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Enhancement of phosphatidylcholine biosynthesis by angiotensin-(1–7) in the rat renal cortex

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Abstract

In the present paper, we investigated the effect of angiotensin-(1–7) (Ang-(1–7)) on phospholipid biosynthesis in the rat renal cortex. A significant increase in phosphatidylcholine (PC) labeling was observed when cortical slices, prelabeled with [^{32}P]orthophosphate, were incubated for 30 min in the presence of Ang-(1–7) (1 pM to 100 nM). Neither the phospholipase C inhibitors, neomycin or db-cAMP nor the protein kinase C inhibitors, chelerythrine or H7, modified the stimulatory effect induced by 0.1 nM Ang-(1–7). The enhancement of PC biosynthesis caused by 0.1 nM Ang-(1–7) was unmodified by either losartan, an AT_1 receptor antagonist, or (1-[[4-(dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazol[4,5-*c*]pyridine-6-carboxylic acid ditrifluoroacetate) (PD 123319), an AT_2 receptor antagonist, but was partially blocked by [D-Ala^7]Ang-(1–7), an Ang-(1–7) specific antagonist. However, losartan potentiated the effect of 100 nM Ang-(1–7) on PC biosynthesis. Losartan by itself increased the *de novo* synthesis of PC. These results suggest that the Ang-(1–7)-mediated increase in PC biosynthesis is independent of AT_1 and AT_2 receptor activation but mediated by a specific Ang-(1–7) receptor. This mechanism is independent of phospholipase C and PKC activation.

Keywords: Angiotensin-(1–7); Phosphatidylcholine; Renal cortex; *De novo* synthesis; Losartan

1. Introduction

It is well known that the renin–angiotensin system has an important role in cardiovascular physiology, fluid homeostasis, and cell function. Angiotensin (Ang) II has been considered the main product of an endocrine system involved in the pathogenesis of hypertension and renal disfunction [1]. Previous studies have demonstrated that Ang-(1–7), a biological end product of the renin–angiotensin system, may either contribute to or oppose the pressor and proliferative actions of Ang II [2,3].

Ang-(1–7) is generated endogenously from both Ang I and Ang II through an independent angiotensin-converting enzyme pathway, which requires the action of tissue-specific endopeptidases [2,4]. Ang-(1–7) concentrations are 6-fold higher in kidney than in plasma, suggesting its intrarenal production [5]. Increased levels of Ang I or treatments with angiotensin-converting enzyme inhibitors enhance Ang-(1–7) production, while reduced levels of the heptapeptide were reported in individuals with untreated essential hypertension [4,6].

Consistent with its site of synthesis, Ang-(1–7) displays an important role in rat renal homeostasis. In contrast to Ang II, Ang-(1–7) increases electrolyte and water excretion with no effect on renovascular resistance [7]. An association between the natriuretic effect of Ang-(1–7) and prostaglandin I_2 release was demonstrated by Hilchey and Bell-Quilley [8]. It was also shown that renal Ang-(1–7) acts in an endocrine fashion to regulate sodium transport by activating phospholipase A_2 [9]. Studies in rabbit aortic smooth muscle cells have shown that both Ang-(1–7) and Ang II promote arachidonic acid release from tissue lipids

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Abbreviations: Ang, angiotensin; db-cAMP, dibutyryl-cyclic AMP; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PKC, protein kinase C; PS, phosphatidylserine; PTA, phosphatidic acid

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by activating phospholipase A_2 via angiotensin receptors [10]. As a consequence of the action of phospholipases on phospholipid catabolism, the *de novo* synthesis of phospholipids is activated to preserve membrane homeostasis [11,12].

We have found that among rat renal zones, the cortex has the lowest phospholipid turnover, reflected by a low phospholipid metabolism [12,13]. Taking into account that Ang-(1–7) (a) is the major product of Ang I metabolism in human and dog kidneys [14], (b) is important in renal function, and (c) induces the activation of phospholipase A_2 [9,10], which is a degradative step that precedes the reconstitution of phospholipids, the aim of the present study was to assess the effect of Ang-(1–7) on the *de novo* synthesis of phospholipids in the rat renal cortex.

