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Effects of angiotensin IV and angiotensin-(1-7) on basal and angiotensin II-stimulated cytosolic Ca^{2+} in mesangial cells

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Abstract

This study analyzed the influence of two main metabolites of angiotensin II, angiotensin IV and angiotensin-(1–7), on basal and angiotensin II-dependent $[Ca^{2+}]_i$ in rat mesangial cells. Angiotensin IV behaved as a weak agonist. Its effects were abolished by angiotensin AT_1 receptor antagonists. Treatment with angiotensin II abolished the effect of a subsequent treatment with angiotensin IV whereas two successive angiotensin IV-dependent $[Ca^{2+}]_i$ peaks were obtained. Angiotensin II increased $[Ca^{2+}]_i$ in a Ca^{2+}-free medium whereas angiotensin IV was inactive. Leucine-valine-valine-hemorphin 7, a hemorphin specific for the angiotensin AT_4 receptor, was devoid of any agonistic or antagonistic effect. In contrast, angiotensin-(1–7), if without influence on basal $[Ca^{2+}]_i$, inhibited angiotensin II- and angiotensin IV-dependent $[Ca^{2+}]_i$ increases. Total inhibition of the angiotensin IV effect was obtained whereas association of angiotensin-(1–7) to 8-(*NN*-diethylamino)-octyl-3,4,5-trimethoxybenzoate, an inhibitor of inositol phosphate-mediated Ca^{2+} release, was necessary to suppress the effect of angiotensin II. These results provide evidence that angiotensin II metabolites may participate in the control of $[Ca^{2+}]_i$ in mesangial cells at the initial stage of binding to the angiotensin AT_1 receptors.

Keywords: Angiotensin IV; Angiotensin-(1–7); Angiotensin AT_1 receptor; Mesangial cell, Ca^{2+} cytosolic

1. Introduction

Angiotensin IV is the N-truncated hexapeptide derived from angiotensin II and angiotensin III by the successive actions of aminopeptidases A and N. It can also be formed from angiotensin I-(3–10) in the presence of converting enzyme (Ardaillou and Chansel, 1998). Angiotensin-(1–7) is the C-truncated heptapeptide derived from angiotensin II by deletion of its eighth (phenylalanine) residue. Several enzymes, namely, neutral endopeptidases 24.11 (neprilysin) and 24.15 and prolylendopeptidase 24–26 are responsible for the production of this metabolite (Ardaillou and Chansel, 1998). Classically, angiotensin II and the heptapeptide angiotensin III were considered to be the only active fragments formed in the cascade of enzymatic events constituting the renin–angiotensin system (Blair-West et al., 1971). More recently, a large body of evidence led to the concept that angiotensin IV and angiotensin-(1–7) are also active products. High-affinity binding sites for angiotensin IV were described with specific characteristics that enabled them to be distinguished from the two main subtypes, AT_1 and AT_2, of the angiotensin II receptor (Hall et al., 1993). It was also shown that angiotensin IV could exert effects that were opposite to those of angiotensin II. For example, infusion of angiotensin IV into the renal artery produced a dose-dependent increase in cortical blood flow (Coleman et al., 1998a), and exposure of chick heart cells to angiotensin IV attenuated angiotensin II-dependent increases in protein and RNA synthesis (Baker and Aceto, 1990). Such effects were attributed to a new class of receptors that were designated AT_4. These receptors exhibit poor affinity for angiotensin II and angiotensin AT_1 or AT_2 receptor antagonists whereas they recognize related peptides, in particular hemorphins with a N terminal sequence similar to that of angiotensin IV (Garreau et al., 1998). However, other studies demonstrated that angiotensin IV could also behave as an angiotensin AT_1 receptor agonist, the main difference between angiotensin II and angiotensin IV being the low affinity of the latter for the angiotensin AT_1 receptor and, consequently, its attenuated biological potency (Li et al.,

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1997). Both types of effects of angiotensin IV (angiotensin AT_4- or angiotensin AT_1 receptor-mediated) can be pharmacologically distinguished using specific antagonists such as losartan or candesartan and divalinal for the angiotensin AT_1- and the angiotensin AT_4 receptor-mediated responses, respectively. In this context, the vasoconstrictor effect of angiotensin IV in the mesenteric and hindlimb vascular beds of the cat (Champion and Kadowitz, 1997) and the rat (Champion et al., 1998) was attributed to angiotensin AT_1 receptor activation because it was abolished by losartan and candesartan whereas the vasodilation following angiotensin IV injection into the cerebral, cochlear or renal circulation was considered to be an angiotensin AT_4 receptor-mediated response because it was suppressed by divalinal-angiotensin IV (Coleman et al., 1998a; Coleman et al., 1998b; Kramar et al., 1997). Recently, we provided functional evidence for a third type of angiotensin IV receptor in rat mesenteric artery whose activation after blockade of both angiotensin AT_1 and AT_2 receptors resulted in a vasoconstrictor effect (Loufrani et al., 1999).

Like angiotensin IV, angiotensin-(1–7) mediates most of its effects through a novel angiotensin non-AT_1–non-AT_2 receptor subtype. Angiotensin-(1–7) behaves as an antihypertensive peptide which counterbalances the actions of angiotensin II. It stimulates the synthesis and the release of nitric oxide and vasodilator prostaglandins and also potentiates the effects of bradykinin. Furthermore, angiotensin-(1–7) decreases tubular sodium and bicarbonate reabsorption, promotes diuresis and produces vasodilation (Ferrario et al., 1998). It also exerts antiproliferative effects on vascular smooth muscle (Tallant et al., 1999). All these effects of angiotensin-(1–7) are inhibited by [D-Ala^7] angiotensin-(1–7), which does not bind to angiotensin AT_1 or AT_2 receptors and, also, by [Sar^1,Thr^8] angiotensin II, a nonselective angiotensin II receptor antagonist, which suggests that angiotensin-(1–7) shares with angiotensin II a receptor distinct from angiotensin AT_1 and AT_2 receptors. However, some of the responses to angiotensin-(1–7) have been shown to be blocked by angiotensin AT_1 or AT_2 receptor antagonists, which makes it possible that angiotensin-(1–7) could, although with low affinity, activate the classical receptors of angiotensin II (Jaiswal et al., 1993).

Angiotensin AT_1 and AT_4 receptor sites are present in the kidney. Interestingly, their localizations are somewhat different. Both receptors are present in the proximal tubule and the outer medulla, but angiotensin AT_4 receptors in contrast to angiotensin AT_1 receptors are not found in the glomeruli (Handa et al., 1998). However, angiotensin IV specifically binds to cultured rat mesangial cells and stimulates intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in these cells (Chansel et al., 1998). Such a coupling of angiotensin IV binding sites to Ca^{2+} signaling has been observed also in rat vascular smooth muscle cells (Dostal et al., 1990), opossum kidney cells (Dulin et al., 1995) and pulmonary artery endothelial cells (Patel et al., 1999). Angiotensin-(1–7) binding at angiotensin AT_1 receptor sites has been recently reported in rat glomerular membranes (Gironacci et al., 1999), but no effect of angiotensin-(1–7) on cytosolic Ca^{2+} levels was observed in any of the preparations studied (Jaiswal et al., 1993; Tallant et al., 1991).

The purpose of the present study was first to analyze the mechanism by which angiotensin IV produces an increase of $[Ca^{2+}]_i$ in rat mesangial cells. We thought it of interest to compare the effects of angiotensin IV and angiotensin II in order to better appreciate whether angiotensin IV acts through stimulation of angiotensin AT_1 receptors or in an independent manner. We also examined whether angiotensin-(1–7) modifies $[Ca^{2+}]_i$ in rat mesangial cells or interferes with the calcium-stimulating effects of angiotensin II and angiotensin IV.

