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Angiotensin-(3–4) counteracts the Angiotensin II inhibitory action on renal Ca^{2+}-ATPase through a cAMP/PKA pathway

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ABSTRACT

We recently demonstrated that Angiotensin-(3—4) [Ang-(3—4)], an Ang II-derived dipeptide, overcomes inhibition of plasma membrane Ca^{2+}-ATPase promoted by nanomolar concentrations of Ang II in basolateral membranes of renal proximal tubule cells, with involvement of a so far unknown AT_2R-dependent and NO-independent mechanism. The present study investigates the signaling pathway triggered by Ang-(3—4) that is responsible for counteracting the inhibitory effect of Ang II, and attempts to elucidate the functional interaction of the dipeptide with Ang II at the level of AT_2R. Stimulation by cholera toxin of $G_s\alpha$ protein structurally linked to AT_2R — as revealed by their co-immunoprecipitation — mimicked the effect of Ang-(3—4) on Ca^{2+}-ATPase activity. Furthermore, addition of dibutyril-cAMP (db-cAMP) mimicked Ang-(3—4), whereas the specific PKA inhibitor, $PKAi_{(5–24)}$ peptide, suppressed the counter-regulatory effect of Ang-(3—4) and the AT_2R agonist, CGP42112A. Membrane-associated PKA activity was stimulated by Ang-(3—4) or CGP42112A to comparable levels as db-cAMP, and the Ang-(3–4) effect was abrogated by the AT_2R antagonist PD123319, whereas the AT_1R antagonist Losartan had no effect. Ang-(3—4) stimulated PKA-mediated phosphorylation of Ca^{2+}-ATPase and activated PKA to comparable levels. Binding assays demonstrated that Ang-(3—4) could not displace ^{3}H-Ang II from HEK 293T cells expressing AT_2R, but 10^{-10} mol/L Ang-(3—4) resulted in the appearance of a probable higher-affinity site (picomolar range) for Ang II. The results presented herein demonstrate that Ang-(3—4), acting as an allosteric enhancer, suppresses Ang II-mediated inhibition of Ca^{2+}-ATPase through an AT_2R/cAMP/PKA pathway, after inducing conformational changes in AT_2R that results in generation of higher-affinity sites for Ang II.

1. Introduction

Recently, new components of the renin angiotensin system (RAS) have been described including bioactive shorter peptides derived from Angiotensin II (Ang II), such as Ang III, Ang IV and Ang-(1—7) (for review, see [1]). These metabolites exert their effects by interacting with the classic receptors described for Ang II, the AT_1 and AT_2 receptors (AT_1R and AT_2R), or acting through their own receptors, e.g. AT_4R and $AT_{(1—7)}R$ (MAS), which are specific for Ang IV and Ang-(1—7), respectively [2,3]. Actions that are similar or opposite to those generated upon binding of Ang II to its receptors can be observed, depending on the type of receptor being activated.

Recently, we found parallel pathways in the renal cortex that can generate a very short, potent Ang II-derived metabolite, Ang-(3—4) [4]. Importantly, this Ang II-derived peptide displays antihypertensive actions *in vivo* and *in vitro* [5,6]. Furthermore, we demonstrated [7] that Ang-(3—4) counteracts – with femtomolar affinity – the inhibition that 10^{-10} mol/L Ang II promotes — *via* PKC — on Ca^{2+}-ATPase resident in

Abbreviations: ACE, angiotensin converting enzyme; Ang II, angiotensin II; Ang-(1—7), angiotensin-(1—7); Ang-(3—4), Val^3—Tyr^4; AT_1R, type 1 angiotensin II receptors; AT_2R, type 2 angiotensin II receptors; $AT_{(1—7)}R$(MAS), angiotensin-(1—7) receptors; AT_4R, angiotensin IV receptors; BSA, bovine serum albumin; CGP4211A, (2S,3S)-2-[[(2S)-1-[(2S)-2-[[(2S)-6-[[(2S)-5-[bis(azanyl)-methylideneamino]-2-(phenylmethoxy-carbonylamino) pentanoyl]amino]-2-[[(2S)-3-(4-hydroxyphenyl)-2-(pyridin-3-ylcarbonylamino)-propanoyl] amino]-hexanoyl]-amino]-3-(1H-imidazol-5-yl)propanoyl]pyrrolidin-2-yl]carbonylamino]-3-methyl-pentanoic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; db-cAMP, dibutyril cyclic AMP; DMEM, Dulbecco's modified Eagle's medium; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); IBMX, 3-isobutyl-1-methylxanthine; losartan, 2-Butyl-4-chloro-1-[[2′(1H-tetrazol-5-yl)[1,1′-biphenyl]-4-yl]methyl]-1H-imidazole-5-methanol; PKA, cyclic AMP-dependent protein kinase; PKAi, the PKA inhibitor peptide $PKAi_{(5–24)}$; PKC, protein kinase C; PD123319, (6S)-1-[[4-(dimethylamino)-3-methylphenyl]methyl]-5-(2,2-diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, TRIS-buffered saline; TCA, trichloroacetic acid; TRIS, 2-amino-2-hydroxymethyl-propane-1,3,diol.

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the basolateral membranes of kidney proximal tubule cells [8]. As this ATPase is responsible for the fine tuning of cytosolic Ca^{2+}, and proximal reabsorption of fluid is modulated by the intracellular activity of this cation [9], the influence of Ang-(3—4) on transepithelial Ca^{2+} fluxes emerges as a novel physiological process for controlling the volume of body fluid compartments, and consequently, of arterial pressure. To date, the signaling cascade triggered by this potent dipeptide remains unknown.

