhibitor, Ang II and Ang-(1–5), and use of short proteolysis times, indicate that Ang-(1–7)→ Ang-(1–5)→ Ang-(1–4)→ Ang-(3–4) is a major route. When Ang III was combined with the same inhibitors, the following pathway was demonstrated: Ang III→ Ang IV→ Ang-(3–4). Ca^{2+}-ATPase assays with different Ang II concentrations and different peptidase inhibitors confirm the existence of these pathways in BLM and show that a prolyl-carboxypeptidase may be an alternative catalyst for converting Ang II to Ang-(1–7). Overall, we demonstrated that BLM have all the peptidase machinery required to produce Ang-(3–4) in the vicinity of the Ca^{2+}-ATPase, enabling a local RAS axis to effect rapid modulation of active Ca^{2+} fluxes.

1. Introduction

In a recent paper [1] we demonstrated that: (i) femtomolar Ang-(3–4) (Val^3-Tyr^4) completely abolishes the inhibition of basolateral membrane Ca^{2+}-ATPase by 100 pmol/L Ang II in kidney proximal tubule cells; (ii) Ang-(3–4) is formed when Ang II is incubated with a basolateral membrane-enriched fraction; (iii) Ang-(1–7) is an intermediate in the pathway that produces Ang-(3–4). In addition, we observed that inhibition of the Ca^{2+} pump is progressively cancelled if the initial Ang II concentration is increased to 10 nmol/L, and the activity remained at the control level in the presence of micromolar Ang II concentrations. The recovery of Ca^{2+}-ATPase is accompanied by metabolization of Ang II and generation of Ang-(3–4). These observations, especially those related to the plasma membrane Ca^{2+}-ATPase, indicate a novel physiological phenomenon, corroborating the view that the effect of Ang II is counteracted by small peptides derived from Ang II metabolism within the renin–angiotensin system (RAS) [2,3]. Importantly, physiological Ang II levels are high (~5 nmol/L) in the peritubular fluid of proximal tubules [4–7]. At this concentration, plasma membrane Ca^{2+}-ATPase activity is restored and the production of active Ang II metabolites appears to be favored [1].

The possible systemic relevance of Ang-(3–4) emerged more than ten years ago when Matsufuji, Saito and coworkers demonstrated its antihypertensive effect in spontaneously hypertensive rats [8,9]. More recently, Pentzien and Meisel [10] showed that this dipeptide is remarkably stable in human blood serum. With respect to possible intrarenal (local) effects, the influence of Ang-(3–4) on the basolateral membrane Ca^{2+}-ATPase suggests a significant role in transepithelial transport processes in the proximal tubule. Cytosolic Ca^{2+} fluctuations in renal cells — which are partly controlled by the plasma membrane Ca^{2+} pump [11] — have been implicated in the modulation of fluid reabsorption in this nephron segment [12]. Thus, Ang-(3–4) could be considered a local counter-regulator of Ang II action in fine-tuning cytosolic Ca^{2+} levels in proximal tubule cells.

Ang-(3–4) is formed from Ang I, Ang II and Ang-(1–7), as shown by previous investigations using three different preparations: a crude membrane fraction from kidney cortex, a cortex fraction enriched in apical membranes, and a preparation of isolated proximal tubules [13,14]. These studies identified several enzymes involved in proteolytic cascades leading to the formation of small RAS peptides. Angiotensin-converting enzyme (ACE) and ACE2 appeared to play a central role in

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these proteolytic pathways, evidence — at the tissue level — that they share complementary functions, as proposed previously [15–17].

The aim of the present work was to investigate possible pathways for the formation of Ang-(3–4) from Ang II and Ang III in basolateral membranes. An attempt was also made to study how inhibition of these peptidase-mediated routes may influence the recovery of the neighboring Ca^{2+}-ATPase activity when the Ang II concentration is high.

2. Materials and methods

2.1. Animal care

Animal care and the control of health in the sources of kidneys were as described in [1]. The study was approved by the local ethics committee (at the Federal University of Rio de Janeiro) in agreement with National Institutes of Health recommendations.

2.2. Materials

Buffers, bovine serum albumin, Ang II and Ang III were purchased from Sigma. Ang IV, Ang-(1–5), Ang-(3–7) and the Ang-(3–4) standards were synthesized by EZBiolab, and Percoll was from GE Healthcare. DX600 and Plummer's inhibitor were purchased from Phoenix Pharmaceutical Inc. and Calbiochem, respectively. Z–pro–prolinal, bestatin, thiorphan and PCMB were generous gifts from Dr. Luiz Juliano (Department of Biophysics, Federal University of São Paulo, Brazil). The specific fluorescence energy transfer substrates for ACE, ACE2 and neprilysin (NEP), viz. Abz-FRK(Dnp)P-OH, Abz-APK (Dnp)-OH and Abz-rGL-EDDnp, respectively, were synthesized in an automated solid-phase peptide synthesizer (Shimadzu) as previously described [18–20]. The fluorogenic substrate for aminopeptidases (AP), F-MCA, was a generous gift from Dr. Maria Aparecida Juliano (Department of Biophysics, Federal University of São Paulo, Brazil). The purity of the synthesized peptides was controlled by amino acid analysis and mass determination as described in [19]. The $^{32}P_i$ and [γ-^{32}P]ATP were obtained as in [1]. Acetonitrile and trifluoroacetic acid were from TEDIA Co. Inc. Distilled water, deionized using Milli-Q resins (Millipore Corp.), was used to prepare all solutions. All other reagents were of the highest purity available.

2.3. Membrane preparation

Basolateral membranes were isolated and purified from kidney proximal tubule cells as described in [1], with particular care to minimize intracellular membrane contamination [21,22]. The preparation contained about 40% unsealed membrane fragments [23], allowing free access of Ang II and its derived peptides, peptidase inhibitors and ATP to their corresponding sites. Protein concentration was measured with Folin-phenol reagent [24]. Since the preparation was devoid of cytosolic components after separation/purification, the metabolizing enzymes analyzed in this study were membrane-bound.

2.4. Measurement of ACE, ACE2, AP and NEP specific activities by hydrolysis of specific fluorescent substrates

The activities of ACE, ACE2, AP and NEP were measured using specific fluorescent substrates in the absence or presence of the inhibitors lisinopril (for ACE), bestatin (for AP) and thiorphan (for NEP). Since no ACE2 activity was detected, its inhibitor DX600 was not used in this group of experiments. Hydrolysis of substrates containing the fluorogenic groups MCA (4-methyl-coumaryl-7-amide) or Abz (ortho-amino benzoic acid) and the fluorescence suppressor group Dnp (dinitrophenyl) or EDDnp (*2,4*-dinitrophenyl-ethylendiamino) was monitored continuously at 37 °C by spectrofluorimetry (Hitachi). For assays using MCA-containing substrates, λ_{ex} and λ_{em} were 380 and 460 nm, respectively; for Abz-containing substrates, λ_{ex} and λ_{em} were 320 and 420 nm, respectively. The initial substrate concentrations indicated in the legend to Fig. 1 were determined by spectrophotometry using the molar extinction coefficient for Dnp ($\varepsilon = 17{,}300\ M^{-1}\ cm^{-1}$). The ACE, ACE2 and AP reaction media were buffered with 100 mmol/L Tris–HCl (pH 7.4) or 50 mmol/L bis-TRIS-propane (pH 9.0), both containing 50 mmol/L NaCl and 10 μmol/L $ZnCl_2$; NEP was measured in 50 mmol/L Tris–HCl (pH 7.4) or 50 mmol/L bis-TRIS-propane (pH 9.0), as indicated in the legend to Fig. 1. The membrane protein concentration in the assays varied as indicated in the legend and insets of Fig. 1.