2. Methods

2.1. Materials

Reagents for these studies were obtained as follows: culture media, antibiotics and cell culture supplies were from Gibco (Paisley, UK); fetal calf serum was from Boehringer (Mannheim, Germany); acetoxymethylester of Fura-2 was from Calbiochem (San Diego, CA); angiotensin II and angiotensin II-(3–8), also referred to as angiotensin IV, and angiotensin-(1–7), and [Sar^1, Thr^8] angiotensin II were from Sigma (St. Louis, MO). Leucine-valine-valine-hemorphin 7 (LVV-H7) was a gift from J.M. Piot (La Rochelle, France). It had been purified from a peptic hemoglobin hydrolysate as previously described (Piot et al., 1992). Losartan and candesartan were donated by Merck, Sharp and Dohme Research Laboratories (West Point, PA) and by Astra Hässle (Stockholm, Sweden), respectively. [^{3}H] myoinositol (27 TBq/mmol) was from the Radiochemical Centre (Amersham, UK) and [^{125}I] [Sar^1, Ile^8] angiotensin II was from NEN Life Science Products (Boston, MA). All other reagents were from Sigma.

2.2. Cell culture

Primary cultures of mesangial cells were obtained from collagenase-treated glomeruli as previously described (Foidart et al., 1980). Kidneys were removed under pentobarbital anesthesia from 100- to 150-g male Sprague–Dawley rats, and glomeruli were isolated by sieving techniques and centrifugation. Collagenase-treated glomeruli were seeded in plastic Petri dishes in the presence of 8 ml of RPMI-1640 medium buffered with 20 mM HEPES, pH 7.4, and supplemented with 10% fetal calf serum, 50 units/ml of penicillin G, 50 μg/ml of streptomycin sulfate and 2 mM of glutamine. Culture medium was changed every 2 days. Mesangial cells began to grow from glomeruli

after 7–8 days. These cells, stellate or fusiform in shape when observed under phase-contrast microscopy, were subcultured at day 21. Confluent cells in primary cultures were used for $[Ca^{2+}]_i$ measurement and confluent cells in the second subculture were used for binding studies and inositol phosphate determination. Cultured cells exhibited the typical morphological and biochemical features of mesangial cells (Foidart et al., 1980).

2.3. Intracellular free calcium ($[Ca^{2+}]_i$) measurement

Subconfluent cells in Petri dishes were loaded for 45 min at 37°C with 5 μM Fura 2/AM dissolved in phosphate buffer at pH 7.4 (NaCl, 135 mM; Na_2HPO_4, 1 mM; KCl, 5 mM; Mg_2SO_4, 0.5 mM; $CaCl_2$, 1.8 mM; glucose, 10 mM; HEPES, 10 mM) supplemented with 1 mg/ml of bovine serum albumin. After being washed, the cells were trypsinized (trypsin, 0.05%, EDTA, 0.02%) and resuspended in the same buffer with or without $CaCl_2$. Two milliliters of the suspension was transferred into a quartz cuvette under constant stirring at 37°C, and fluorescence was monitored in a spectrofluorometer Quanta Master 1 (Photon Technology International) before and after the addition of the agents to be tested. Fura 2 was alternately excited at wavelengths of 340 and 380 nm, and emission was measured at 510 nm. The fluorescence intensities (S at 340 nm and L at 380 nm) were simultaneously recorded. $[Ca^{2+}]_i$ was calculated from the following equation: $[Ca^{2+}]_i = K_d\ [(R - R_{min})/(R_{max} - R)](L_{max}/L_{min})$ where $K_d = 224$ nM, $R = S/L$, and L_{min}, L_{max}, R_{min}, and R_{max} are L and R values at zero and saturating concentrations of calcium, respectively. Both conditions were obtained by calibration with 0.02% Triton × -100 for the maximum value and 10 mM ethylene glycol-bis-aminoethylether-N,N,N',N'-tetra-acetic acid (EGTA) for the minimum value. Felix software 1.1 program was used for $[Ca^{2+}]_i$ calculation (Photon Technology International).

2.4. Measurement of inositol phosphate production

Inositol phosphates were measured as previously described (Grandalano et al., 1993). Subconfluent cells in 6-well plates were incubated for 48 h at 37°C in inositol-deficient Waymouth medium containing 1.1 MBq per well of myo-[^{3}H] inositol. Then, after a 15-min preincubation with 10 mM lithium chloride, cells were exposed to 0.1 μM angiotensin II or 1 μM angiotensin IV, with or without 10 μM losartan, for 1 min. The medium was discarded, and 2 ml of ice-cold 5% trichloracetic acid was added. Cells were scraped away from the wells and were washed once more with 5% trichloracetic acid. The aqueous phase was extracted twice with 2 vol. of diethylether. Then, the samples were adjusted to pH 7 with 5 mM sodium tetraborate and loaded onto 2 ml columns of Dowex AG1-× 8 resin (Biorad). The columns were washed with 10 ml of water and 10 ml of 5 mM sodium tetraborate. Inositol phosphates were then eluted with 10 ml of increasing concentrations of ammonium formate (0.2–0.8 M) in formic acid (0.1 M). Five milliliters of each collected fraction was mixed with scintillation fluid and counted in a beta counter. After correction for quenching, inositol phosphate values were expressed as percentages of control (ratio of inositol phosphate content in angiotensin II- or angiotensin IV-treated cells to inositol phosphate content in cells exposed only to buffer). Three separate wells were studied in each condition.

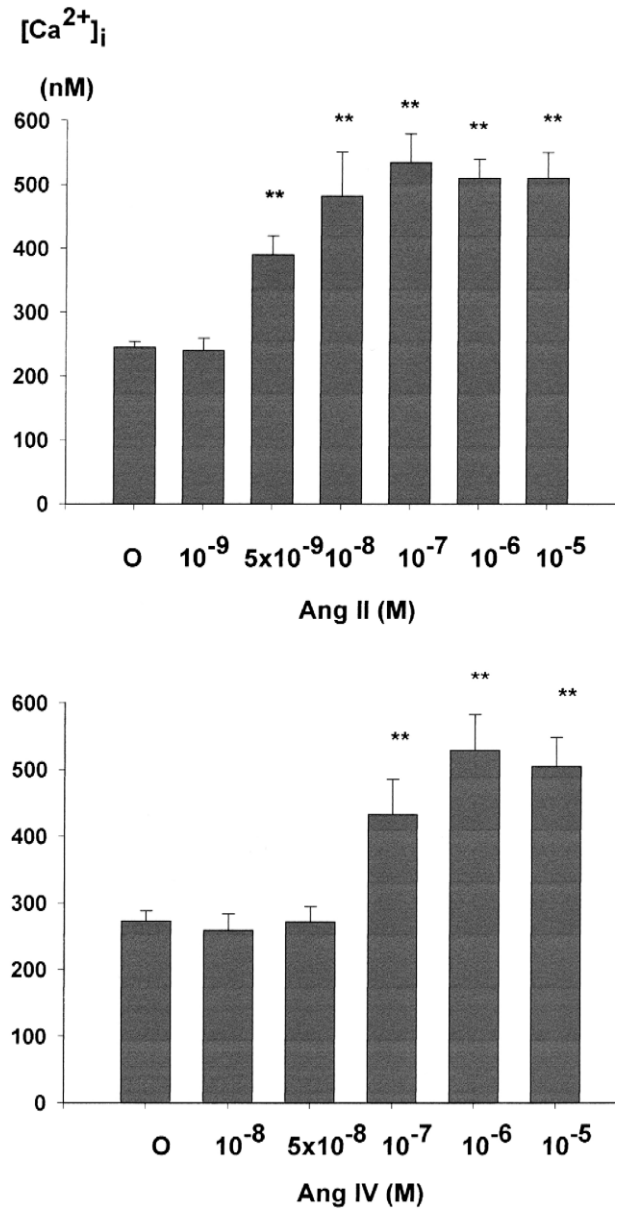

Fig. 1. Dose dependence of average change in cytosolic calcium concentration ($[Ca^{2+}]_i$) from baseline in rat mesangial cells treated with angiotensin II (upper part) or angiotensin IV (lower part). Values are means ± S.E. ($N = 6$). Changes in $[Ca^{2+}]_i$ were statistically significant from 5 nM angiotensin II and 100 nM angiotensin IV ($P < 0.05$); ** = $P < 0.01$ vs. zero concentration.