2. Materials and methods

2.1. Materials

Carrier-free [^{32}P]orthophosphate (sp. act. 25 mCi/mmol) was obtained from New England Nuclear, and X-ray film for autoradiography from Eastman Kodak. HPTLC silica gel plates, neomycin (Neo), db-cAMP, and chelerythrine (Che) were purchased from the Sigma Chemical Co. All other reagents and chemicals were of analytical grade (Merck or Mallinckrodt) and purchased from local suppliers.

2.2. Synthesis of Ang-(1–7) and [D-Ala^7]Ang-(1–7)

The Merrifield solid-phase procedure [15] was used with Boc-amino acid derivatives. The crude peptide was purified and characterized as a single component by HPLC. It showed the correct amino acid composition and sequence. The peptide purity was confirmed by matrix-assisted laser desorption mass spectrometry.

2.3. Isolation of rat renal cortex

Male Wistar rats (body weight: 250–270 g) were decapitated, and both kidneys were immediately removed and placed in ice-cold 10 mM Tris–HCl, pH 7.4, containing 5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgSO_4$, 140 mM NaCl, and 5.5 mM glucose (incubation buffer). After decapsulation, the kidney was cut along its longitudinal axis, the cortex was dissected, and 0.5-mm thick slices were obtained with a Stadie–Riggs microtome.

2.4. Phospholipid biosynthesis assay

A phospholipid biosynthesis assay was carried out as previously described [12]. Briefly, slices of rat renal cortex (5 mg wet weight) were incubated for 60 min in 200 μL of incubation buffer at 37° with 14 μCi [^{32}P]orthophosphate. Then Ang-(1–7) was added, and the cortical slices were incubated for an additional 30 min. The reaction was stopped on ice by adding 2 mL of chloroform:methanol (2:1, v/v), and tissue samples were homogenized in glass tubes with a Teflon pestle at 1000–1500 g. Phases were separated by adding 0.6 mL of chloroform and 0.6 mL of water; the chloroform phase containing the lipids was removed and dried at 25° under a nitrogen stream. This extraction procedure ensures an $85 \pm 9\%$ lipid recovery.

Lipid extracts were redissolved in chloroform and applied onto HPTLC plates precoated with silica gel G. Phospholipids were separated by one-dimensional two-solvent system chromatography. The first solvent system used was chloroform:methanol:acetic acid:water (40:10:10:1, by vol.); the second solvent system was chloroform:methanol:acetic acid:water (120:46:19:3, by vol.). R_f values were 0.20, 0.30, 0.47, 0.55, 0.70, and 0.88 for sphingomyelin, PC, PI, PS, PE, and PTA, respectively. Phospholipid fractions were detected with iodine vapors, and radioactivity incorporated into each phospholipid was visualized by autoradiography. Plate zones corresponding to PC and PI fractions were scraped off and quantified by liquid scintillation counting.

2.5. Statistical analysis

Data were analyzed by Student's *t*-test. Statistical analysis was performed with SigmaStat (Jandel Scientific) and the Instat (GraphPad Software Inc.) programs. *P* values lower than 0.05 were considered significant.

3. Results

3.1. Phospholipid biosynthesis in the rat renal cortex

To examine phospholipid biosynthesis, slices of rat renal cortex were incubated with [^{32}P]orthophosphate for 60 min. Steady-state equilibrium was reached at 45 min with no changes beyond this time, up to 120 min (data not shown). At 60 min most of the radioactivity was found associated with PC ($62.5 \pm 2.7\%$), followed by PI ($12.9 \pm 0.9\%$) and then PE ($7.0 \pm 0.6\%$), while no radioactivity was incorporated into PS. In terms of specific activity, the highest value corresponded to PC, followed by PI and then by PE (data not shown). No changes in phospholipid content were observed during the experimental period.