We have demonstrated that cAMP-dependent protein kinase (PKA) activates the plasma membrane Ca^{2+}-ATPase in proximal tubules [10]. PKA activation antagonized the influence of PKC on renal $(Na^+ + K^+)$ATPase [11] and the recently cloned and purified [12] ouabain-insensitive Na^+-ATPase [13] through interacting with pathways that involve Ang II receptors. Therefore, we hypothesized that Ang-(3—4)-induced reactivation of the Ang II-inhibited renal Ca^{2+}-ATPase involves triggering of the PKA-signaling pathways in proximal tubule cells.

The present work investigated whether Ang-(3—4) modifies Ang II binding at the level of AT_2R in a renal cell line that only expresses this class of receptors, and if a PKA pathway downstream of AT_2R and PKA-mediated phosphorylation of renal Ca^{2+}-ATPase is associated with reactivation of the renal Ca^{2+}-ATPase that is inhibited by physiological Ang II concentrations.

2. Experimental procedures

2.1. Animal care

Santa Inês sheep were raised on ranches in Jequié (BA, Brazil) and Montes Claros (MG, Brazil) that are under supervision of the Brazilian Ministry of Agriculture. The health of the animals was certified by a licensed veterinarian from an authorized slaughterhouse. Sheep were used due to the fact that their kidneys produce abundant levels of Ca^{2+}-ATPase and because their kidneys are frequently discarded as wastage. This avoids use of experimental animals such as rats, which would be sacrificed for their kidneys only. This study was approved by the local ethics committee for the use of animals in biomedical assays (IBCCF No.104), which follows the recommendations of the National Institute of Health (NIH) guide for the care and use of laboratory animals.

2.2. Materials

Human embryonic kidney cells (HEK 293T) were purchased from American Type Culture Collection. ^{3}H-Ang II was obtained from GE Healthcare. Unlabeled Ang II, the AT_2R agonist CGP42112A, cholera toxin (CTX), dibutyril cyclic AMP (db-cAMP), histone H8 and the AT_2R antagonist PD123319 were bought from Sigma-Aldrich; the PKA inhibitor peptide (PKAi) was purchased from Calbiochem. Ang-(3—4) was synthesized by EZBiolab. Losartan was from Merck, Sharp & Dohme, and Protein A/G PLUS-agarose from Santa Cruz Biotechnology. The 5f10 antibody and phospho-(Ser/Thr) PKA substrate antibodies were purchased from Thermo Scientific and Cell Signaling Inc., respectively. The anti-AT_2R (anti-goat and anti-rabbit) and anti-$G_s\alpha$ protein antibodies were obtained from Santa Cruz Biotechnology and Calbiochem, respectively. ^{32}P-labeled orthophosphate ($^{32}P_i$) was purchased from the Brazilian Institute of Energy and Nuclear Research, and used to prepare [γ-^{32}P]ATP according to Maia and coworkers [14]. The radioactivity recovered in assays was quantified using a liquid scintillation counter.

2.3. Cell culture and transfections

HEK 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum, gentamycin sulfate (50 μg/ml) and glutamine (2 mmol/L) in a 5% CO_2/air atmosphere. Transient transfections of the cells were performed using the calcium phosphate precipitation method described by Gether and coworkers [15].

2.4. Binding assays

HEK 293T cells transfected with the AT_2R expression plasmid were transferred to 12-well culture plates (3×10^5 cells/well) 24 h before binding assays were carried out. One day after plating, cells were washed briefly with cold 25 mmol/L Tris–HCl buffer (pH 7.4) containing 140 mmol/L NaCl, 5 mmol/L $MgCl_2$ and 0.1% bovine serum albumin (BSA). Binding experiments were performed at 4 °C, and initiated by adding 4×10^{-12} mol/L ^{30}H-Ang II and increasing concentrations (10^{-11} to 10^{-6} mol/L) of non-radioactive Ang II or Ang-(3—4) as competitors. In one series of experiments, binding was initiated with ^{3}H-Ang II 4×10^{-12} mol/L and displacement with unlabeled Ang II was assayed in the presence of a fixed Ang-(3—4) concentration (10^{-10} mol/L). The binding buffer consisted of 25 mmol/L Tris–HCl (pH 7.4), 5 mmol/L $MgCl_2$, 0.1% BSA and 100 μg/ml bacitracin. The competition profiles and IC50 values were calculated based on one- or two-site competition profile analysis tools using GraphPad Prism 4.0 software.

2.5. Preparation of basolateral membranes from kidney proximal tubule cells

Membranes were isolated from the outer cortex (*cortex corticis*) of sheep kidneys [7]. More than 90% of the cell population in this region comprises proximal tubules [16]. The basolateral membrane-enriched fraction was isolated and purified using the Percoll gradient method [10,17]. Controls for enrichment with basolateral membranes and for minimal residual contamination with other intracellular membranes have been described elsewhere [17].

2.6. Plasma membrane Ca^{2+}-ATPase activity assays

Ca^{2+}-ATPase activity determinations and calculation of total $CaCl_2$ needed for the required free Ca^{2+} concentration were as described previously [7]. Briefly, membrane suspensions (0.2 mg/ml in 250 mmol/L sucrose) were preincubated for 30 min at 37 °C with 1 mmol/L ouabain and supplied with a 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 (resulting in 20 μmol/L free Ca^{2+}). Other additions are indicated in the corresponding figure legends. The plasma membrane Ca^{2+}-ATPase activity was calculated as the difference between the activities in the absence and presence of 2 mmol/L EGTA. The reaction was started by adding 5 mmol/L [γ-^{32}P]ATP (specific activity ~1 Ci/mol), and after 20 min at 37 °C, it was stopped by adding 2 vol of activated charcoal (in 0.1 mol/L HCl). The suspensions were centrifuged at 18,000 ×*g* and aliquots of the clear supernatants were counted using a liquid scintillation counter. Specific additions of other reagents were detailed in the corresponding figures or figure legends.