2.5. Proteolysis assays and HPLC

Proteolysis of Ang II, Ang III, Ang IV, Ang-(1–5) and Ang-(3–7) was assayed in the presence of peptidase inhibitors as described in the corresponding figure legends. Before addition of Ang II or Ang II-related peptides (30 μmol/L), the membranes (1 mg/mL) were preincubated for 20 min at 37 °C in 250 mmol/L sucrose (at pH 7.4) with the different inhibitors to ensure that the peptidases were inactivated. Except when otherwise indicated (Fig. 4), proteolysis reactions were continued at the same temperature for 30 min. Since Ca^{2+}-ATPase activity in the presence of Ang II was measured at pH 9.0 ([1] and Fig. 11), Ang II metabolization was also studied in 20 mmol/L bis-TRIS-propane buffer (pH 9.0). All other experimental details and HPLC measurements were exactly as in [1]. Assays were undertaken at least three times and the elution profiles of the different peptides were reproducible. However, external conditions may influence HPLC results, so synthetic peptides were always used as standards to determine and compare the retention times of the products precisely in different sets of experiments.

2.6. Determination of basolateral plasma membrane Ca^{2+}-ATPase activity

Measurement of Ca^{2+}-ATPase activity in the presence of Ang II and peptidase inhibitors in the combinations and concentrations shown in Fig. 10 were as in [1]. Briefly, membranes (0.2 mg/mL final protein concentration) were preincubated for 30 min at 37 °C with a solution containing 250 mmol/L sucrose and 1 mmol/L ouabain — the latter to guarantee complete inhibition of (Na^++K^+)-ATPase activity — in the absence or presence of the peptidase inhibitors. The membrane suspension was then mixed with Ang II (100 pmol/L, 1 μmol/L or 10 μmol/L) and a 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+}). Assays were initiated by adding 5 mmol/L [γ-^{32}P]ATP ($\approx$1 Ci/mol), carried out at 37 °C and stopped after 20 min with activated charcoal [25]. The total $CaCl_2$ needed to achieve 20 μmol/L free Ca^{2+} was calculated as described by Sorenson and coworkers [26]. Ca^{2+}-ATPase was calculated as the difference between the total activity and that determined in the presence of 2 mmol/L EGTA.

2.7. Statistics

Ca^{2+}-ATPase activities were expressed as means ± SE. Differences between mean values in the different combinations and concentrations of Ang II and peptidase inhibitors were assessed by ANOVA, followed by Newman–Keuls analysis.

3. Results

3.1. Peptidases resident in basolateral membranes and Ang II metabolization

The following experiments (Fig. 1) show the activities of ACE, ACE2, AP and NEP, at pH 7.4 or pH 9.0, in basolateral membranes from kidney proximal tubule cells, being ACE activity independent of pH (Fig. 1A). The time course of hydrolysis of their respective specific

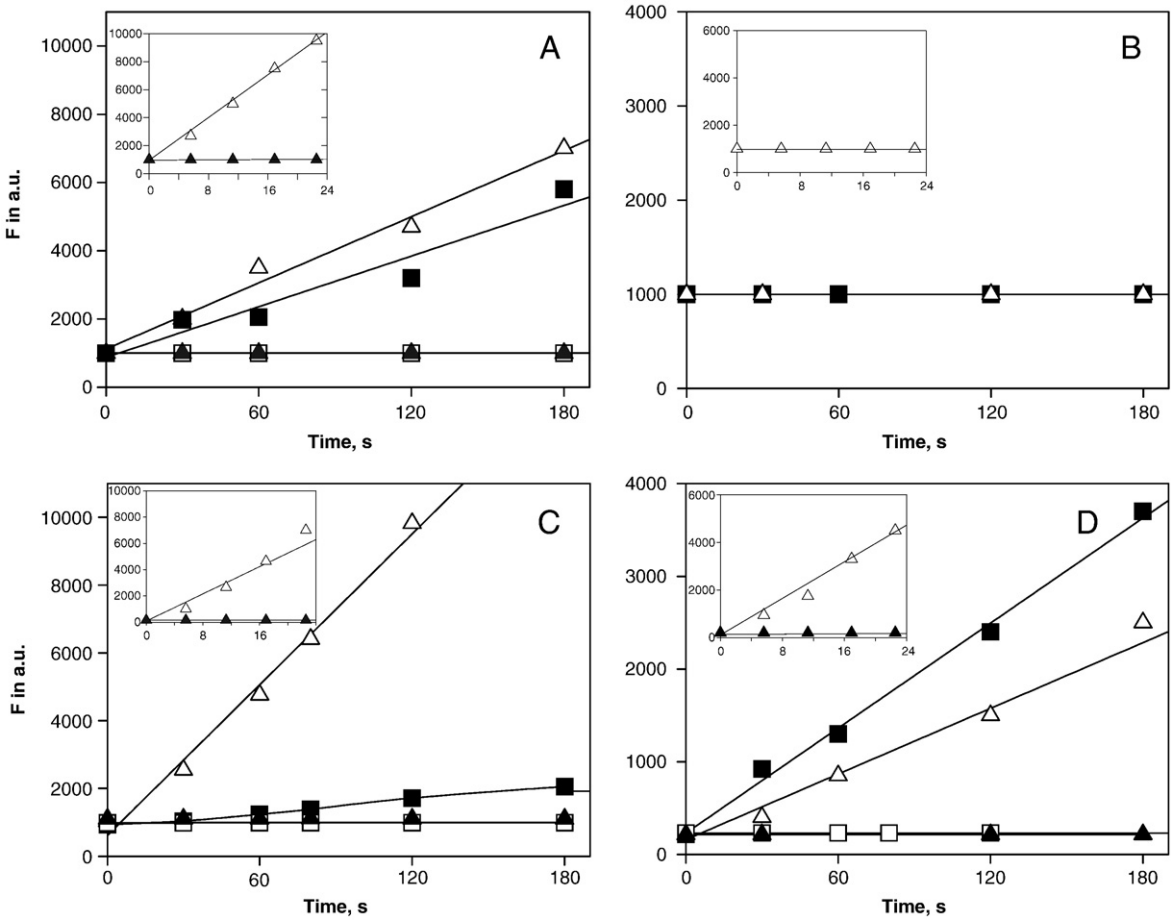

Fig. 1. Time course of peptidase activities in basolateral membranes from kidney proximal tubule cells. The activities of ACE (A), ACE2 (B), AP (C) and NEP (D) were assayed in the presence (10 μmol/L) of the fluorescent substrates Abz-FRK(Dnp)P-OH, Abz-APK(Dnp)-OH, F-MCA and Abz-rGL-EDDnp, as described under Materials and methods. Assays were carried out at pH 7.4 (Δ,▲) or pH 9.0 (□, ■), in the absence (■, Δ) or presence (□,▲) of their respective inhibitors: 2 μmol/L lysinopril (for ACE), 10 μmol/L bestatin (for AP) and 100 nmol/L thiorphan (for NEP). Membrane protein concentration was 16 μg/mL (ACE, ACE2 and NEP assays) and 30 μg/mL (AP assays). Since there was no detectable ACE2 activity, its inhibitor DX600 was not assayed. The data points show that the fluorescence intensity (in arbitrary units, a.u.) increases as long as hydrolysis of the substrates progresses. The insets show the dependence of hydrolysis on membrane protein concentration at pH 7.4 in the absence (Δ) or presence (▲) of inhibitors. The ordinate of the insets indicates fluorescence (in a.u.) and the abscissa protein concentration (in μg/mL).

fluorescence energy transfer substrates, Abz-FRK(Dnp)P-OH, Abz-APK-(Dnp)-OH, F-MCA and Abz-rGL-EDDnp [18–20] in saturating concentrations (10 μmol/L), was followed in the absence or presence of the specific inhibitors, except for ACE2, which was barely detectable (Fig. 1B). AP activity decreased (Fig. 1C) and NEP activity increased (Fig. 1D) when the pH was changed from 7.4 to 9.0. The insets show the dependence of hydrolytic activity on protein concentration. Fig. 2 shows that Ang II is metabolized to the same end-products, Tyr and Ang-(3–4), at pH 7.4 (Fig. 2A) and 9.0 (Fig. 2B).