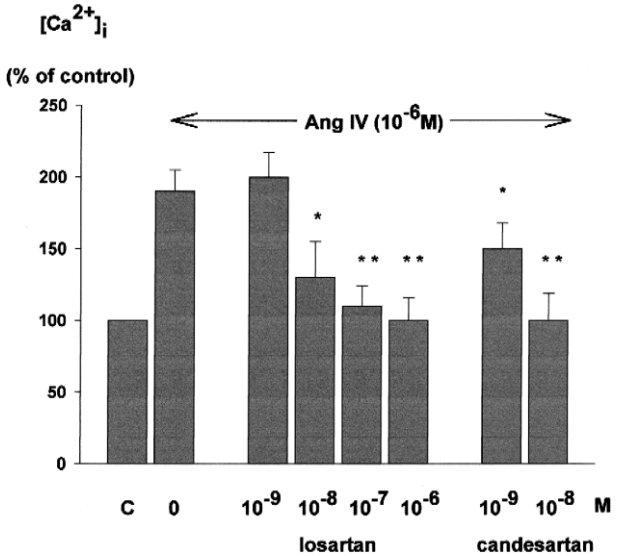

Fig. 2. Effect of two specific angiotensin AT_1 receptor antagonists, losartan and candesartan, on angiotensin IV-dependent cytosolic calcium concentration ($[Ca^{2+}]_i$) in rat mesangial cells. These two drugs were added to the cell suspension 2 min before treatment with 1 μM angiotensin IV. Values are means ± S.E. ($N = 4$). Losartan and candesartan significantly reduced angiotensin IV-dependent $[Ca^{2+}]_i$. C = control; * = $P < 0.05$ and ** = $P < 0.01$ vs. zero concentration.

2.5. Binding studies

Binding studies with mesangial cells were performed using confluent cell monolayers in 12-well plates as previously published (Chansel et al., 1994). The culture medium was removed and cells were washed with 20 mM Tris–HCl buffer, pH 7.4, containing (in mM) 5 glucose, 135 NaCl, 10 KCl and 10 $NaCH_3COO$ (Buffer A). They were then incubated for 45 min at 22°C in the same medium with 80 pM ^{125}I [Sar^1, Ile^8] angiotensin II and increasing concentrations (100 pM–10 μM) of various unlabelled peptides including angiotensin II, [Sar^1, Ile^8] angiotensin II, angiotensin II-(1–7) and [Sar^1, Thr^8] angiotensin II, a nonselective inhibitor of angiotensin II receptor sites which has been shown to inhibit the biological effects of angiotensin II-(1–7) (Ferrario et al., 1998). Incubation was terminated by aspiration of the medium followed by three washes with 1 ml of 0.15 M NaCl each. Cells were then dissolved in 500 μl of 1 M NaOH and counted for ^{125}I radioactivity in a gamma automatic counter with 65% efficiency (LKB, Bromma, Sweden). Binding was first calculated as fmol bound per mg of protein and then expressed as a percentage of control. Protein concentration was determined according to Bradford (1976), using bovine serum albumin as a standard.

2.6. Statistics

Data are presented as mean values ± S.E. Statistical comparisons of the mean values were done using Student's *t* test or two-way analysis of variance.

3. Results

3.1. Effects of angiotensin II and angiotensin IV on $[Ca^{2+}]_i$ in rat mesangial cells

Basal $[Ca^{2+}]_i$ in rat mesangial cells was 245 ± 92 nM ($n = 34$). $[Ca^{2+}]_i$ release was immediate after stimulation by angiotensin II or angiotensin IV. As previously de-

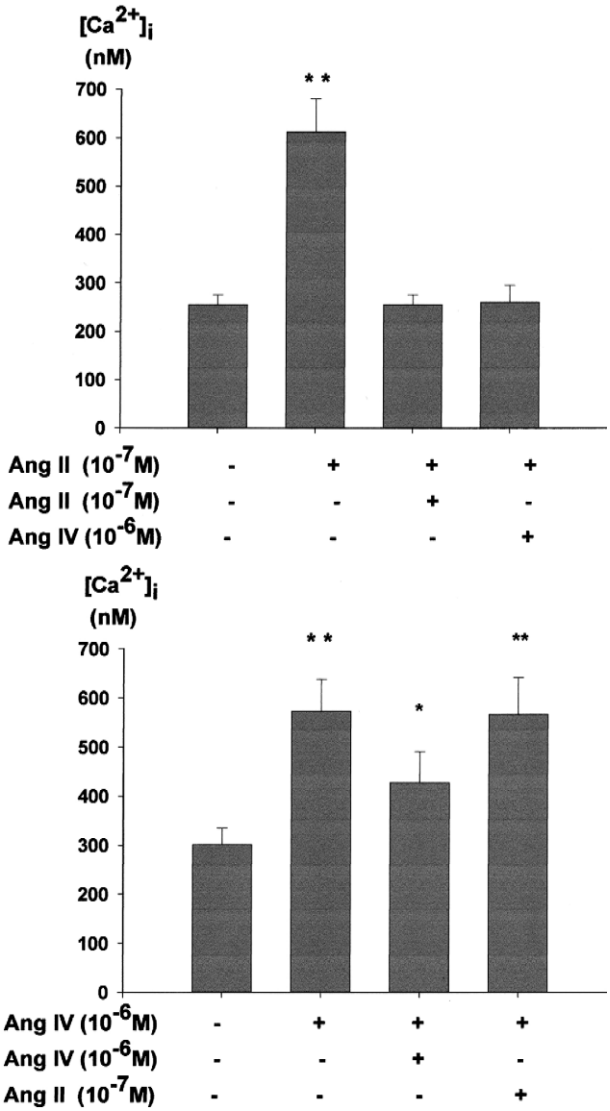

Fig. 3. Cytosolic calcium ($[Ca^{2+}]_i$) response to angiotensin II alone (0.1 μM) and angiotensin II (0.1 μM) or angiotensin IV (1 μM) in rat mesangial cells 2 min after the first stimulation with angiotensin II (0.1 μM) in the upper part; $[Ca^{2+}]_i$ response to angiotensin IV (1 μM) alone and angiotensin IV (1 μM) or angiotensin II (0.1 μM) in rat mesangial cells 2 min after the first stimulation with angiotensin IV (1 μM) in the lower part. Note that the second bar from the left corresponds to the first $[Ca^{2+}]_i$ peak whereas the third and fourth bars illustrate the amplitude of the second response. Values are means ± S.E. ($N = 5$–7). Pretreatment with angiotensin II suppressed the effect of a subsequent treatment with angiotensin II or angiotensin IV. Pretreatment with angiotensin IV did not prevent a subsequent significant effect of angiotensin IV or angiotensin II; * = $P < 0.05$ and ** = $P < 0.01$ vs. control.