2.7. Determination of cAMP-dependent protein kinase activity

The PKA activity was measured through the incorporation of the γ-phosphoryl group of [γ-^{32}P]ATP into histone in the absence or presence of PKAi, a specific PKA inhibitor [10,18]. The basolateral membranes (0.7 mg/ml in 0.1 ml final volume) were added to medium containing 20 mmol/L HEPES-TRIS (pH 7.0), 4 mmol/L $MgCl_2$, 10 nmol/L 3-isobutyl-1-methylxanthine (IBMX) and 1.5 mg/ml histone H8, with or without 100 nmol/L PKAi. Other additions are indicated in the corresponding figure legends. After mixing, histone phosphorylation was initiated through the addition of 10 μmol/L [γ-^{32}P]ATP (10 Ci/mmol), carried out at 37 °C for 10 min and arrested by adding 0.1 ml trichloroacetic acid (TCA) (40%, w/v). After vigorous stirring, samples were filtered under vacuum (Millipore filters, 0.45 μm pore diameter). The filters were washed with 8 ml 20% TCA

and 9 ml 0.1 mol/L phosphate buffer (pH 7.0) to remove unused [γ-^{32}P] ATP, and counted using a liquid scintillation counter.

2.8. Immunoprecipitation, SDS-PAGE and immunodetection of the $AT_2R/G_s\alpha$ complex

Membranes (1 mg/ml) were solubilized in a sucrose solution containing 0.1% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) for 30 min at room temperature. Ten microliters of primary anti-goat polyclonal antibody of AT_2R was mixed with 1 ml membrane suspension and incubated at 4 °C for 1 h, supplemented with 20 μL of protein A/G PLUS-agarose and incubated overnight with gentle stirring. The samples were centrifuged at 1000 *g* for 5 min and the supernatant retained as a control for the immunoprecipitation procedure. The pellet was washed three times with Tris-buffered saline (TBS) before being heated in a water-bath to 100 °C for 4 min with 40 μL SDS-PAGE sample buffer. After final centrifugation at 16,100 *g* for 2 min, the supernatant was subjected to SDS-PAGE followed by Western blotting.

Sample proteins were separated using SDS-PAGE (10%) and transferred to nitrocellulose membranes at 350 mA. The membranes were incubated with 5% non-fat milk in TBS (pH 7.6) for 1 h to prevent non-specific binding and probed with rabbit polyclonal anti-$G_s\alpha$ antibody (1:1000 dilution) for 1 h at room temperature under gentle stirring, washed three times with TBS containing 0.1% Tween 20 (TBST), exposed to the secondary antibody, washed again and visualized using ECL™. For a control of the efficiency of immunoprecipitation, AT_2R was immunodetected using an anti-rabbit polyclonal antibody (1:500) after stripping of the nitrocellulose membrane. AT_2R and $G_s\alpha$ have a molecular mass close to that of the heavy chain of IgG. Therefore, the strategy was to immunoprecipitate the receptors using an anti-goat polyclonal antibody, followed by a rabbit $G_s\alpha$ or AT_2R polyclonal antibody for the Western blotting, to minimize the appearance of unspecific bands. A negative control (Western blotting without membrane proteins) was used for precise localization of the IgG bands.

2.9. PKA-mediated phosphorylation of Ca^{2+}-ATPase activity

Ca^{2+}-ATPase was phosphorylated and immunoprecipitated as follows: basolateral membranes (1 mg/ml) were incubated in a reaction medium (final volume 1 ml) containing 50 mmol/L bis-TRIS-propane buffer (pH 7.4), 0.5 mmol/L ouabain, 5 mmol/L $MgCl_2$, 10 mmol/L NaF, CaEGTA buffer (60 μmol/L Ca^{2+}) and 10 nmol/L IBMX, supplemented with 10^{-10} mol/L Ang-(3—4), 10^{-7} mol/L db-cAMP and 10^{-7} mol/L PKAi in the combinations detailed in the corresponding figure legend. Phosphorylation was initiated by adding a mixture of 120 mM KCl and 5 mmol/L unlabeled ATP. After 10 min at 37 °C, the reaction was stopped using 0.1% (w/v) CHAPS. The tubes were supplemented with 10 μL anti-Ca^{2+}-ATPase antibody (5f10 anti-mouse antibody; 1:500 dilution) and incubated for 1 h at 4 °C before 20 μL A/G PLUS-agarose was added. The mixtures were maintained under gentle agitation at 4 °C overnight, and centrifuged at 1000 *g* for 5 min at the same temperature. The sediments were washed three times using TBS, and the final pellets were resuspended in 40 μL SDS-PAGE sample buffer before being heated to 100 °C for 4 min. After centrifugation at 16,100 *g* for 2 min, the supernatant was subjected to SDS-PAGE followed by Western blotting.

Sample proteins were separated using SDS-PAGE (10%) and transferred to nitrocellulose membranes at 350 mA. The membranes were incubated with 5% non-fat milk in TBS (pH 7.6) for 1 h to prevent non-specific binding and probed with anti-Ca^{2+}-ATPase antibody 5f10 (1:500 dilution) overnight at 4 °C. The nitrocellulose membranes were washed five times for 3 min using TBS with 0.1% Tween 20, probed using anti-mouse secondary monoclonal antibody (1:2500 dilution) for 1 h and developed using ECL™. The membranes were stripped using 0.2 mol/L glycine (pH 2.2) for 1 h, incubated with polyclonal anti-rabbit phospho-(Ser/Thr) PKA substrate antibody (1:1000 dilution) and treated as above, with the exception that a secondary anti-rabbit polyclonal antibody was used (1:5000 dilution).