3.2. Effect of Ang-(1–7) on PC biosynthesis in the rat renal cortex

To determine the role of Ang-(1–7) in the regulation of PC biosynthesis, cortical slices were prelabeled with ^{32}P, and then incubated with Ang-(1–7) for 30 min. As shown

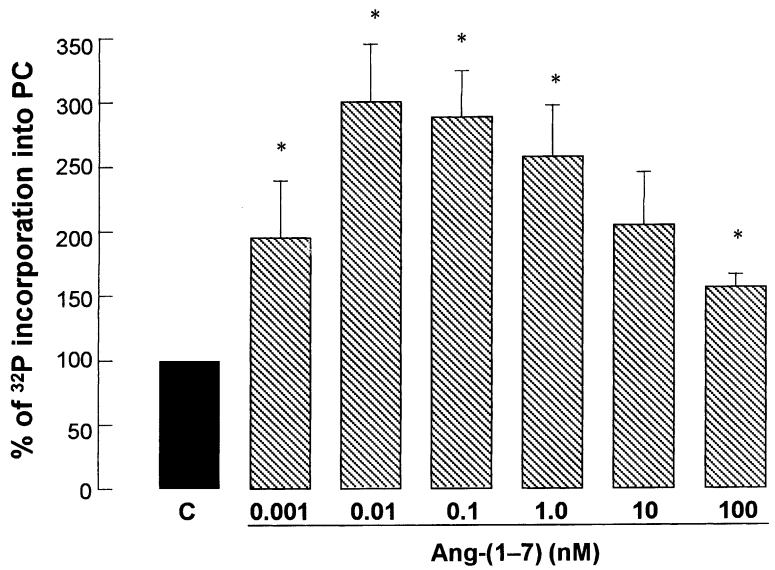

Fig. 1. Effect of Ang-(1–7) on PC biosynthesis in the rat renal cortex. Renal cortical slices were incubated for 60 min with [^{32}P]orthophosphate, and then were stimulated for 30 min with increasing concentrations of Ang-(1–7) (hatched bars). Phospholipids were extracted and separated as described in Section 2. Results are the means ± SEM of four different determinations and are expressed as percentage of ^{32}P incorporated into the PC fraction, compared with the control (C; black bar), i.e. in the absence of peptide (equal to 100%). The asterik (∗) is a value significantly different from the control value, $P < 0.05$.

in Fig. 1, the heptapeptide produced a concentration-dependent stimulation of ^{32}P incorporation into PC; a maximal increase of 200% above control was observed in the 0.01–0.1 nM range. Higher concentrations produced a smaller increase in ^{32}P incorporation into the PC fraction, i.e. in the presence of 100 nM Ang-(1–7) a 60% increase above the control was obtained.

3.3. Effect of signalling mechanism inhibitors on the increase of PC biosynthesis induced by Ang-(1–7) in the rat renal cortex

Since AT_1 receptors have been associated with phospholipase C-dependent phosphoinositide degradation [16], we studied the role of this signal transduction pathway in the Ang-(1–7)-mediated stimulation of PC biosynthesis. Prelabeled slices were preincubated with inhibitors of phospholipase C or PKC and then incubated in the presence of 0.1 nM Ang-(1–7) for an additional 30 min. Neomycin (1 mM) and db-cAMP (1 mM) concentrations that block renal cortical phospholipase C activation [17,18] did not affect the stimulatory effect of Ang-(1–7) (Fig. 2A). Thus, Ang-(1–7) stimulation appeared to be independent of phospholipase C activation. Both inhibitors by themselves increased PC biosynthesis, suggesting a role for phospholipase C in the basal production of PC. On the other hand, radioactivity incorporated into PC in the presence of 0.1 nM Ang-(1–7) was potentiated in prelabeled slices incubated in the presence of either 0.6 μM chelerythrine or 100 μM H7 (Fig. 2B). However, both chelerythrine and H7 individually elicited an increase in the *de novo* synthesis of PC that was similar to that obtained after coincubation of Ang-(1–7) with these inhibitors (Fig. 2B).