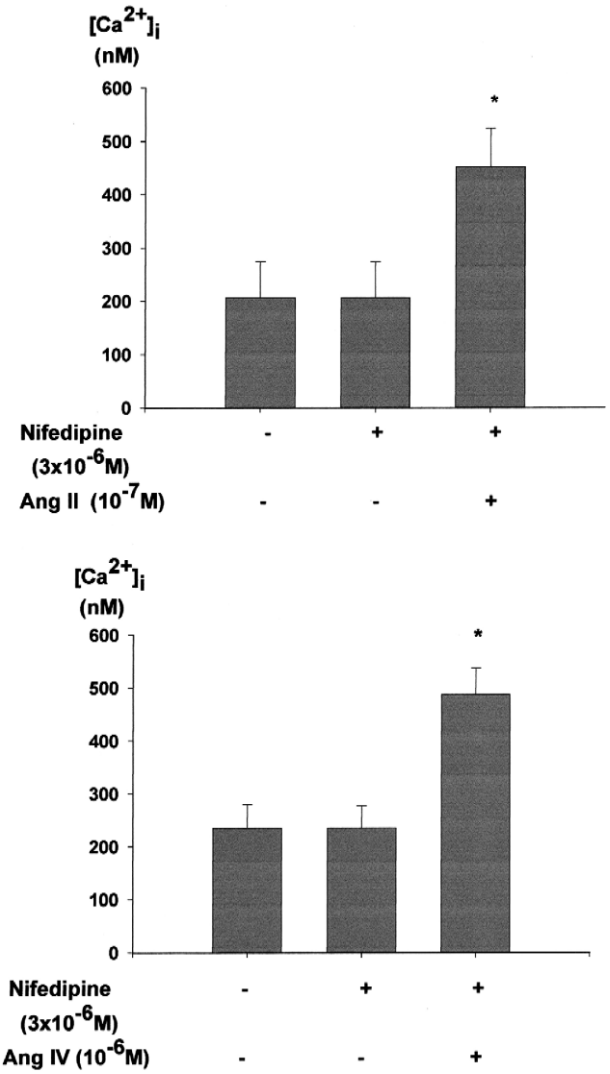

Fig. 4. Effect of nifedipine, a voltage-dependent Ca^{2+} channel blocker, on angiotensin II (0.1 μM)- or angiotensin IV (1 μM)-dependent cytosolic calcium concentration ($[Ca^{2+}]_i$) in rat mesangial cells. Values are means ± S.E. ($N = 4$) Cells were pretreated with 3 μM nifedipine for 3 min before peptide addition. Nifedipine did not prevent the stimulatory effect of both peptides; * = $P < 0.05$ vs. control.

scribed (Chansel et al., 1998), the $[Ca^{2+}]_i$ response to both peptides was biphasic, with a peak followed by a low-level but sustained phase. The bars shown in the figures correspond to the $[Ca^{2+}]_i$ value of the peak. Angiotensin II induced a dose-dependent increase of $[Ca^{2+}]_i$ with a threshold at 5 nM. A plateau of approximately twice the basal value was reached at 100 nM. No further increase was observed up to 10 μM, which was the highest concentration studied. Angiotensin IV also stimulated $[Ca^{2+}]_i$, but only from 100 nM with a plateau at 1 μM (Fig. 1). Losartan (1 μM) and candesartan (10 nM) alone had no effect on $[Ca^{2+}]_i$, but completely inhibited ($P < 0.01$) the response to 1 μM angiotensin IV (Fig. 2), thus indicating that the stimulator effect of angiotensin IV needed previous angiotensin AT_1 receptor stimulation. Successive addition of two doses of angiotensin II (0.1 μM) or of angiotensin II (0.1 μM) and angiotensin IV (1 μM) with a 2-min interval failed to elicit a second significant $[Ca^{2+}]_i$ peak. Pretreatment with angiotensin IV (1 μM) did not significantly modify the subsequent effect of angiotensin II (0.1 μM), but attenuated ($P < 0.05$) that of angiotensin IV (1 μM) (Fig. 3).

To further investigate the mechanism of Ca^{2+} mobilization by angiotensin IV in rat mesangial cells, we examined the effects of different drugs, each acting at a definite step in $[Ca^{2+}]_i$ regulation. Nifedipine (3 μM), a L-type Ca^{2+} channel blocker, did not modify either $[Ca^{2+}]_i$ baseline

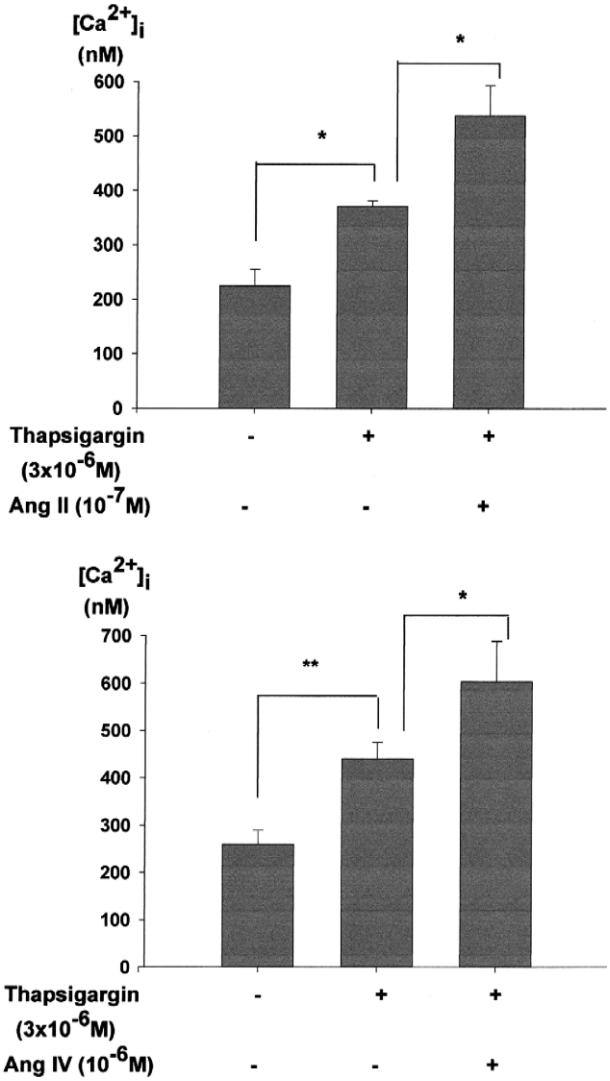

Fig. 5. Effect of thapsigargin, an endoplasmic reticulum Ca^{2+}-ATPase inhibitor, on angiotensin II (0.1 μM)- or angiotensin IV (1 μM)-dependent cytosolic calcium concentration ($[Ca^{2+}]_i$) in rat mesangial cells. Note that the second bar from the left corresponds to the first $[Ca^{2+}]_i$ peak whereas the third bar illustrates the amplitude of the second response. Values are means ± S.E. ($N = 6$). Angiotensin II significantly increased $[Ca^{2+}]_i$ in thapsigargin-treated cells ($P < 0.05$). Cells were incubated with 3 μM thapsigargin for 3 min before peptide addition; * = $P < 0.05$ and * * = $P < 0.01$.