2.10. Statistical analysis

The data are presented as mean ± SE. Differences were analyzed using one-way ANOVA, followed by a Newman–Keuls post test. Differences were considered significant at $p<0.05$.

3. Results

3.1. Involvement of cAMP-mediated pathway in the recovery of the Ang II-inhibited Ca^{2+}-ATPase activity

During experiments where single concentrations of Ang-(3–4) and Ang II were assayed to demonstrate counteracting effects on Ca^{2+}-ATPase, the concentrations used were 10^{-14} mol/L and 10^{-10} mol/L, respectively. We crossed these concentrations with dose–response curves obtained previously and demonstrated: (i) 10^{-14} mol/L Ang-(3–4) completely reactivated the Ca^{2+}-ATPase inhibited by 10^{-10} mol/L Ang II [7]; and that (ii) the maximal inhibition of Ca^{2+}-ATPase is attained using 10^{-10} mol/L Ang II [8].

The experiments depicted in Fig. 1 investigated whether a cAMP-mediated pathway is involved in maintaining normal Ca^{2+}-ATPase activity in the presence of an inhibitory concentration (10^{-10} mol/L) of Ang II. Fig. 1A demonstrates that stimulation of the membrane-associated adenylyl cyclase by CTX mimicked the counteracting effect of Ang-(3—4) on the inhibition of Ca^{2+}-ATPase by Ang II, whereas the toxin alone had no effect. In addition, Fig. 1B demonstrates that addition of db-cAMP abrogated the inhibition of the Ca^{2+} pump by Ang II, and had no influence when used in isolation.

3.2. $G_s\alpha$ protein is associated with AT_2R in basolateral membranes

G_s proteins are linked with stimulation of CTX-stimulated cAMP-mediated pathways [19]. Therefore, we investigated a possible structural link between AT_2R and G_s proteins in basolateral membranes (probed against the α subunit). Fig. 2 demonstrates that $G_s\alpha$ protein (A) and AT_2R (C) are present in basolateral membranes, and more importantly, clearly demonstrates that almost all originally membranous $G_s\alpha$ are present in immunoprecipitates obtained using the antibody against AT_2R (B). A negative control of immunoprecipitation using an anti AT_2R antibody without basolateral membranes, followed by Western blotting for AT_2R, allowed visualization of the two IgG bands (heavy and light) (D), with molecular masses different from that seen for AT_2R (compare C and D).

3.3. Specific blockade of PKA impairs Ca^{2+}-ATPase reactivation by Ang-(3—4) or by the AT_2R agonist CGP42112A

Fig. 3 demonstrates that recovery of control of Ca^{2+}-ATPase activity by Ang-(3—4) or CGP42112A did not occur in the presence of the specific PKA inhibitor PKAi peptide, indicating that PKA participates downstream of AT_2R in the reactivation of the Ca^{2+}-ATPase inhibited by 10^{-10} mol/L Ang II.

3.4. Ang-(3—4) stimulates the membrane-associated PKA in basolateral membranes

The results presented in Fig. 4A demonstrate that Ang-(3—4) stimulated the PKA that is associated to basolateral membranes of proximal tubules [20] even in subfemtomolar concentrations; furthermore, 10^{-14} mol/L Ang-(3—4) stimulated PKA to the same extent as that obtained with db-cAMP (Fig. 4B).

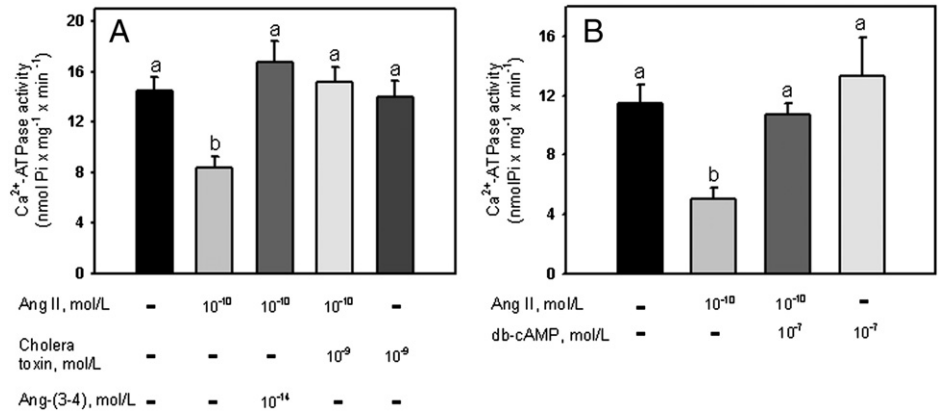

Fig. 1. Reactivation by Ang-(3—4) of the Ang II-inhibited renal Ca^{2+}-ATPase activity. (A) Ca^{2+}-ATPase activity was assayed as described in Experimental procedures in the presence of Ang II, Ang-(3—4) and CTX in the combinations and concentrations shown on the abscissa. Data are mean ± SE (n = 7). (B) Ca^{2+}-ATPase activity was assayed in the presence of Ang II and dibutyryl cyclic AMP (db-cAMP) in the combinations shown on the abscissa. Data are mean ± SE (n = 3). Different lowercase letters above bars indicate statistically different mean values ($p<0.05$).