3.2. ACE2 is not involved in the generation of Ang-(3–4) in basolateral membranes: participation of other carboxypeptidases

That ACE2 is not involved in generating Ang-(3–4) was confirmed by the experiment shown in Fig. 3A. When the membranes were incubated with Ang II and 1 μmol/L DX600 (an inhibitor of ACE2), Ang-(3–4) was still formed. In order to identify the enzyme(s) involved in the first step of Ang II proteolysis, Ang II was added to membrane suspensions previously preincubated with 20 nmol/L Plummer's inhibitor — considered a potent inhibitor of carboxypeptidase N (CPN) [27] (Fig. 3B) — or 100 nmol/L Z–pro–prolinal — a prolyl carboxypeptidase (PCP) inhibitor (Fig. 3C). Ang II was not metabolized in the presence of Plummer's inhibitor, but was still metabolized to Ang-(3–4) and Tyr when Z–pro–prolinal was used.

3.3. Ang II metabolization at short incubation times: characterization of intermediate products

Ang II is rapidly cleaved to Ang-(3–4), with Ang-(1–7) as a transient intermediate, and Ang-(1–7) can also form Ang-(3–4) [1]. To identify the peptide intermediates beyond Ang-(1–7), and thus the pathway by which Ang-(3–4) is formed, Ang II metabolization was assayed at 15 s, 1 min and 2 min. Fig. 4A,B,C shows its conversion to Ang-(3–4) and Tyr with Ang-(1–7), Ang-(1–5) and Ang-(1–4) as intermediates. The small peak of Ang-(1–7) at the shortest time (15 s) reflects the high turnover of ACE to form Ang-(1–5). Proteolysis of Ang-(1–7) at a short time (90 s) also yielded Ang-(1–5), Ang-(1–4), Ang-(3–4) and Tyr peaks (Fig. 4D), and further confirmation that Ang-(1–5) and Ang-(1–4) are downstream intermediates was obtained by incubating the membranes with Ang-(1–5) for 15 s and 3 min. Fig. 4E,F shows the rapid proteolysis of Ang-(1–5) and the gradual and sequential appearance of Ang-(1–4) and Ang-(3–4).

A small and not well resolved Ang III peak is seen at 1 and 2 min (Fig. 4B,C) and residual Ang III is detected by HPLC coupled to mass spectrometry after nearly complete Ang II hydrolysis [1], suggesting that the early step in which Asp^1 is removed could also lead to Ang-(3–4). As Ang III has already been shown to be a substrate for Ang-(3–4) formation in the plasma of spontaneously hypertensive rats (SHR) [9], it seems that there are at least two possible routes for Ang-(3–4)

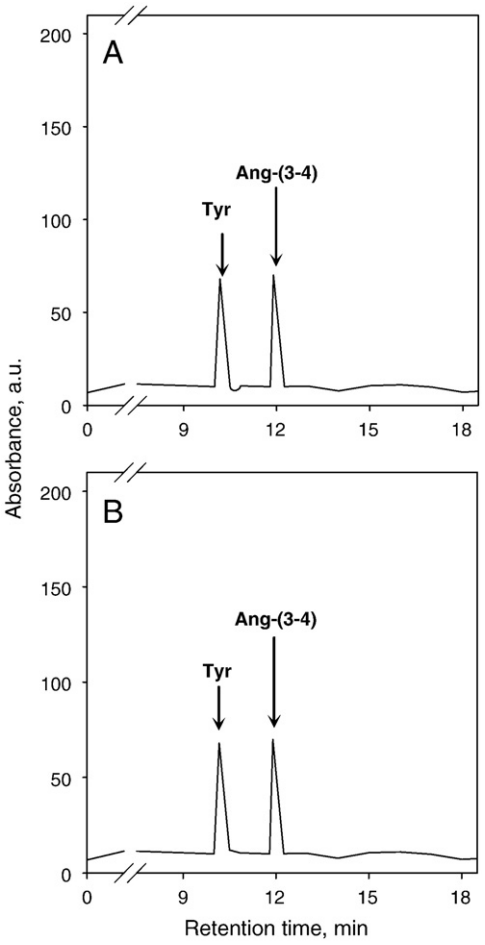

Fig. 2. Ang II is metabolized to Ang-(3–4) and Tyr in basolateral membranes at either pH 7.4 or 9.0. Basolateral membranes (1 mg/mL) were incubated with Ang II (30 µmol/L) for 30 min in a medium containing 250 mmol/L sucrose (pH 7.4) (A) or 20 mmol/L bis-TRIS-propane buffer (pH 9.0) (B). Other experimental details are given under Materials and methods and in [1]. The different proteolysis products are indicated with arrows.

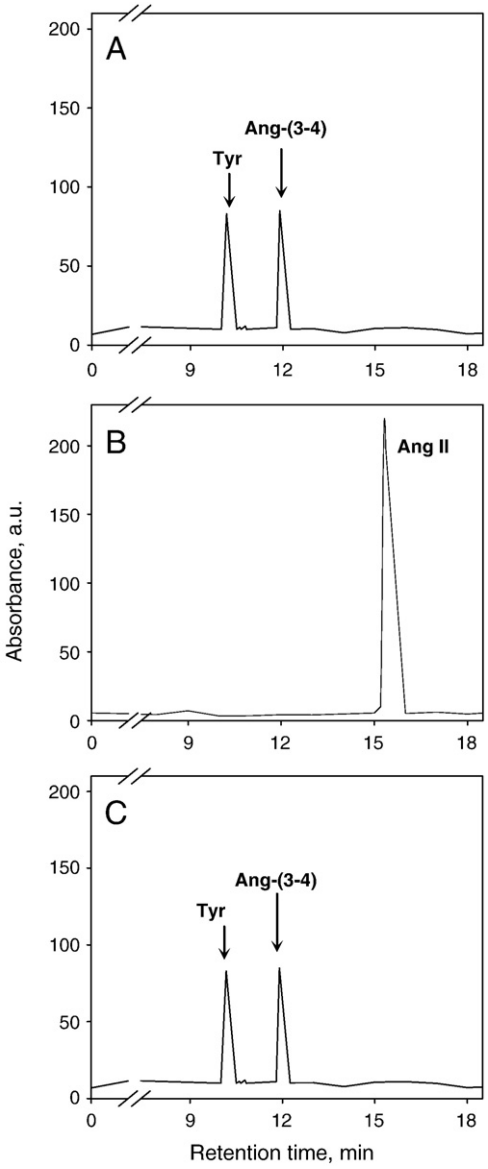

Fig. 3. Plummer's inhibitor, but not DX600 or Z—pro—prolinal, blocks Ang II hydrolysis. The basolateral membranes were preincubated for 20 min in the presence of 1 µmol/L DX600 (A), 20 nmol/L Plummer's inhibitor (B) or 100 nmol/L Z—pro—prolinal (C). After addition of Ang II, the chromatograms were obtained after 30 min incubation as described under Materials and methods and in [1].

formation from Ang II, one of which has Ang-(1–7) as intermediate while the other has Ang III (Fig. 5).