concentration or $[Ca^{2+}]_i$ increase in the presence of angiotensin II (0.1 μM) or angiotensin IV (1 μM) (Fig. 4). This demonstrates that neither peptide influences voltage-dependent L-type Ca^{2+} channels. Incubation of rat mesangial cells with 3 μM of thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, increased basal $[Ca^{2+}]_i$ as expected, but did not prevent a subsequent increase of $[Ca^{2+}]_i$ after stimulation with 0.1 μM angiotensin II or 1 μM angiotensin IV indicating that both peptides can act even when reloading of the endoplasmic reticulum with Ca^{2+} is blocked (Fig. 5). However, emptying of the endoplasmic reticulum Ca^{2+} stores by inhibition of refilling with thapsigargin could contribute to Ca^{2+} entry via the putative capacitative mechanism when extracellular Ca^{2+} is available. Therefore, we studied also the effects of the two peptides following exposure of the cells to thapsigargin after Ca^{2+} had been removed from the extracellular medium by the addition of 1 mM EGTA to a nominally Ca^{2+}-free medium. Under such conditions, both angiotensin II and angiotensin IV were inactive, thus demonstrating the role of Ca^{2+} influx in the effect observed. A similar conclusion was reached in the studies of mesangial cells incubated with 8-(*NN*-dietylamino)-octyl-3,4,5-trimethoxybenzoate (TMB-8) (0.1 mM), an inhibitor of inositol phosphate-mediated Ca^{2+} release from intracellular stores, in the usual Ca^{2+}-containing medium. Angiotensin IV (1 μM) and angiotensin II (0.1 μM) were still able to produce a $[Ca^{2+}]_i$ peak 3 min after the TMB-8-dependent Ca^{2+} increase, suggesting the role of Ca^{2+} influx (Results not shown). Angiotensin II- and angiotensin IV-induced $[Ca^{2+}]_i$ increases were not affected by pretreatment of the cells with pertussis toxin (100 ng/ml, 15 h), thus eliminating a role for pertussis toxin-sensitive G proteins in the signaling pathways. Finally, we examined the effect of Ca^{2+} in the incubation medium. Addition of EGTA (1 mM) to a Ca^{2+}-free medium decreased $[Ca^{2+}]_i$ baseline and completely suppressed the response to 1 μM angiotensin IV. In contrast, it attenuated but did not suppress the response to 0.1 μM angiotensin II (Fig. 6).

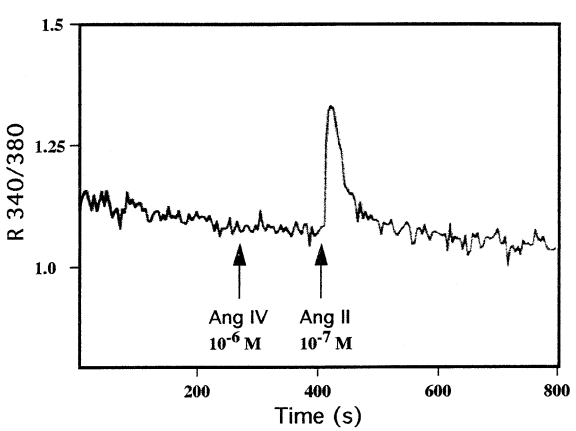

Fig. 6. Representative tracing of the effect of prolonged maintenance of rat mesangial cells in a zero Ca^{2+} medium containing 1 mM EGTA on 1 μM angiotensin IV- and on 0.1 μM angiotensin II-induced $[Ca^{2+}]_i$ increases. The ratio of fluorescence intensities at 340 and 380 nm is plotted against time. No effect of angiotensin IV was observed whereas angiotensin II was still stimulatory.

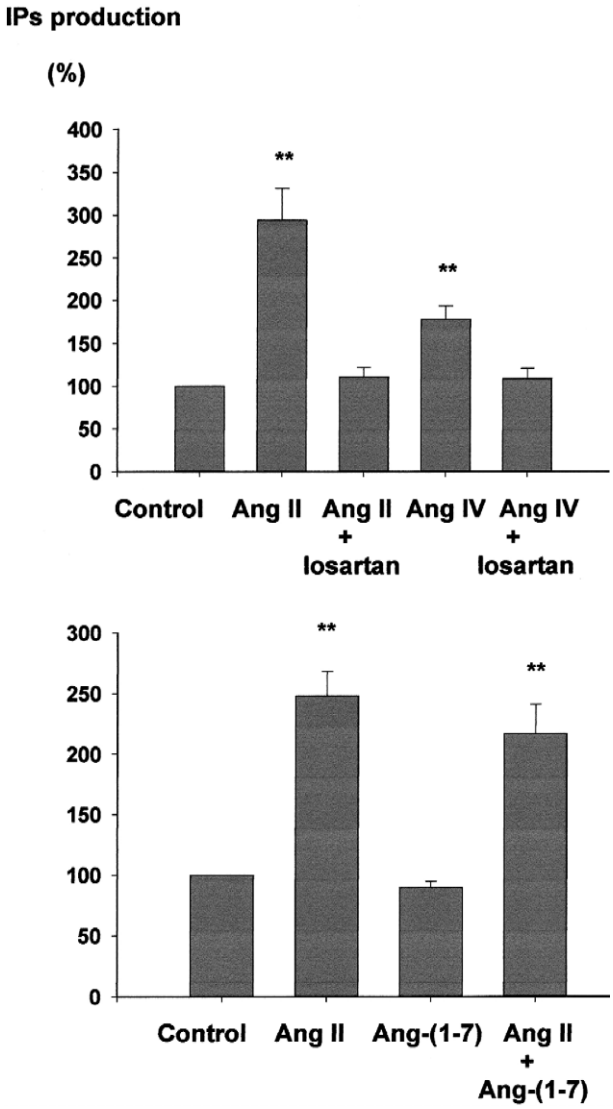

Fig. 7. Total inositol phosphate (IP) production in rat mesangial cells which had been stimulated with angiotensin II (0.1 μM) or angiotensin IV (1 μM) with or without the addition of 10 μM losartan (upper part) and total inositol phosphate production after stimulation with angiotensin II (0.1 μM) with or without angiotensin-(1–7) (lower part). The sum of inositol monophosphate, inositol bisphosphate and inositol trisphosphate production after exposure of the cells to the agents tested for 1 min is shown. Values are means ± S.E. (Five different experiments with triplicates in each.) Angiotensin II and angiotensin IV, but not angiotensin-(1–7), significantly stimulated inositol phosphate production. This stimulation was suppressed in the presence of losartan but not of angiotensin-(1–7). $^{**} = P < 0.01$ vs. control.

3.2. Effects of angiotensin II, angiotensin IV and angiotensin-(1–7) on inositol phosphate production in rat mesangial cells

Intracellular Ca^{2+} mobilization is often mediated by inositol (1, 4, 5) trisphosphate formation. Incubation of the