3.5. AT_2R, but not AT_1R, is linked to the cAMP→PKA pathway that reactivates Ca^{2+}-ATPase in basolateral membranes

Additional evidence concerning the participation of AT_2R in reactivation by Ang-(3—4) of the Ang II-inhibited Ca^{2+}-ATPase *via* PKA is presented in Fig. 5A. First, the AT_2R antagonist PD123319 blocked stimulation of membrane-associated PKA activity by Ang-(3—4); second, the AT_2R agonist CGP42112A reproduced the effect of Ang-(3—4). Furthermore, losartan (a selective AT_1R antagonist) was unable to abolish activation of the kinase by the dipeptide (Fig. 5B).

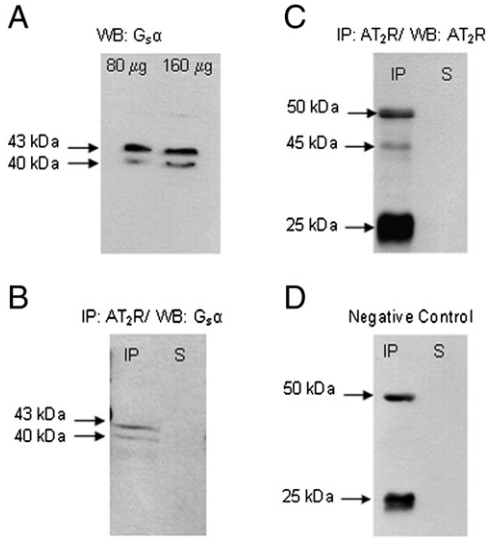

Fig. 2. $G_s\alpha$ protein is associated with AT_2R in the basolateral membranes of proximal tubule cells. Immunoprecipitation, SDS-PAGE and immunodetection were carried out as described in Experimental procedures. Representative immunodetections of 3 performed with different membrane preparations in the conditions described as follows. (A) Western blotting analysis in basolateral membranes (BLM) demonstrate the presence of $G_s\alpha$ (40–43 kDa). The gel was loaded with the amounts of membrane protein shown. (B) Western blotting of $G_s\alpha$ (40–43 kDa) using a rabbit polyclonal antibody after immunoprecipitation using a goat polyclonal antibody against AT_2R. IP: immunoprecipitate, S: supernatant. (C) Immunodection of AT_2R (45 kDa) using a rabbit polyclonal antibody after immunoprecipitation of AT_2R with the use of a goat polyclonal antibody against AT_2R. IP: immunoprecipitate, S: supernatant. (D) Negative control showing that there is no AT_2R band when the immunodetection procedure was carried out in the absence of basolateral membranes. The 25 and 50 kDa bands correspond to the IgG light and heavy chains, respectively.

3.6. Ang-(3—4) stimulates phosphorylation of renal Ca^{2+}-ATPase that is recognized by a phospho-(Ser/Thr) PKA antibody

Fig. 6 demonstrates that Ang-(3—4) stimulates basal phosphorylation of the Ca^{2+}-ATPase recognized by a specific antibody against small amino acid sequences in proteins phosphorylated by PKA. This hyperphosphorylation obtained with Ang-(3–4) is comparable to that observed in the presence of db-cAMP, and the increase in phosphorylation correlates with the stimulus of PKA activity *in vitro* (Fig. 4B). When PKAi is added, the effect of Ang-(3—4) on Ca^{2+}-ATPase phosphorylation is almost completely abrogated.

3.7. Binding of Ang-(3—4) to the AT_2R generates a probable higher-affinity site for Ang II

Despite the aforementioned evidence that Ang-(3—4) exerts its effects *via* AT_2R, the peptide could not displace ^{3}H-Ang II over a wide range of concentrations (Fig. 7A, □) in HEK 293T cells expressing AT_2R. Unlabeled Ang II exhibited a competition profile where the following equation was adjusted to the experimental points (■):

$$\%^3\text{H—Ang II bound} = \text{bottom} + \left\{(\text{top} - \text{bottom})/\left(1 + 10^{X\ -\ \log IC50}\right)\right\} \quad (1)$$

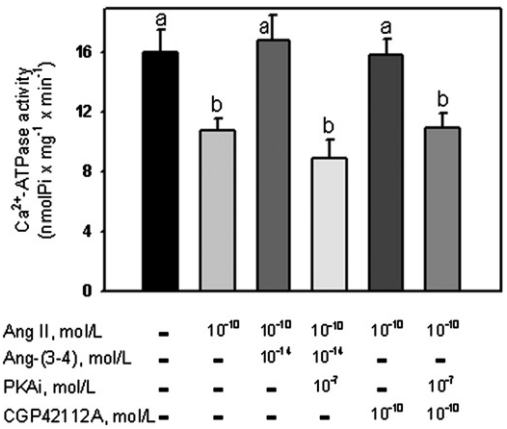

Fig. 3. The specific PKA inhibitor, PKAi peptide, suppresses the reactivating effect of Ang-(3—4) and the AT_2R agonist, CGP42112A, on the Ca^{2+}-ATPase inhibited by Ang II. Assays were carried out in the presence of Ang II, Ang-(3—4), PKAi and CGP42112A, in the combinations shown on the abscissa. Data are mean ± SE (n = 6). Different lowercase letters above bars indicate statistically different mean values ($p<0.05$).