3.4. Effect of angiotensin receptor antagonists on Ang-(1–7)-stimulated PC biosynthesis in the rat renal cortex

At least two major receptor types for Ang II, namely AT_1 and AT_2, are known to exist on the cell surface in various target organs [16]. To determine the involvement of these receptor types in the stimulatory effect of Ang-(1–7) on PC biosynthesis, the effects of losartan, a selective AT_1 receptor antagonist, and PD 123319, a selective antagonist for AT_2 receptors, were examined. Neither losartan (0.1–10 nM) nor PD 123319 (0.1–10 nM) modified the enhancement of PC biosynthesis caused by 0.1 nM Ang-(1–7) (Fig. 3A), suggesting that Ang-(1–7)-stimulated PC biosynthesis is independent of AT_1 or AT_2 receptor activation. However, the Ang-(1–7) specific antagonist [D-Ala^7]Ang-(1–7) [19] (0.1–10 nM) partially blocked the increase in the *de novo* synthesis of PC caused by 0.1 nM Ang-(1–7) (Fig. 4), suggesting that this effect is mediated, at least in part, by a specific Ang-(1–7) receptor. The Ang-(1–7) specific antagonist, by itself, increased PC biosynthesis (Fig. 5).

Interestingly, the stimulatory response to 100 nM Ang-(1–7) increased to 180% above the basal value in the presence of 10 μM losartan, while it was not affected by 10 μM PD 123319 (Fig. 3B).

Since losartan enhances the ability of Ang-(1–7) to stimulate PC biosynthesis, we determined the effect of losartan by itself on PC biosynthesis. Prelabeled slices were incubated in the presence of losartan for an additional 30 min. As depicted in Fig. 3, losartan stimulated PC biosynthesis in a concentration-dependent manner: 10 nM losartan increased PC biosynthesis by 65% over the