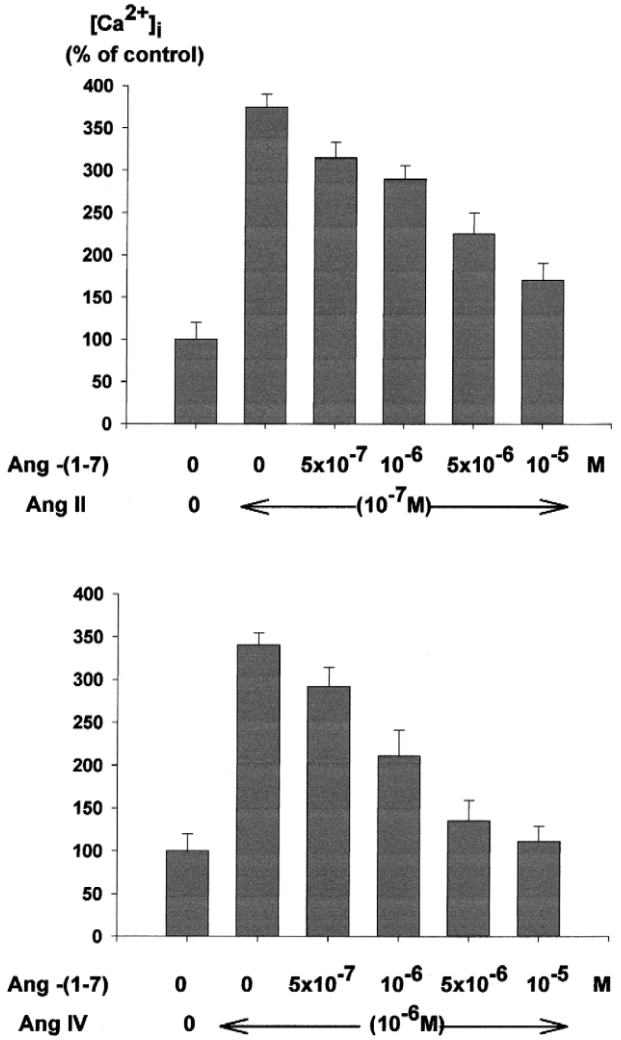

Fig. 8. Dose dependence of average changes in cytosolic calcium concentration ($[Ca^{2+}]_i$) in rat mesangial cells treated with 0.1 μM angiotensin II (upper part) or 1 μM angiotensin IV (lower part) in the presence of increasing concentrations of angiotensin-(1–7). Values are means ± S.E. ($N = 4$). Changes in $[Ca^{2+}]_i$ are statistically significant from 1 μM angiotensin-(1–7) for both the effects of angiotensin II and angiotensin IV ($P < 0.05$).

cells for 1 min with 0.1 μM angiotensin II increased the formation of inositol monophosphate, inositol bisphosphate and inositol trisphosphate to nearly three times the basal value. Angiotensin IV (1 μM) was less active since it induced an increase of about 60% above basal value. In both cases, losartan totally abolished the stimulatory effects (Fig. 7, upper part). Angiotensin-(1–7) did not influence basal or angiotensin II-stimulated inositol phosphate formation (Fig. 7, lower part).

3.3. Interference of angiotensin-(1–7) with the Ca^{2+}-stimulating effects of angiotensin II and angiotensin IV

Angiotensin-(1–7) in concentrations up to 10 μM had no effect on $[Ca^{2+}]_i$ in rat mesangial cells. However, this metabolite inhibited the $[Ca^{2+}]_i$ increase obtained in the presence of angiotensin II or angiotensin IV. For both peptides, the inhibitory effect was apparent with concentrations of angiotensin-(1–7) between 0.5 and 10 μM. A more marked effect was noted on the angiotensin IV-dependent than on the angiotensin II-dependent $[Ca^{2+}]_i$ increase. Indeed, the stimulatory influence of 1 μM angiotensin IV was abolished with 10 μM angiotensin-(1–7) whereas a 50% increase persisted when the same concentration of angiotensin-(1–7) was studied in combination with 1 μM angiotensin II (Fig. 8). [Sar^1, Thr^8] angiotensin II (10 μM), a nonselective inhibitor of angiotensin II receptors that has been shown to inhibit the biological effects of angiotensin II (1–7) (13), also abolished the $[Ca^{2+}]_i$ peak when added just before angiotensin II or angiotensin IV (results not shown).

In order to evaluate whether angiotensin-(1–7) inhibited the Ca^{2+} influx or the Ca^{2+} release from the intracellular stores, we examined the effects of angiotensin-(1–7) and TMB-8, studied separately or in combination, on the angiotensin II-dependent $[Ca^{2+}]_i$ increase. TMB-8 (100 μM) and angiotensin-(1–7) (10 μM), used alone, inhibited partially the effect of angiotensin II. When used in combination, the $[Ca^{2+}]_i$ increase obtained with 0.1 μM angiotensin II was totally abolished (Fig. 9). Statistical analysis of these results using the 2-way ANOVA method confirmed there were significant ($P < 0.05$) effects of angiotensin-(1–7) and TMB-8. No interaction between the

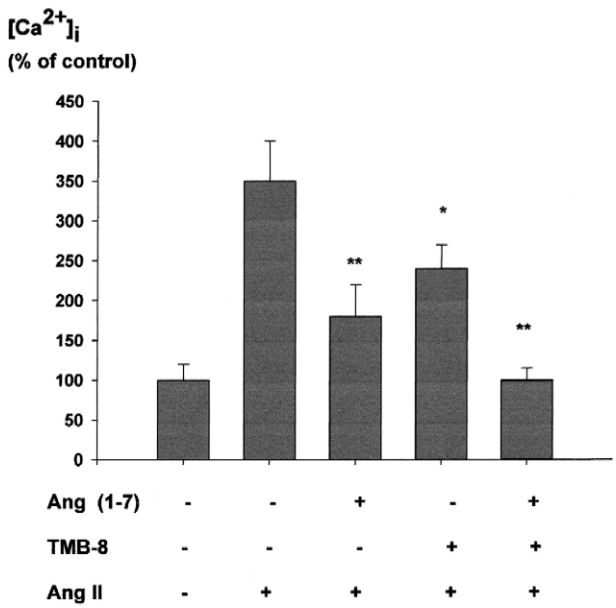

Fig. 9. Effects of 10 μM angiotensin-(1–7) and 100 μM TMB-8, an inhibitor of inositol phosphate-mediated Ca^{2+} release from intracellular stores, studied separately or in combination on angiotensin II-dependent $[Ca^{2+}]_i$ increase in rat mesangial cells. Values are means ± S.E. ($N = 4$). Two-way ANOVA indicated a significant effect of angiotensin-(1–7), TMB-8 and their association; $^{*} = P < 0.05$, $^{**} = P < 0.01$ vs. angiotensin II alone.

agents could be demonstrated, suggesting that TMB-8 and angiotensin-(1–7) acted via independent mechanisms.

3.4. Absence of effects of LVV-H7 on $[Ca^{2+}]_i$ in rat mesangial cells

LVV-H7 (10 μM) did not modify $[Ca^{2+}]_i$ in rat mesangial cells. In addition, it did not influence the subsequent stimulatory effects of either angiotensin IV (1 μM) or angiotensin II (0.1 μM). Therefore, this agent exhibited neither agonistic nor antagonistic effects (Fig. 10). This suggests that LVV-H7 did not occupy the same binding sites as angiotensin II or angiotensin IV.

3.5. Binding studies

To confirm that the agonistic effects of angiotensin IV and the antagonistic effects of angiotensin-(1–7) depended on the prior binding of both peptides to the angiotensin AT_1 receptor, we performed competitive binding inhibition studies with ^{125}I [Sar^1, Ile^8] angiotensin II and rat mesangial cells (Fig. 11). There was a rapid displacement of the radioactive ligand in the presence of angiotensin II and

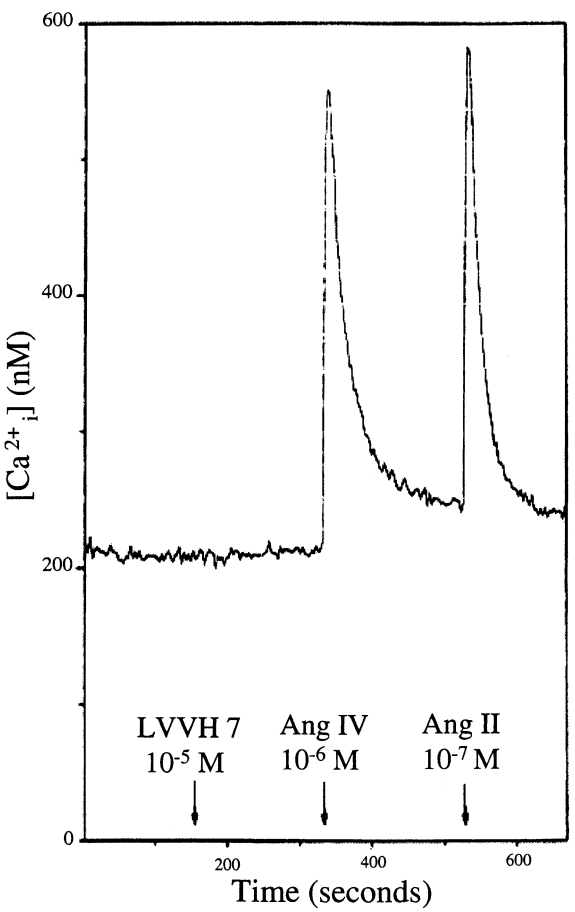

Fig. 10. Typical record of $[Ca^{2+}]_i$ in rat mesangial cells treated successively with LVV-H7 (10 μM), angiotensin IV (1 μM) and angiotensin II (0.1 μM).