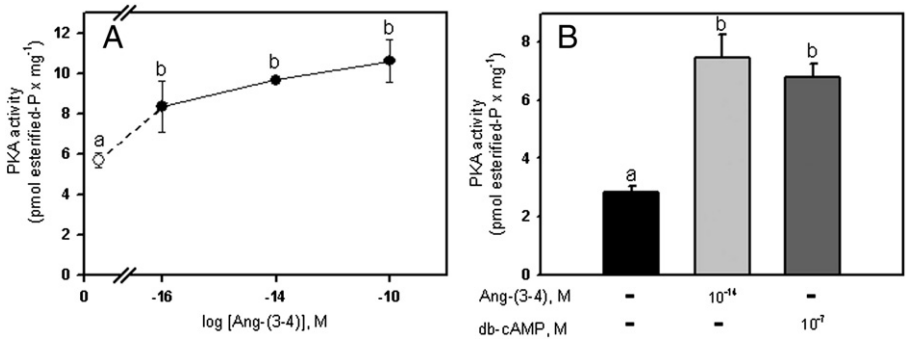

Fig. 4. Ang-(3−4) stimulates the membrane-associated PKA. PKA activity in basolateral membranes was assayed in the presence of the Ang-(3−4) (A) and db-cAMP concentrations (B) shown on the abscissae. Different lowercase letters above bars indicate statistically different mean values ($p<0.05$). Data are mean ± SE ($n=7$ and $n=5$ in A and B, respectively).

where "bottom" and "top" are the minimum and maximal values of the curve, "X" is the logarithm of the concentration of unlabeled Ang II, and "IC50" is the concentration of unlabeled Ang II that is effective for displacing 50% of the ^{3}H-Ang II initially bound.

When competition binding assays for Ang II were performed in the presence of a constant concentration (10^{-10} mol/L) of Ang-(3−4), we observed the appearance of a probable higher-affinity site for Ang II, with an IC50 approximately four orders of magnitude lower than in the absence of Ang-(3−4) (Fig. 7B). In this case, the competition profile was obtained using an equation derived for two classes of sites:

$$\%^{3}\mathrm{H-Ang\ II\ bound} = \mathrm{bottom} + (\mathrm{top} - \mathrm{bottom}) \times \left\{ \left[\mathrm{Fraction\ 1} / \left(1 + 10^{\mathrm{X - log\ IC50\ of\ fraction\ 1}} \right) \right] + \left[(1 - \mathrm{Fraction\ 1}) / \left(1 + 10^{\mathrm{X - log\ IC50\ of\ fraction\ 2}} \right) \right] \right\} \quad (2)$$

where "bottom", "top" and "X" have the definitions outlined above. "Fraction 1" corresponds to parcel of receptors with high affinity for Ang II, and "Fraction 2" corresponds to receptors (sites) with lower affinity for Ang II. The IC50 values for these two classes of sites are represented by Eq. (2).

The binding affinity data calculated from these equations are presented in Table 1.

4. Discussion

Signaling pathways coupled to AT_1R receptors are relatively well documented, whereas those linked to AT_2R remain poorly understood [21,22]. Herein, we present evidence that Ang-(3−4), in addition to its reported inhibition of the angiotensin converting enzyme (ACE) [5,23–25], can interact with AT_2R coupled to G_s proteins in the basolateral membranes of kidney proximal tubule cells. From a physiological point of view, the concentrations of Ang-(3−4) used during the present study are less than those present in human plasma and renal tissue, where the dipeptide can accumulate [26,27]. Downstream adenylyl cyclase and PKA activation occurs after interaction of Ang-(3−4) with AT_2R/G_s and culminates in the reactivation of the neighboring plasma membrane Ca^{2+}-ATPase inhibited by picomolar Ang II concentrations. Most important in terms of direct evidence concerning the interaction of Ang-(3−4) with the membrane-associated PKA is the stimulation – not previously described – of the kinase by subfemtomolar Ang-(3−4) concentrations (see Fig. 4). The results presented in Fig. 6 demonstrating stimulation of the regulatory phosphorylation of Ca^{2+}-ATPase, together with those showing involvement of AT_2R, provide further evidence that Ca^{2+}-ATPase is the final target of a reactivating pathway, $AT_2R \rightarrow G_s \rightarrow$adenylyl cyclase$\rightarrow$cAMP$\rightarrow$PKA, in which Ang-(3−4) is a primary messenger.

Isoform 1 of Ca^{2+}-ATPase, which predominates in renal proximal tubule cells and can be phosphorylated by PKA [10], possesses a specific site for this kinase, localized downstream of the calmodulin binding domain [28,29]. However, several isoforms of Ca^{2+}-ATPase have a threonine residue target for PKC in the pump sequence corresponding to the calmodulin binding domain [29]. It may be speculated that the inhibitory PKC-mediated phosphorylation of the latter in the presence of Ang II [8] is counteracted by the PKA-mediated stimulatory phosphorylation of the former site elicited by Ang-(3−4) (Figs. 4B and 6). If this were the case, opposite phosphorylations elicited by Ang II and Ang-(3−4) could act by modulating binding of the