3.4. Investigating the steps catalyzed by aminopeptidases and carboxypeptidases downstream of Ang-(1–7)

Fig. 1C shows an AP activity in the basolateral membranes. Since 10 µmol/L bestatin, an inhibitor of AP, partially blocks the formation of Ang-(3–4) from Ang II (Fig. 6A), it is clear that this class of enzymes participates in some catalytic steps, as expected. Ang II was metabolized more slowly when a higher bestatin concentration was used (100 µmol/L) and the single Tyr peak was barely detectable (Fig. 6B). NEP — which was also detected in the membranes (Fig. 1D) — could collaborate in this pathway. This was confirmed by the experiment depicted in Fig. 7A, showing that thiorphan (a specific NEP inhibitor) partially blocked the conversion of Ang II to Ang-(3–4) and Tyr, indicating a downstream thiorphan-sensitive step in the cascade. Plummer's inhibitor also partially prevented the proteolysis of Ang-(1–5) (Fig. 7B).

3.5. Exploring the pathway through Ang III

The next experiments were undertaken to verify that Ang III can generate Ang-(3–4). Ang III was completely cleaved to Ang-(3–4) and Tyr when incubated with the membranes (Fig. 8A). Proteolysis of Ang III was partially prevented (Fig. 8B) by 100 µmol/L bestatin, and inhibition was less prominent when the bestatin concentration was lowered to 10 µmol/L (data not shown), evidence that AP is necessary for removing Arg^2 in the pathway depicted on the left side of Fig. 5. Proteolysis of Ang III was complete (i) in the presence of 20 (Fig. 8C) or 200 (not shown) nmol/L Plummer's inhibitor, and (ii) in the presence of 100 nmol/L thiorphan (Fig. 8D), so no CPN and NEP activities are apparently involved in Ang-(3–4) formation along this branch. Ang IV, the peptide succeeding Ang III, can generate Ang-(3–4) and Tyr (Fig. 8E); HPLC revealed a small peak of Ang-(3–7) and a residual amount of Ang IV (Fig. 8F) when Ang IV was hydrolyzed in the presence of thiorphan. Since Ang-(3–7) yields Ang-(3–4) and Tyr (data not shown), Ang-(3–7) could be the next intermediate in the cascade initiated by Ang III, arising from Ang IV [28]. However, this does not appear to be the only pathway because Fig. 9A shows that no Ang-(3–4) was formed from Ang-(3–7) in the presence of the thiorphan concentration employed in Fig. 8D. Also, 100 µmol/L *p*-chloro-mercury-benzoate (PCMB) — which would inhibit Phe^8 removal from Ang IV — did not impair Ang-(3–4) generation from Ang III (Fig. 9B).

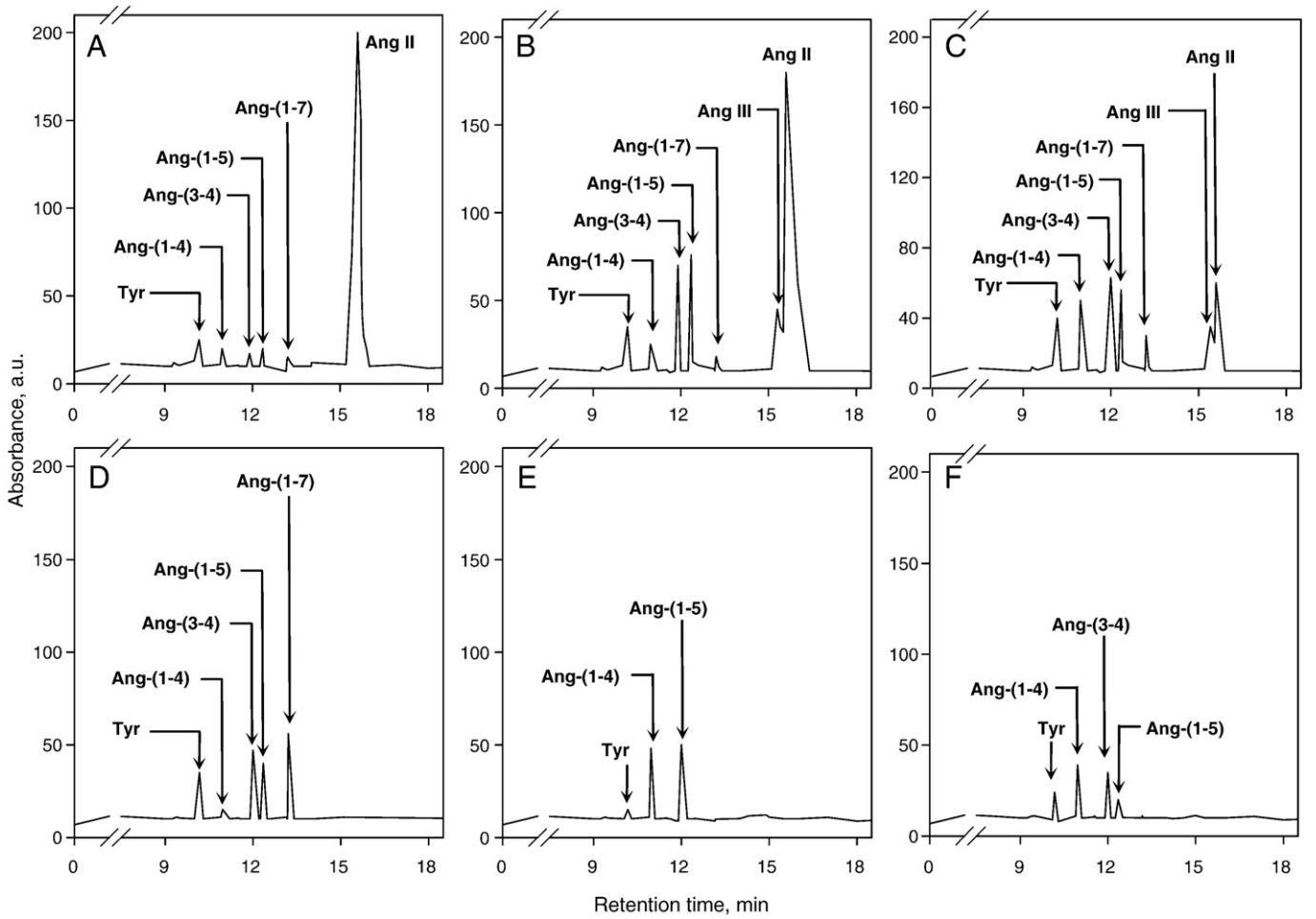

Fig. 4. Metabolization of Ang II, Ang-(1–7) an Ang-(1–5) during brief incubation with the membranes. Upper panels show progressive Ang II proteolysis after 15 s (A), 1 min (B) and 2 min (C). Lower panels show Ang-(1–7) proteolysis after 90 s (D); and progressive Ang-(1–5) proteolysis after 15 s (E) and 3 min (F). The synthetic standards, assayed as parallel controls without incubation with the membranes or incubated with previously denaturated membrane proteins (see [1]), allowed identification of the peaks.