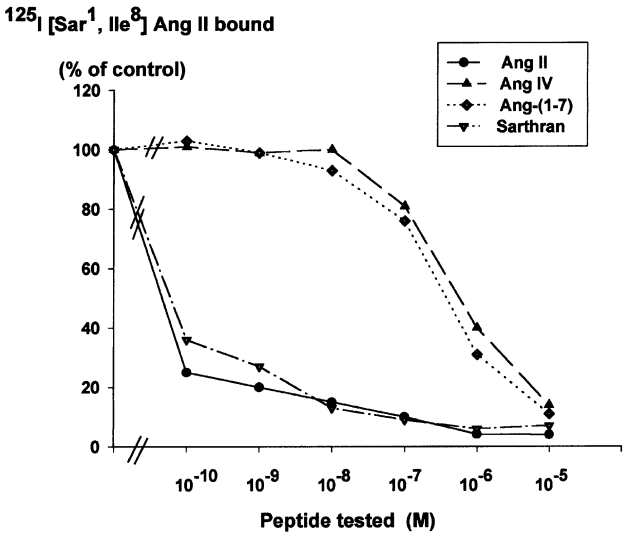

Fig. 11. Competitive inhibition of ^{125}I [Sar^1, Ile^8] Ang II binding to rat mesangial cells in the presence of increasing concentrations of angiotensin II, angiotensin IV, angiotensin-(1–7) and [Sar^1, Thr^8] angiotensin II (Sarthran). Each point is the mean of three values.

[Sar^1, Thr^8] angiotensin II. Inhibitions of 70–80% were obtained with 0.1 nM of each of them. In contrast, angiotensin IV and angiotensin II-(1–7) were markedly less potent. For both of them, only 10% inhibition was observed at 0.1 μM and the concentration giving 50% inhibition was close to 0.5 μM.

4. Discussion

The present study provides novel information on the interactions between angiotensin II and two of its metabolites, the carboxy terminal fragment, angiotensin IV, and the amino terminal fragment, angiotensin-(1–7), in the regulation of $[Ca^{2+}]_i$. The mechanisms by which angiotensin II stimulates $[Ca^{2+}]_i$ in mesangial cells have been extensively described (Ardaillou et al., 1999). Binding of angiotensin II to its AT_1 receptor results in the activation, in the presence of guanyl nucleotides, of the α subunit of a heterotrimeric G protein which, in turn, activates phospholipase C. The latter enzyme hydrolyzes phosphatidylinositol 4,5 bisphosphate to give diacylglycerol and inositol 1,4,5-trisphosphate. This product releases Ca^{2+} from intracellular stores, mainly the endoplasmic reticulum, after binding to specific inositol trisphosphate receptors. In addition to this mechanism, angiotensin II also stimulates Ca^{2+} influx from the extracellular medium through Ca^{2+} channels which are either voltage-dependent or receptor-operated. Activation of voltage-dependent Ca^{2+} channels is preceded by stimulation of a Ca^{2+}-dependent chloride conductance. The resulting chloride efflux produces mesangial cell membrane depolarization and triggers extracellular Ca^{2+} entry. Therefore, in mesangial cells, as in vascular smooth muscle cells, angiotensin II influences

cell contraction by a combination of Ca^{2+} mobilization and Ca^{2+} influx. The respective roles of both events can be evaluated by using pharmacological tools, for example, TMB-8 or thapsigargin to block intracellular Ca^{2+} mobilization and EGTA to chelate extracellular Ca^{2+} and thus inhibit its entry into the cell (Ruan and Arendshorst, 1996).

The glomerulus possesses the enzymatic equipment needed to produce angiotensin IV and angiotensin-(1–7). Glomerular epithelial cells express aminopeptidases A and N (Stefanovic et al., 1992a) and neprilysin (Ardaillou et al., 1992). Mesangial cells express aminopeptidase N (Stefanovic et al., 1992a,b) and, possibly, aminopeptidase A (Troyanovskaya et al., 1996). It is thus likely that angiotensin II and its metabolites are all present in the mesangial cell environment. This prompted us to examine whether both metabolites influence $[Ca^{2+}]_i$ directly or by interfering with angiotensin II.

Angiotensin IV behaves identically to angiotensin II since it stimulates $[Ca^{2+}]_i$ in rat mesangial cells after binding to the angiotensin AT_1 receptor, as shown by the inhibitory effect of losartan and candesartan, two angiotensin AT_1 receptor antagonists. This stimulatory effect of angiotensin IV on $[Ca^{2+}]_i$ in rat mesangial cells is in accordance with a previously published preliminary report (Chansel et al., 1998). It is also consistent with similar findings observed by Dostal et al. (1990) for rat vascular smooth muscle cells. The angiotensin AT_1 receptor-mediated effect of angiotensin IV occurs at higher concentrations than that of angiotensin II (0.1 μM vs. 5 nM), suggesting a weak affinity of angiotensin IV for the angiotensin AT_1 receptor. This appears clearly from the data shown in Fig. 10. The concentrations providing 50% inhibition of maximum binding of ^{125}I [Sar^1, Ile^8] angiotensin II to rat mesangial cells were about 0.5 μM for angiotensin IV and less than 0.1 nM for angiotensin II. Interestingly, the $[Ca^{2+}]_i$ stimulating effect of angiotensin IV and its capacity to bind angiotensin AT_1 receptors were observed in the same range of concentrations. The fact that angiotensin IV stimulates angiotensin AT_1 receptors and thus acts as a vasoconstrictor agent has been demonstrated in several models, in particular in the mesenteric and hindlimb vascular beds of the cat (Champion and Kadowitz, 1997; Garrison and Kadowitz, 1996; Garrison et al., 1995) and of the rat (Champion et al., 1998; Gardiner et al., 1993). Recently, we also reported that angiotensin IV exerted vasoconstrictor effects on normal or phenylephrine-precontracted rat mesenteric arteries, effects that were abolished by losartan and candesartan (Loufrani et al., 1999). As in mesangial cells, angiotensin IV had a much lower affinity for angiotensin AT_1 receptors than its precursor angiotensin II in all of these models. LVV-H7, a hemorphin which recognizes the angiotensin AT_4 but not the angiotensin AT_1 receptor, exhibited neither an agonistic effect on the $[Ca^{2+}]_i$ increase when administered alone nor an antagonistic effect when given just before angiotensin IV or angiotensin II. This result thus confirms that the stimulatory effect of angiotensin IV on $[Ca^{2+}]_i$ in rat mesangial cells is angiotensin AT_1 receptor-, but not angiotensin AT_4 receptor-dependent. Indeed, we previously demonstrated that LVV-H7 is a potent inhibitor of ^{125}I angiotensin IV binding to angiotensin AT_4 receptor sites in rabbit collecting duct cell membranes (Garreau et al., 1998). The mechanisms by which angiotensin IV and angiotensin II increase $[Ca^{2+}]_i$ in mesangial cells are, however, not entirely the same. Two differences can be noted. First, the initial exposure of mesangial cells to 0.1 μM angiotensin II totally prevented the subsequent effect of both 0.1 μM angiotensin II and 1 μM angiotensin IV on $[Ca^{2+}]_i$ whereas pretreatment with 1 μM angiotensin IV did not modify the stimulatory effect of 0.1 μM angiotensin II and only attenuates that of 1 μM angiotensin IV. The latter result is in accordance with our previous report that, in contrast with angiotensin II, repeated angiotensin IV (1 μM)-dependent contractions can be observed in the same vessel (Loufrani et al., 1999). Secondly, incubation of mesangial cells in a Ca^{2+}-free medium totally abolished the effect of angiotensin IV on $[Ca^{2+}]_i$ whereas it partially decreased that of angiotensin II.