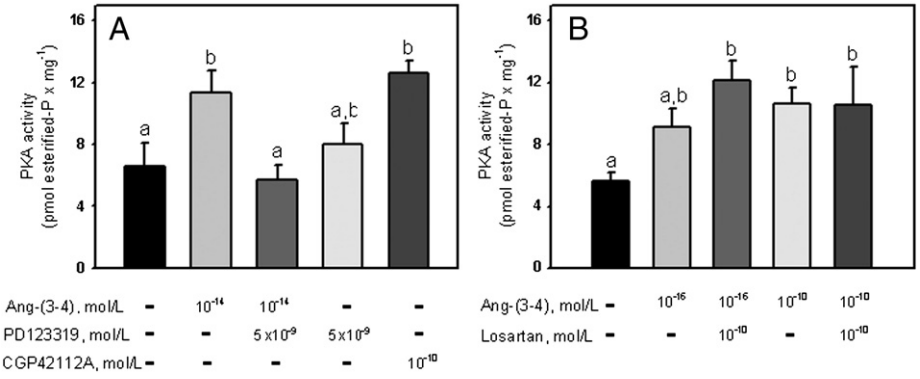

Fig. 5. PKA is functionally linked to AT_2R in basolateral membranes. (A) PKA activity in basolateral membranes was assayed in the presence of Ang-(3−4), the AT_2R antagonist, PD123319, and the AT_2R agonist, CGP42112A, in the combinations shown. Data are mean ± SE ($n=8$). (B) PKA activity in the presence of Ang-(3−4) and losartan, in the combinations shown. Assays were performed in the presence of Ang-(3−4) and losartan in the combinations shown. Data are mean ± SE ($n=5$). In both panels different lowercase letters above bars indicate statistically different mean values ($p<0.05$).

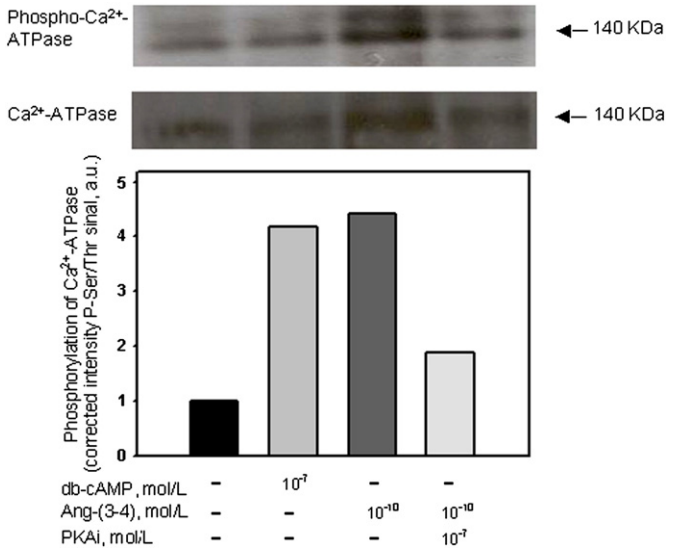

Fig. 6. PKA-mediated phosphorylation of Ca^{2+}-ATPase *in vitro*, using the combinations of db-cAMP, Ang-(3−4) and PKA inhibitor (PKAi) presented on the abscissa. Upper panel: phospho-immunosignal detected using the phospho-(Ser/Thr) PKA substrate antibody. Middle panel: immunodetection of Ca^{2+}-ATPase using the 5f10 antibody. Lower panel: quantification of the ratio between phospho-immunosignal and intensity of Ca^{2+}-ATPase immunodetection. The value obtained without additions was taken as a reference arbitrarily fixed in 1.

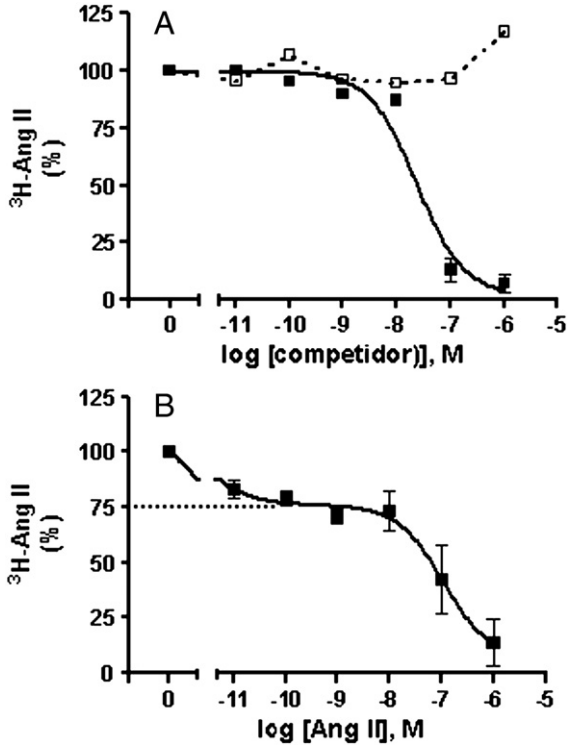

Fig. 7. Binding of Ang-(3−4) to AT_2R generates a probable high affinity site for Ang II. Binding assays were carried out in HEK 293T cells transfected with the AT_2R, as described in Experimental procedures, using ^{3}H-Ang II as the radioligand. (A) Competition binding assays in the presence of the increasing concentrations of unlabeled Ang II (■) or Ang-(3−4) (□) shown on the abscissa. (B) Competition binding assays in the presence of increasing concentrations of unlabeled Ang II and a constant concentration of Ang-(3−4) (10^{-10} mol/L). Note that the presence of Ang-(3−4) generates a probable high-affinity site for Ang II in the range of 10^{-12} mol/L. The competition profiles were generated using the software Prism GraphPad by non-linear regression equations fitting for 1 (A) or 2 (B) binding sites. The dashed line in A was traced by hand. The dotted line in B separates the populations of AT_2R with different affinities for Ang II. The data points are mean ± SE from 3 independent experiments performed in duplicate (n = 3).

Table 1
Competition binding assays in cells expressing the AT_2R.