3.6. Peptidase inhibitors prevent recovery of Ca^{2+}-ATPase at high Ang II concentrations, depending on the Ang II/inhibitor ratio

As shown recently [1], femtomolar Ang-(3–4) completely reverses the inhibition of basolateral membrane Ca^{2+}-ATPase by picomolar concentrations of Ang II; the same recovery starts at 1 nmol/L Ang II, is near complete when Ang II reaches 10 nmol/L and is maintained in the micromolar concentration range. To investigate whether this recovery is associated with the Ang II proteolysis and Ang-(3–4) formation seen in the preceding experiments, Ca^{2+}-ATPase was assayed in the presence of high concentrations of Ang II and different combinations of peptidase inhibitors. Fig. 10 shows that the Ang II/peptidase inhibitor ratio determines whether the recovery of Ca^{2+}-ATPase activity is blocked. When Ca^{2+}-ATPase was assayed in the presence of Plummer's inhibitor, the restoration of enzyme activity by a high Ang II concentration (1 µmol/L) and metabolization of the peptide were prevented (Fig. 3B). Adding 100 nmol/L of the PCP inhibitor Z–pro–prolinal to the assay medium in the presence of 1 µmol/L Ang II did not change the 50% inhibition of Ca^{2+}-ATPase. However, inhibition was overcome with 10 µmol/L Ang II, a condition in which the metabolites were formed (Fig. 3C). A similar profile was obtained when the medium was supplemented with bestatin to inhibit the AP-mediated steps of Ang II breakdown. Finally, using thiorphan — which partially inhibits the possibly NEP-mediated Ang-(1–4)→ Ang-(3–4) conversion — the expected recovery of Ca^{2+}-ATPase became evident, since Ang-(3–4) was still formed (Fig. 7A).

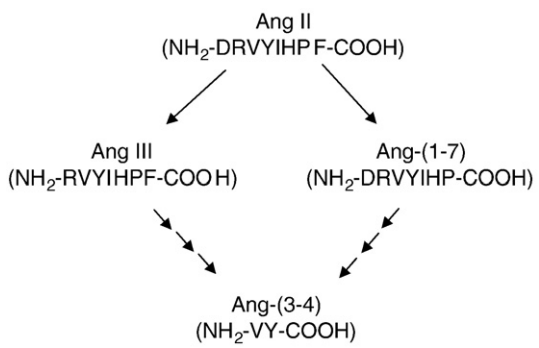

Fig. 5. Proposed main pathways for the formation of Ang-(3–4) in kidney basolateral membranes. The scheme shows pathways with Ang-(1–7) or Ang III as key intermediates (see text).

4. Discussion

4.1. Intrarenal formation of Ang-(3–4) from Ang II

In the present study we searched for membrane-associated peptidases that could supply the neighboring kidney plasma membrane Ca^{2+}-ATPase with Ang-(3–4), formed from the high concentrations of Ang II and Ang III in the peritubular fluid [4–7]. This biochemical network within the RAS could be implicated in the physiological reactivation of the Ca^{2+} pump after inhibition by

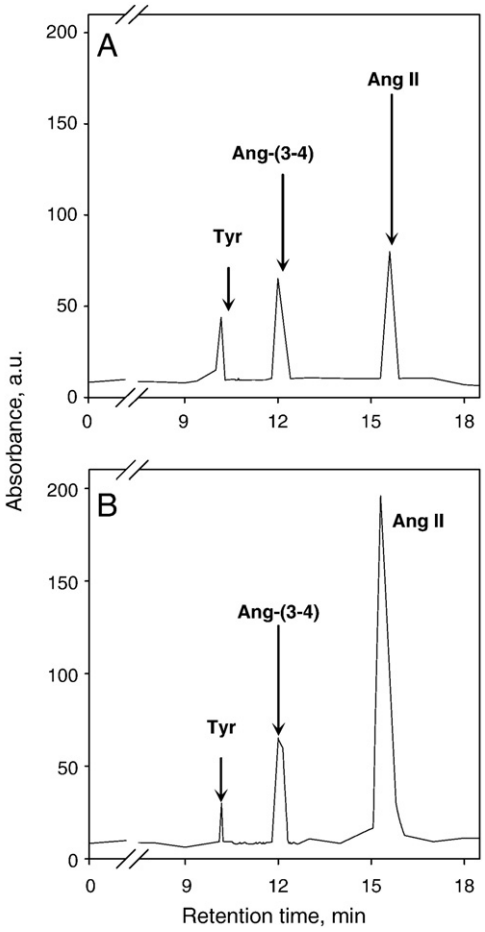

Fig. 6. Partial inhibition of Ang II hydrolysis by bestatin. The membranes were preincubated for 20 min in the presence of 10 (A) or 100 (B) μmol/L bestatin. After addition of Ang II to the membrane suspension, the chromatograms were obtained after an additional 30 min incubation.

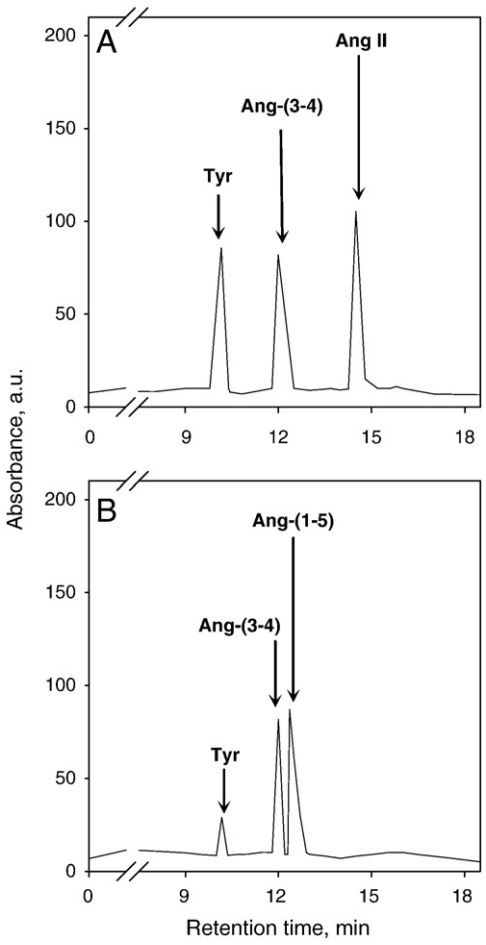

Fig. 7. Thiorphan and Plummer's inhibitor partially block the formation of Ang-(3–4) from Ang II and from Ang-(1–5), respectively. The membrane suspensions were preincubated for 20 min in the presence of 100 nmol/L thiorphan (A) or 20 nmol/L Plummer's inhibitor (B) and then supplied with 30 μmol/L of either Ang II (A) or Ang-(1–5) (B). The chromatograms were obtained after an additional 30 min incubation. The mixed synthetic Ang-(3–4) and Ang-(1–5) standards, assayed as parallel controls, allowed identification of the peaks.

picomolar Ang II [1,29], as well as other Ang II-modulated processes. The potential physiological role of Ang-(3–4) emerged more than ten years ago when its formation and its possible hypotensive effect were reported by Matsufuji and coworkers [9]. Evidence that it participates in different physiological processes has accumulated during the past decade [10,30–35]. The large amounts of Ang-(3–4) that accumulate in kidneys [32], its high potency in counteracting the inhibition of renal plasma membrane Ca^{2+}-ATPase by Ang II [1] and its unexpected resistance to proteolysis [10] support the view that it may be an important physiological modulator within the RAS axis in kidney tissue.

In principle, 13 peptidases could participate in the formation of various shorter peptides from Ang II [13,14,28,36]. Three peptidases were demonstrated in isolated basolateral membranes using specific fluorescent substrates (Fig. 1), and their participation in Ang II or Ang III metabolism was confirmed by HPLC after the peptides and peptidase inhibitors were incubated with the membranes (Figs. 2–4 and 6–8). There is a need for caution in comparing the activities shown in Fig. 1 — and especially in extrapolating them to cellular conditions — because the concentrations of the respective physiological substrates could be below or above saturation, and the K_m and k_{cat} values for synthetic and natural substrates are certainly different. However, the results of combining these specific substrates with their inhibitors allow us to conclude that the membranes have ACE > NEP > AP activities at both pH 7.4 and 9.0, with no detectable sign of ACE2 activity.