Moreover, the stimulatory effect of angiotensin IV on $[Ca^{2+}]_i$ as well as that of angiotensin II persisted after thapsigargin treatment when the cells were incubated in the presence of 1.8 mM Ca^{2+} whereas the stimulatory effect was abolished in a Ca^{2+}-free medium. These results demonstrate that angiotensin IV essentially influences the influx-related component of the $[Ca^{2+}]_i$ increase, but they do not lead to the conclusion that angiotensin IV only acts on Ca^{2+} entry into the cell because this peptide also slightly but significantly increased inositol phosphate production suggesting it also produces mobilization of Ca^{2+} from its cellular stores. However, it is likely that the role of Ca^{2+} entry in the $[Ca^{2+}]_i$ peak after angiotensin IV treatment is most important. Almost similar results were observed by Dulin et al. (1995) in opossum kidney cells. Angiotensin IV failed to influence inositol phosphate formation in this preparation but, as we also found, EGTA completely abolished the angiotensin IV-induced increase of $[Ca^{2+}]_i$, which is consistent with an exclusive role on Ca^{2+} influx. Whereas in rat mesangial cells, the stimulatory effect of angiotensin IV on $[Ca^{2+}]_i$ implies angiotensin AT_1 receptor activation, an angiotensin AT_1 receptor-independent $[Ca^{2+}]_i$ increase has been observed in lung endothelial cells (Patel et al., 1999). In this preparation, angiotensin IV stimulated $[Ca^{2+}]_i$ even in a Ca^{2+}-free medium and in the presence of angiotensin AT_1 and AT_2 receptor antagonists. All of these results underline the complexity of the effects of angiotensin IV due to the multiplicity of its receptor types (Loufrani et al., 1999).

Angiotensin-(1–7), in contrast with angiotensin IV, behaved as an antagonist of angiotensin II at the angiotensin AT_1 receptor binding site. There was a progressive inhibition of the $[Ca^{2+}]_i$ peak obtained with 0.1 μM angiotensin

II or 1 μM angiotensin IV when increasing concentrations of angiotensin-(1–7) were simultaneously added. Total inhibition and 74% inhibition were obtained with 10 μM of angiotensin-(1–7) for the angiotensin IV and the angiotensin II effect, respectively. Of note, these inhibitory concentrations (0.5–10 μM) of angiotensin-(1–7) corresponded exactly to those displacing ^{125}I [Sar^1, Ile^8] angiotensin II from the angiotensin AT_1 receptor binding sites of rat mesangial cells. The mechanism of the residual stimulation of $[Ca^{2+}]_i$ observed with 0.1 μM angiotensin II and 10 μM angiotensin-(1–7) in combination cannot be due to angiotensin-(1–7) because the latter did not exhibit an agonistic effect, even at this high concentration. It can only result from the activation of a small fraction of the angiotensin AT_1 receptors still occupied by angiotensin II. There is accumulating evidence that angiotensin-(1–7) opposes the actions of angiotensin II in many systems and, particularly, in the kidney (Ferrario et al., 1998; Tallant et al., 1999; Jaiswal et al., 1993; Ferrario et al., 1997). The mechanism of this counterregulation may involve the production of vasodilator mediators, such as nitric oxide or prostanoids, or may be direct. The latter process has been shown to operate in the vasculature (Mahon et al., 1994) and seems also to be implicated in the angiotensin II- or angiotensin IV-dependent $[Ca^{2+}]_i$ increase in rat mesangial cells. The fact that angiotensin-(1–7) did not modify basal $[Ca^{2+}]_i$ of rat mesangial cells also suggests that, in this preparation, it cannot influence nitric oxide or prostanoid production via pathways involving the mobilization of $[Ca^{2+}]_i$. We used pharmacological tools to analyze the mechanism by which angiotensin-(1–7) inhibits the angiotensin II-dependent $[Ca^{2+}]_i$ increase. TMB-8 (0.1 mmol/l), an inhibitor of inositol phosphate-mediated release of Ca^{2+} from its intracellular stores, did not inhibit entirely the $[Ca^{2+}]_i$ increase observed in the presence of 0.1 μM angiotensin II, demonstrating that the angiotensin II effect is maintained in part when the mobilization of intracellular Ca^{2+} is blocked. Angiotensin-(1–7), although used at a high concentration (10 μM), blunted but did not abolish both the angiotensin II-dependent $[Ca^{2+}]_i$ peak and the angiotensin II-dependent inositol phosphate production. Interestingly, the combined treatment with angiotensin-(1–7) and TMB-8 inhibited totally the effect of angiotensin II on $[Ca^{2+}]_i$. This suggests that angiotensin-(1–7) acts on Ca^{2+} influx rather than on $[Ca^{2+}]_i$ mobilization which was blocked in the presence of TMB-8. Such a hypothesis would also explain the observation that angiotensin-(1–7) completely inhibited the angiotensin IV-dependent $[Ca^{2+}]_i$ increase which was mainly due to Ca^{2+} entry from the extracellular medium. Ueda et al. (2000) showed recently that angiotensin-(1–7) attenuated vasoconstriction evoked by angiotensin II in human forearm resistance vessels, thus confirming the antagonistic action of angiotensin-(1–7) on the $[Ca^{2+}]_i$-mediated effects of angiotensin II.

Whether angiotensin IV and angiotensin-(1–7) generation can interact in vivo with the effects of angiotensin II on glomerular function remains questionable. Our results demonstrate that angiotensin IV and angiotensin-(1–7) acted on $[Ca^{2+}]_i$ or angiotensin II-dependent $[Ca^{2+}]_i$ at concentrations 20 and 100 times greater than those of angiotensin II. The agonistic effect of angiotensin IV was thus observed at high concentrations, making it unlikely that this peptide plays a role in the physiological control of $[Ca^{2+}]_i$ in mesangial cells, even if the marked levels of aminopeptidases A and N in the kidney (Stefanovic et al., 1992a,b) suggest the rapid degradation of angiotensin II into this metabolite. Of note, the circulating levels of angiotensin-(1–7) increase 25- to 50-fold during converting enzyme inhibition (Luque et al., 1996). Moreover, the demonstration of high amounts of angiotensin-(1–7) in urine (Ferrario et al., 1997) suggests that the kidney is an important source of angiotensin-(1–7), which is in agreement with the predominant distribution of neprilysin in the renal tissue. It is thus possible that concentrations of angiotensin-(1–7) that are high enough to interact with angiotensin II are reached in the kidney.

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