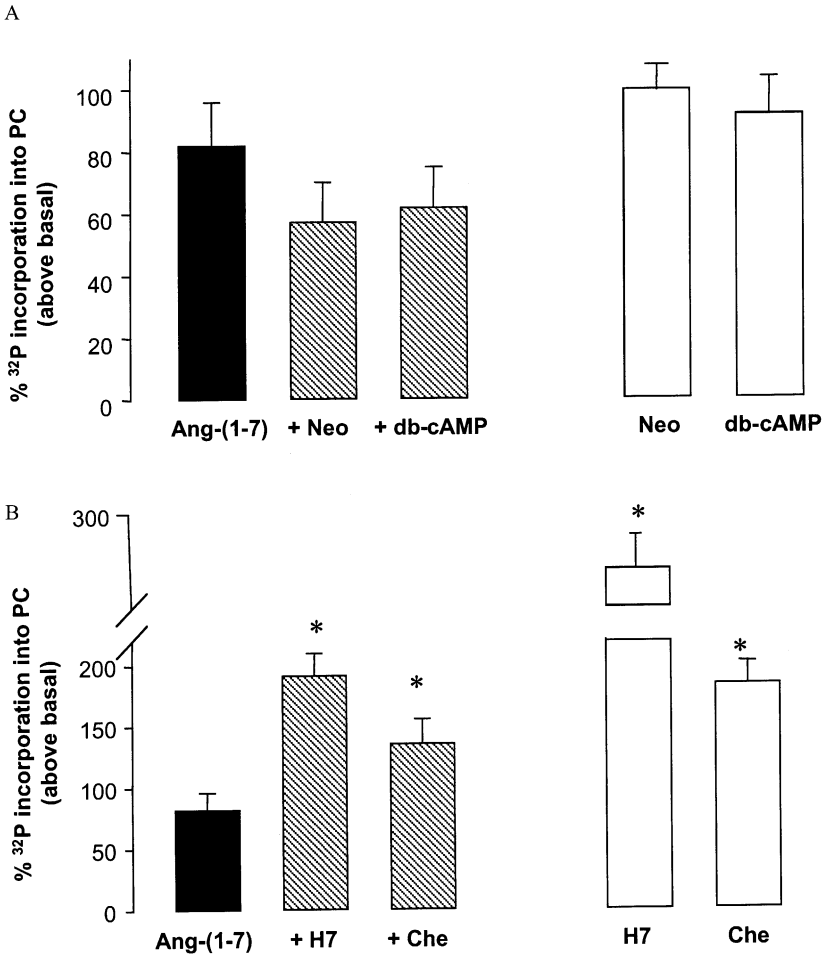

Fig. 2. Effects of Neo or db-cAMP (A) and Che or H7 (B) on the enhancement of PC biosynthesis caused by 0.1 nM Ang-(1–7). Renal cortical slices were incubated for 60 min with [^{32}P]orthophosphate, and then were stimulated for 30 min with 100 pM Ang-(1–7) (black bars) or the inhibitor alone (open bars). When indicated, 1 mM Neo, 1 mM db-cAMP, 0.6 μM Che, or 100 μM H7 was added simultaneously with Ang-(1–7) (hatched bars). Phospholipids were extracted and separated as described in Section 2. Results are the means ± SEM of four different determinations and are expressed as the percentage of ^{32}P incorporated, above the basal value, into the PC fraction. The asterik (∗) is a value significantly different from Ang-(1–7) treatment ($P < 0.05$).

basal level (Fig. 3A, last column), whereas 10 μM losartan increased biosynthesis by 120% (Fig. 3B, last column).

4. Discussion

It is widely accepted that although receptor-mediated activation of different phospholipases promotes phospholipid hydrolysis [20], the endogenous phospholipid content of cellular membranes is maintained by reconstitution through *de novo* synthesis [11]. Our results show that Ang-(1–7) increases PC biosynthesis in the rat renal cortex. As the Ang-(1–7) concentration was increased above 0.01 nM, PC biosynthesis was attenuated (Fig. 1), probably due to tachyphylaxis or desensitization of angiotensin peptide receptors. Another possibility is that increasing the Ang-(1–7) concentration stimulates phospholipase A_2 activity which breaks down [^{32}P]PC causing a competing reaction and resulting in reduced PC production. Our findings agree with a previously reported study showing that Ang-(1–7)-stimulated arachidonic acid release and prostacyclin production in rabbit aortic smooth muscle cells were also diminished at higher Ang-(1–7) concentrations [10]. It has been demonstrated that agonist-induced desensitization occurs within minutes of incubation with Ang II, was maximal at saturating concentrations of the agonist, and may be PKC-mediated [21]. Ang-(1–7) did not fulfill these conditions in our system since the increased stimulatory effect of Ang-(1–7) on PC production in the presence of either chelerythrine or H7, both PKC inhibitors, was similar to that obtained when the tissue was incubated in the presence of the inhibitors alone (Fig. 2). This suggests that desensitization is mediated by a non-PKC pathway. Furthermore, and despite the fact that phospholipase C down-regulates PC biosynthesis, the mechanism by which Ang-(1–7) increases PC production seems to be independent of phospholipase C activation, since the enhancement of the *de novo* synthesis of PC by Ang-(1–7) was unaffected by either neomycin or