Taken as a whole, the data obtained in this work suggest three different routes in kidney basolateral membranes for producing Ang-(3–4) from Ang II, with Ang-(1–7) and Ang III as first intermediates (Fig. 11). For convenience, the right and middle pathways from Ang-(1–7) are denoted branches A and B, respectively. A connection between branch C and branch B leading towards the same end product, Ang-(3–4), is supported by the detection of Ang-(3–7) when Ang IV hydrolysis is retarded with thiorphan (Fig. 8F).

4.2. The Ang-(1–7) pathway

The first step in the production of Ang-(3–4) from Ang II in kidney basolateral membranes, the formation of Ang-(1–7), is ACE2-independent (Fig. 3A), since ACE2 is barely detectable (Fig. 1B). Kidney tubule ACE2 is confined to the apical aspect of the cells [37], where the proteolysis of filtered Ang II may be important for recovering the constituent amino acids. Other relevant roles can be attributed to ACE2 in other parts of the kidney; deletion of the ACE2 gene is associated with fibrillar collagen deposition in the glomeruli and the development of glomerulosclerosis, evidence that the generation of Ang II peptides by ACE2 is critically important for preserving renal structure and function [38].

Although ACE2 is important in Ang II metabolization in renal tubules, Ang-(1–7) and other small peptides are still formed when ACE2 is inhibited [14], suggesting that other CPs participate in the earlier step of

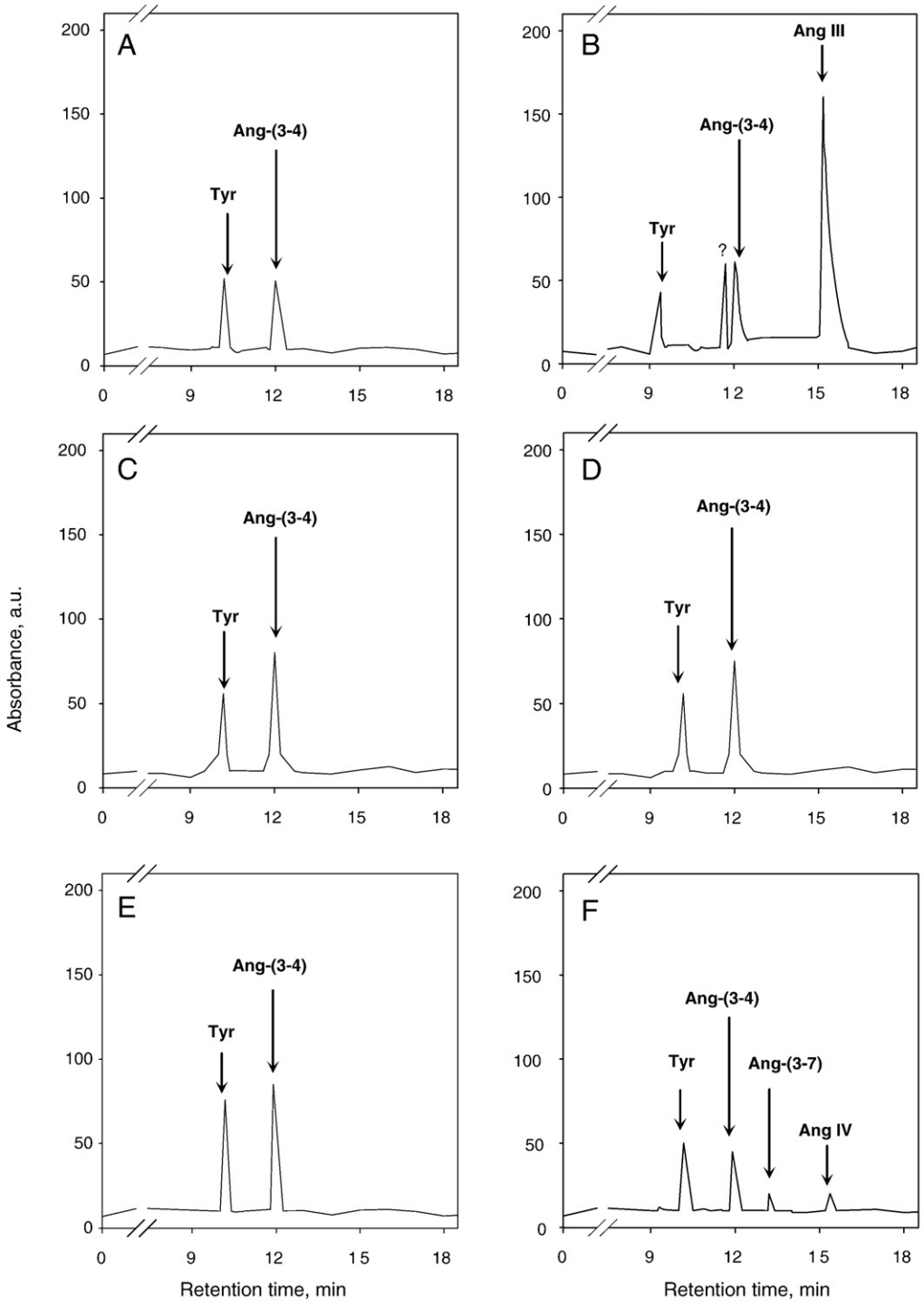

Fig. 8. Hydrolysis of Ang III and Ang IV by peptidases in kidney basolateral membranes. The membrane suspensions were preincubated for 20 min in the presence of 100 µmol/L bestatin (B), 20 nmol/L Plummer's inhibitor (C) or 100 nmol/L thiorphan (D, F); A,E: control of Ang III and Ang IV metabolization without inhibitors, respectively. They were then supplemented with 30 µmol/L Ang III or Ang IV and the chromatograms were obtained after a further 30 min incubation. In (B) the chromatograms repeatedly show an unidentified peak with a retention time of 11.7 min (indicated by "?"). This peak probably corresponds to a side-product of Ang III hydrolysis when some AP-catalyzed step is retarded by bestatin (see text). The synthetic Ang-(3–7) standard, assayed as a parallel control, allowed indentification at 13.1 min.

Ang II cleavage in basolateral membranes of proximal tubule cells. Here, the results of experiments on proteolysis and Ca^{2+}-ATPase activity using Plummer's inhibitor (Figs. 3B and 10) and Z—pro—prolinal (Figs. 3C and 10) indicate that a CPN and a PCP are involved. Plummer's reagent is considered to block CPN in nanomolar concentrations [27] similar to those used in the assays of Figs. 3B and 10. PCP has no apparent role in the conversion Ang II→ Ang-(1–7) because Z—pro—prolinal does not impair Ang-(3–4) formation from 30 µmol/L Ang II (Fig. 3C), but its participation is revealed because, with 1 µmol/L Ang II, the PCP inhibitor prevents the recovery of Ca^{2+}-ATPase (Fig. 10). It can therefore be concluded that a Plummer's-sensitive CP (probably a CPN) is a key enzyme in the hydrolysis of Ang II to Ang-(1–7), removing Phe^8 in the first step towards Ang-(3–4). This can be considered further evidence that an effective proteolytic cascade towards Ang-(3–4) goes through Ang-(1–7), as previously shown [1] and confirmed in Fig. 4A,B,C,D. PCP could be also important, as a redundant catalyst, when its activity is