Peptides	Ang II	Ang-(3−4)	Ang II + Ang-(3−4)
Cell line/receptor	IC_{50} ± SE	IC_{50} ± SE	IC_{50} ± SE
HEK-AT_2R	33.7 ± 9.1 nmol/L	NCP	2.9 ± 0.2 pmol/L 114.0 ± 5.2 nmol/L

Competition assays using HEK-AT_2R cells were performed with ^{3}H-Ang II in the presence of increasing concentrations of Ang II (Fig. 7A, ■), Ang-(3−4) (Fig. 7A, □) or Ang II plus a constant concentration of Ang-(3−4) (Fig. 7B, ■). Values are mean ± SE of 3 independent experiments performed in duplicate with cells from different cultures. NCP, no competition profile. The binding affinity data were obtained using Eqs. (1) and (2) (see text).

autoinhibitory domain to the catalytic domain of the pump, a role for phosphorylating processes that was proposed several years ago [30].

The physiological relevance of the coupled Ang-(3−4)→PKA→Ca^{2+}-ATPase regulatory mechanism in terms of homeostasis of liquid compartments emerges from the fact that cytosolic Ca^{2+} and intracellular Ca^{2+} sparks are finely controlled by the plasma membrane Ca^{2+} pump [30–33], and these oscillations regulate proximal fluid reabsorption [9]. Therefore, the effect of Ang-(3−4) *via* AT_2R→G_s→adenylyl cyclase→PKA can be considered another pathway in the Ang II-mediated Ca^{2+} signaling triggered as a result of Ang II binding to AT_1R [34], and/or from Ang II direct interaction with ACE [35].

Evidence confirming the relationship between AT_2R, Ang-(3−4) and the PKA cascade in counteracting the effect of Ang II was obtained from experiments carried out in the presence of PKAi, the specific blocker of the α-catalytic subunit of PKA [10,18]. The inhibitor impairs reactivation of the Ang II-inhibited Ca^{2+} pump by either Ang-(3−4) or the AT_2R agonist, CGP42112A (Fig. 3). This was supported by the finding that the AT_2R agonist stimulates PKA to a comparable extent as cAMP and Ang-(3−4). The close structural relationship of AT_2R/G_s, demonstrated by complete coprecipitation of the $G_s\alpha$ protein and the receptor, could be an essential feature guaranteeing their tight functional coupling in proximal tubule cells. AT_2R and G_s participate in downregulation of the ouabain-resistant Na^+-ATPase activity by Ang-(1−7) [13]. Therefore, diverse peptides of the RAS family may interact to regulate active ion translocation in proximal tubules *via* AT_2R in addition to AT_1R [9,20]. The combined targeting of active transporters by locally-formed Ang II-derived peptide may represent a sensitive method of ensuring a perfect paracrine adjustment of ion reabsorption and fluid homeostasis of body fluids by Ang II [36].

The importance of PKC and PKA-catalyzed phosphorylations in Ang II-mediated regulatory pathways that impact fluid reabsorption across the proximal epithelium has been confirmed recently through phosphoproteomic analysis [37]. Of note, cAMP and Ang-(3−4) reverse the inhibition of Ca^{2+}-ATPase by Ang II without any effect when assayed alone, despite their stimulatory influence on the membrane-associated PKA activity (Figs. 4 and 5) and in the phosphorylation of the Ca^{2+}-ATPase molecule itself (Fig. 6). Therefore, as aforementioned, it is plausible that Ang II-triggered PKC-mediated inhibitory phosphorylation activates long-range intramolecular communication [38] within the Ca^{2+} pump, promoting conformational changes at sites phosphorylated by PKA. As a result, interaction of the autoinhibitory domain and catalytic domain could decrease [30] when reactivation of the pump was physiologically required.

Finally, a pivotal question that emerges from such results concerns the type of interaction that Ang-(3−4) plays in the Ang II-AT_2R axis. In experimental conditions where Ang-(3−4) coexists with Ang II, there is dissociation of AT_1R/AT_2R heterodimers [7], and this seems to be an obligatory requirement to overcome the doubly losartan- and PD123329-sensitive inhibition of plasma membrane Ca^{2+}-ATPase promoted by 10^{-10} mol/L Ang II [8]. The appearance of a

second binding site, with affinity for Ang II in the 10^{-12} mol/L range (IC50 2.9 pmol/L), *i.e.* four orders of magnitude higher than that observed without Ang-(3–4) (IC50 33.7 nmol/L), is accompanied of a moderate reduction in affinity of the latter site to 114.0 nmol/L (Table 1). Such alterations in affinity and in the competition profile for Ang II are probably representative of a subpopulation of AT_2R with altered conformation due to the allosteric interference of Ang-(3—4) binding, consequently responsible for complete reversing of the Ang II-induced inhibition of the Ca^{2+} pump [7]. Therefore, Ang-(3—4) would act as an allosteric modulator when bound to the AT_2R. Therefore, Ang-(3—4) would not directly compete with Ang II, but would be responsible for inducing conformational changes in the receptors, resulting in modification of the Ang II binding profile. Allosteric modulators can modify affinity of the primary agonist for G protein-coupled receptors either when in their monomeric [39] or dimeric states [40]. Therefore, in native proximal tubule membranes, a similar behavior of conformational modification due to the allosteric interference of Ang-(3—4) could occur with AT_2R/AT_1R heterodimers [7]. We speculate that this is a key event linked to the counter-regulatory action of Ang-(3—4). This event would correspond to the structural modifications induced by Ang-(3—4) in receptors that functionally couple a primed, but silent, AT_2R/G_s complex linked to a downstream PKA pathway.

5. Conclusions

In conclusion, this study has revealed new partners of AT_2R in renal cells: the components of the cAMP-stimulated kinase pathway resident in basolateral membranes and Ang-(3—4), the latter acting as an allosteric modulator [40]. This expands our understanding concerning AT_2R functionality and its association with other receptors that participate in renal ion and fluid handling.

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