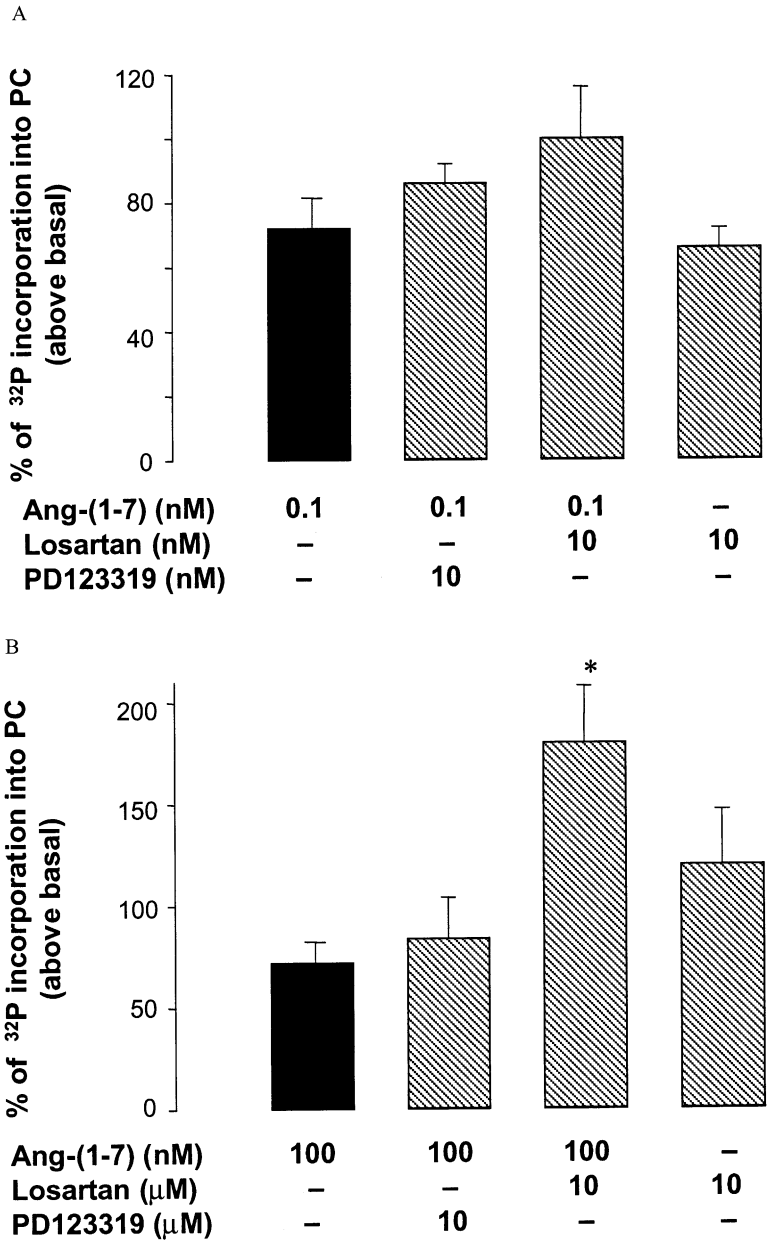

Fig. 3. Effects of losartan and PD 123319 on the enhancement of phospholipid biosynthesis caused by Ang-(1–7). Renal cortical slices were incubated for 60 min with [^{32}P]orthophosphate, and then were stimulated for 30 min with 0.1 nM (A) or 100 nM (B) Ang-(1–7) (black bars). When indicated, losartan or PD 123319 was added simultaneously with Ang-(1–7) (hatched bars). Phospholipids were extracted and separated as described in Section 2. Results are the means ± SEM of four different determinations and are expressed as the percentage of ^{32}P incorporated, above the basal value, into the corresponding phospholipid fraction. The asterik (∗) is a value significantly different from Ang-(1–7) treatment ($P < 0.05$).

db-cAMP, both phospholipase C inhibitors. Since phospholipase C and PKC regulate basal PC biosynthesis and not Ang-(1–7)-stimulated PC production (Fig. 2), it is possible that under Ang-(1–7) stimulation a different pool of PC is being degraded and resynthesized.

Both AT_1 and AT_2 receptor types exist within the renal system, although they are not distributed uniformly [16]. In adult rat and human kidneys, expression of the AT_1 type receptor predominates over the expression of the AT_2 receptor [22]. Although, we have demonstrated previously that Ang-(1–7) has high affinity for AT_1 receptors in the rat renal cortex [23], in this study we found that the increase in ^{32}P incorporation into PC caused by physiological renal concentrations of Ang-(1–7) (0.1 nM) was not modified by either losartan, the AT_1 receptor antagonist, or PD 123319, the AT_2 receptor antagonist, suggesting that the Ang-(1–7)-mediated stimulation of PC biosynthesis is independent of AT_1 and AT_2 receptors. Similarly, Tran *et al.* [24] have demonstrated that the enhancement of PC biosynthesis caused by Ang II in H9c2 cells is independent of AT_1 or AT_2 receptor activation.

Accumulating evidence suggests that the effects of Ang-(1–7) are mediated by a unique angiotensin receptor [4,6]. Both the *in vivo* vasodepressor effects of Ang-(1–7) and the