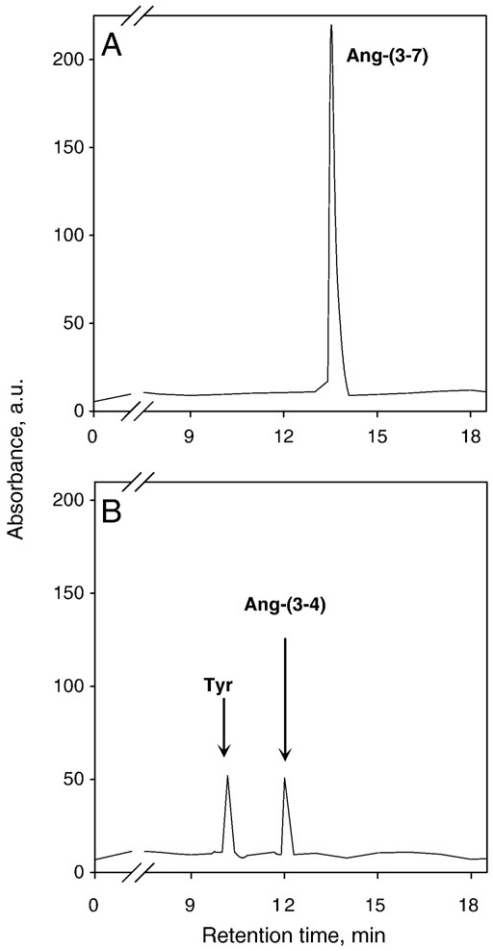

Fig. 9. Proteolysis assay in the presence of Ang-(3–7) plus thiorphan or Ang III plus PCMB. The membrane suspensions were preincubated for 20 min in 100 nmol/L thiorphan (A) or 100 μmol/L PCMB (B). They were then supplemented with 30 μmol/L of either Ang-(3–7) (A) or Ang III (B).The chromatograms were obtained after an additional incubation for 30 min.

enhanced by different stimuli in either physiological or pathophysiological conditions [36].

Besides the Plummer's-sensitive CP and the Z–pro–prolinal-sensitive PCP, branch A in Fig. 11 sequentially includes (i) ACE [1], (ii) other Plummer's-inhibited but less sensitive CP — which participates in the removal of Ile^5 during the conversion Ang-(1–5)→ Ang-(1–4) (Figs. 4E, F and 7B) — and possibly (iii) the combination of an aminopeptidase (Fig. 6) and neprilysin (Fig. 7A) in the final cleavage of the N-terminus to yield Ang-(3–4). From the measurements of Ang II, Ang-(1–7) and Ang-(1–5) proteolysis over short times (Fig. 4) together with the bestatin, thiorphan and Plummer's data (Figs. 6 and 7) and captopril data [1], the most probable intermediates in the right branch depicted in Fig. 5 are: Ang-(1–7)→ Ang-(1–5)→ Ang-(1–4)→ Ang-(3–4). It is remarkable that ACE-mediated formation of Ang-(1–5) from Ang-(1–7) (Fig. 4A,D) is a rapid reaction in several compartments including blood, renal tubules and pulmonary membranes [13,14,39]. Thus, the pathway found in kidney basolateral membranes (branch A, Fig. 11) could be a ubiquitous metabolic route for supplying systemic and local renin–angiotensin systems with Ang-(3–4). It is interesting to observe (Fig. 6) that the higher the bestatin concentration, and the higher the residual Ang II, the lower the Tyr peak, supporting the view that Ang II and also Ang-(1–7) [13] protect Ang-(3–4) against complete degradation to Val and Tyr [1] and contribute to its long tissue and systemic half life [10,32].

The sequential reaction A in Fig. 11 appears to be more important — at least *in vitro* — than that indicated as branch B because Ang-(3–4) formation from Ang-(1–7) is barely detectable in the presence of captopril, used to block the ACE-mediated conversion of Ang-(1–7) to Ang-(1–5) [1]. This is also supported by the observation that, at shorter times (Fig. 4), Ang II proteolysis yields the intermediates Ang-(1–5) and Ang-(1–4) (branch A) but not Ang-(2–7) or Ang-(3–7) (branch B). However, Ang-(3–7) may be an important source of Ang-(3–4) in basolateral membranes through a thiorphan-sensitive reaction (Fig. 9A), as long as it is provided from another source such as Ang IV. This point will be further discussed below.

4.3. The Ang III pathway

Ang III has long been implicated in the synthesis of Ang-(3–4) in plasma [9]. It is also a source of the dipeptide in renal proximal tubule

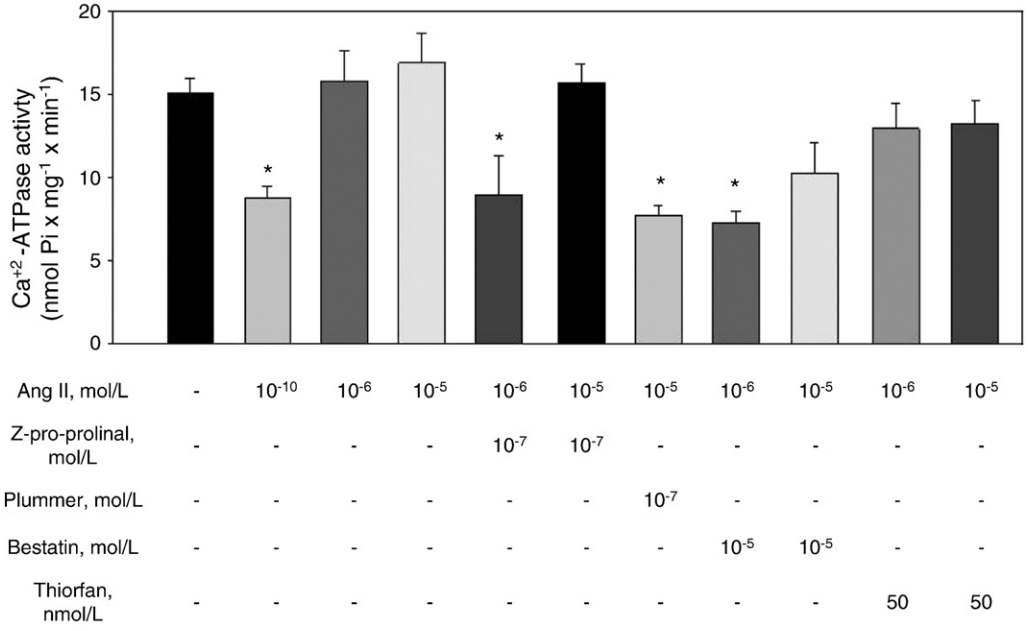

Fig. 10. Ca^{2+}-ATPase activity in the presence of Ang II and peptidase inhibitors. Ca^{2+}-ATPase activity was assayed as described under Materials and methods in the absence or presence of Ang II and peptidase inhibitors in the combinations and concentrations shown on the abscissa. Data bars indicate means ± SE of at least four triplicate determinations using different membrane preparations. *: statistically different from control with no additions ($p < 0.05$).