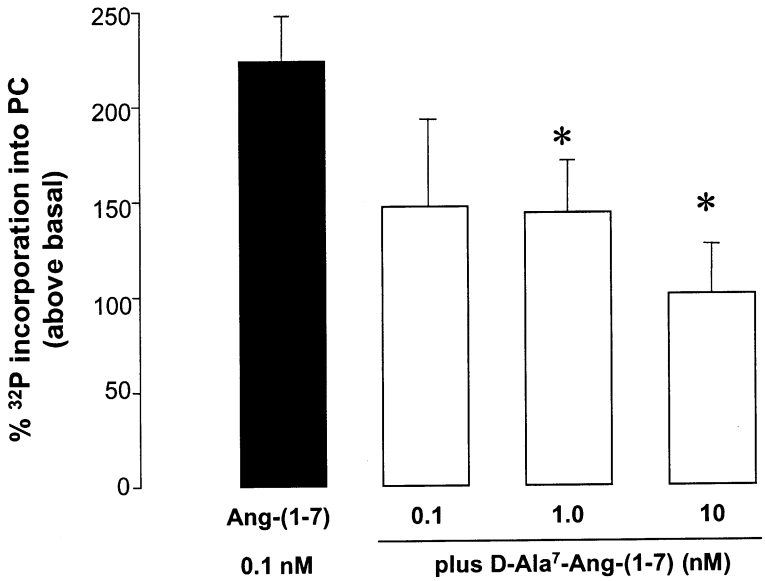

Fig. 4. Effect of [D-Ala^7]Ang-(1–7) on the enhancement of phospholipid biosynthesis caused by Ang-(1–7). Renal cortical slices were incubated for 60 min with [^{32}P]orthophosphate, and then were stimulated for 30 min with 0.1 nM Ang-(1–7) (black bar). When indicated, [D-Ala^7]Ang-(1–7) was added simultaneously with Ang-(1–7) (open bars). Phospholipids were extracted and separated as described in Section 2. Results are the means ± SEM of four different determinations and are expressed as the percentage of ^{32}P incorporated, above the basal value, into the corresponding phospholipid fraction. The asterik (∗) is a value significantly different from Ang-(1–7) treatment ($P < 0.05$).

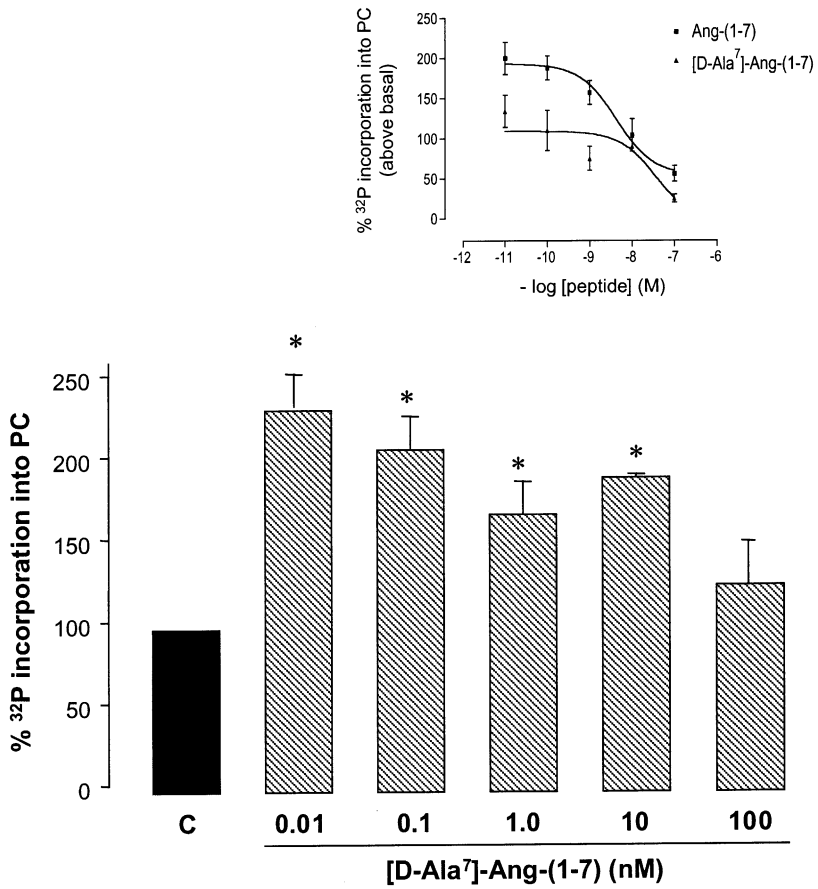

Fig. 5. Effect of [D-Ala^7]Ang-(1–7) on PC biosynthesis in rat renal cortex. Renal cortical slices were incubated for 60 min with [^{32}P]orthophosphate, and then were stimulated for 30 min with 0.01 to 100 nM [D-Ala^7]Ang-(1–7) (hatched bars). Phospholipids were extracted and separated as described in Section 2. Results are the means ± SEM of four different determinations and are expressed as the percentage of ^{32}P incorporated into the corresponding phospholipid fraction compared with the control (C; black bar) equal to 100%. The asterik (∗) is a value significantly different from the control group ($P < 0.05$). Insert: comparison between the effects of Ang-(1–7) and [D-Ala^7]Ang-(1–7) on PC biosynthesis in the rat renal cortex.

stimulation of prostaglandin release caused by the peptide are mediated via a non-AT_1/AT_2 receptor [4,6]. However, it should be noted that under certain conditions the effects of the heptapeptide may be blocked by losartan or to a variable extent by an AT_2 receptor antagonist [2,4,25]. Our present results show that the enhancement of PC biosynthesis caused by Ang-(1–7) was partially blocked by [D-Ala^7]Ang-(1–7), the Ang-(1–7) specific antagonist [19], suggesting that Ang-(1–7) specific receptors are coupled. In agreement with these results, it has been demonstrated that [D-Ala^7]Ang-(1–7) also blocks the Ang-(1–7)-mediated release of arachidonic acid in rabbit aortic vascular smooth muscle cells [10], the antidiuretic action of Ang-(1–7) in water-loaded Wistar rats [19], the cardiovascular effects produced by central administration of this heptapeptide [19,26], and the Ang-(1–7)-induced inhibition of norepinephrine release [25]. Despite the fact that [D-Ala^7]Ang-(1–7) by itself blocked Ang-(1–7)-stimulated PC biosynthesis, it enhanced PC production. This heptapeptide may be acting as a partial agonist, as was previously suggested for saralasin, another peptidic angiotensin antagonist [27]. In fact, although [D-Ala^7]Ang-(1–7) did not produce the maximal effect elicited by Ang-(1–7), it did have a similar behaviour pattern as it induced a lesser response at higher concentrations (Fig. 5), i.e. desensitization may be occurring.

On the other hand, we found that at higher Ang-(1–7) concentrations the joint addition of losartan potentiated the stimulatory effect of the heptapeptide on PC biosynthesis, probably as the result of additive effects. Conversely, it has been reported that losartan alone inhibits PC biosynthesis in H9c2 cells by reducing choline uptake, although CTP:phosphocholine cytidylyltransferase translocation from the cytosolic to the membrane fractions of H9c2 cells was activated [28]. This enzyme catalyzes the rate-limiting step for PC biosynthesis [29]. These apparently conflicting results were attributed by the authors to a compensatory mechanism present in those cells to maintain PC biosynthesis when choline uptake was inhibited. In our system we found that although losartan increased PC production, it inhibited CTP:phosphocholine cytidylyltransferase by 26% (data not shown), probably as a compensatory mechanism. Additional work is needed to elucidate the mechanism by which losartan stimulates PC biosynthesis.

In summary, our results indicate that Ang-(1–7) enhances PC biosynthesis in the rat renal cortex. When used above 0.1 nM, desensitization occurs, a phenomenon frequently observed in angiotensin–receptor interactions [21,30]. Interestingly, physiological Ang-(1–7) concentrations may ensure phospholipid restoration by increasing PC biosynthesis, since the peptide has been shown to stimulate phospholipase A_2, which in turn catalyzes PC hydrolysis [9]. Moreover, Ang-(1–7) seems to act as a renal protective hormone, since, as we have demonstrated previously, an increase in phospholipid turnover is one of the mechanisms by which the renal cortex protects itself against injury [12]. It appears that losartan, which is used pharmacologically in the treatment of hypertension, increases PC biosynthesis and may help to preserve renal cortical membrane homeostasis. This is consistent with the fact that angiotensin-converting enzyme inhibitors, known to increase the renal Ang-(1–7) concentration, and AT_1 blockers, used to decrease blood pressure, have beneficial effects on cardiovascular and renal disease [16,31].

Acknowledgments

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