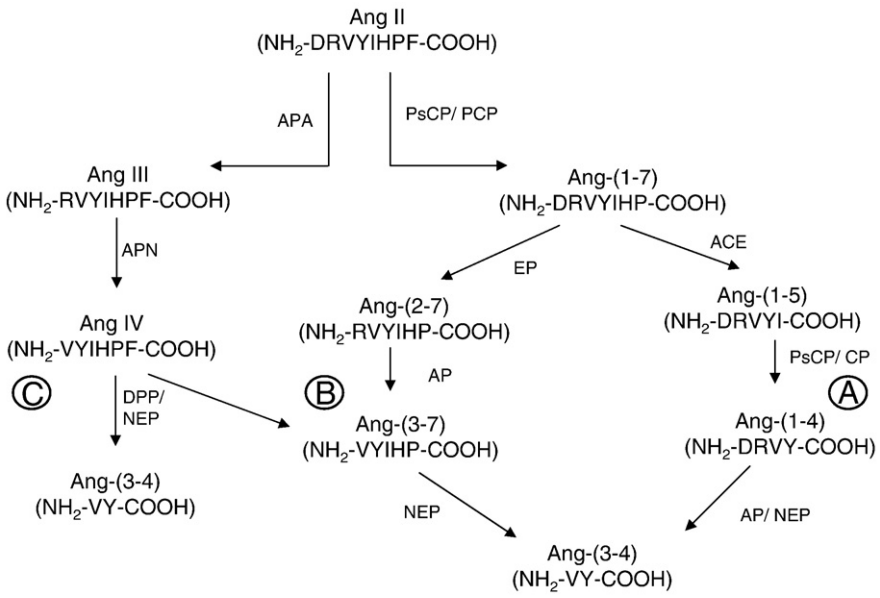

Fig. 11. Proposed pathways for Ang-(3–4) formation from Ang II in kidney basolateral membranes. The scheme shows the intermediates and enzymes proposed from the results in Figs. 1,3,4 and 6–9 (this paper) and in [1]. Peptidases and their abbreviations are: Plummer's sensitive carboxypeptidase (PsCP), prolyl carboxypeptidase (PCP), angiotensin-converting enzyme (ACE), carboxypeptidase (CP), aminopeptidase (AP), neprilysin (NEP), endopeptidase (EP), aminopeptidase A (APA), aminopeptidase N (APN), dipeptidyl aminopeptidase (DPP). The circled letters A, B and C are used throughout the text to denote the pathways that have Ang-(1–7) (A,B) or Ang III (C) as key intermediates after Ang II. Although the reactions could lead to the same end-product, Ang-(3–4), through parallel pathways, the scheme opens the possibility that the Ang-(1–7)- and Ang III-mediated pathways are linked by an Ang IV→ Ang-(3–7) conversion (see text and Fig. 8F).

cell basolateral membranes (Fig. 8A) via a partially bestatin-sensitive pathway (Fig. 8B), represented by branch C in Fig. 10. The sensitivity to bestatin demonstrates the AP-catalyzed steps in the sequential formation of Ang IV, as found in kidney cells [40], and finally Ang-(3–4) plus Ang-(5–8) (Fig. 8E), possibly through a step catalyzed by a bestatin-insensitive membrane-bound dipeptidyl peptidase [28,41,42] together with the thiorphan-sensitive NEP (Fig. 8F). In this work, an AP activity was also demonstrated in the membranes using the fluorescent synthetic AP substrate F-MCA (Fig. 1C), though this is not selective for any individual class of this family of enzymes. This AP activity could be APN, since it is especially abundant in kidney and Ang III is a preferred substrate [40]. The Ang III→ Ang IV transition also appears to have an important physiological role in modulating local Ang II effects in the kidney. Renal APN has been shown to participate in the inhibition of transepithelial Na^+ fluxes [40], so its participation in contra-regulation of the Ang II effects on fluid reabsorption [12] may be considered additional to those exerted by the peptidases of pathway A.

An intriguing result that challenges the importance of pathway C (Fig. 11) in the formation of Ang-(3–4) *via* Ang III in the kidney membranes is shown in Fig. 3B. Inhibition of the step Ang II→ Ang-(1–7) (branch A in Fig. 11) by Plummer's inhibitor did not give rise to detectable Ang-(3–4) *via* Ang III, although picomolar amounts of Ang III were detected by mass spectrometry after 30 min [1] and by HPLC after 1 and 2 min of Ang II metabolization (Fig. 4B,C). On the face of it, this observation might mean that Ang III is not a significant source of Ang-(3–4) in basolateral membranes, since they seem to have very low APA activity for converting Ang II→ Ang III [28]. APA is particularly abundant in the apical membranes of kidney cells [43] and has the same role as ACE2 in the proteolysis of Ang II that precedes the recovery of its amino acids from the ultrafiltrate. It may be proposed, however, that the interstitial fluid surrounding the external surface of the basolateral membrane is the source of Ang III [9] for the ensemble of aminopeptidases in branch C (Fig. 11), providing an alternative pathway for Ang-(3–4) formation. Thus, even if the Ang-(1–7)-mediated pathway is pharmacologically inhibited, Ang-(3–4) could still be formed in the vicinity of the plasma membrane Ca^{2+}-ATPase through a pathway that has Ang III as intermediate (Fig. 8D).

The unidentified peak with retention time 11.7 min when Ang III proteolysis is partially inhibited by bestatin (Fig. 8B) indicates that Ang III may be partially deviated to other pathways in the complex network of membrane-associated peptidases, as shown in different kidney tissue preparations [2,28]. Different Tyr-containing small peptides such as Ang-(3–5) are formed in minute amounts when Ang II is incubated with membranes from kidney tubules [14].

4.4. Is there a connection between the Ang-(1–7) and Ang III pathways?

Even though the Ang III and Ang-(1–7) pathways proceed in parallel towards the same product, Ang-(3–4), there seems to be communication between them. This is corroborated by the Ang-(3–7) peak in the chromatograms when Ang IV proteolysis is assayed in the presence of thiorphan (Fig. 8F), which abolishes the NEP-catalyzed cleavage of Ang-(3–7) (Fig. 9A). Indeed, the CPs that remove Phe^8 from Ang II can catalyze the same reaction with Ang IV as substrate.

4.5. Peptidases and modulation of Ca^{2+}-ATPase by Ang-(3–4)

Finally, the Ca^{2+}-ATPase data (Fig. 10) show the relevance of the key enzymes in Ang II metabolization to the regulation of active Ca^{2+} transport across basolateral membranes. In a previous study [1] it was shown that (i) Ang-(3–4) is a potent reactivator of the renal plasma membrane Ca^{2+}-ATPase after inhibition by picomolar Ang II concentrations, and (ii) reactivation of the pump at high Ang II concentrations occurs due to the substantial formation of Ang-(3–4). The central result in the present work concerning the involvement of peptidases in modulating the Ca^{2+} pump in kidney tubules is that in the presence of Plummer's inhibitor — which completely blocks the formation of Ang-(3–4) from micromolar Ang II (Fig. 3B) — the recovery of Ca^{2+}-ATPase activity is simultaneously blocked. With other inhibitors that partially impair Ang II metabolization, such as Z—pro—prolinal (Fig. 3C) or bestatin (Fig. 8B), the prevention of recovery depends on the Ang II/inhibitor ratio and, therefore, on the Ang II/Ang-(3–4) ratio, supporting the proposal that, at least in kidney tissue, a balance between Ang II and Ang-(3–4) is physiologically more important than absolute

Ang II levels [1]. Fig. 10 also shows that when peptidases such as APs or NEP are partially inhibited, Ca^{2+}-ATPase activity recovers and this recovery clearly depends on the Ang II/bestatin ratio and on the existence of alternatives for the NEP-mediated steps. Ultimately, this may also be possible owing to the high potency of Ang-(3–4) ($pA_{1/2} \approx$ 15.5) in counteracting the Ang II effects [1].

5. Conclusions

In summary, this work allows us to propose possible routes, involving peptidases, for producing Ang-(3–4), a potent reactivator of the plasma membrane Ca^{2+} pump inhibited by picomolar Ang II, in kidney proximal tubule cell basolateral membranes. Furthermore, this and the previous paper [1] reveal that Ang-(3–4) can act with very high affinity within the RAS axis through a mechanism involving Ang II receptors distinct from those reported for ACE inhibition [8–10,30] or in remodeling the vascular bed [32,33].

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