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Intracellular Angiotensin II and cell growth of vascular smooth muscle cells

[1]Catalin M. Filipeanu, [1]Robert H. Henning, [1]Dick de Zeeuw & *,[1]Adriaan Nelemans

[1]Department of Clinical Pharmacology, University of Groningen, A. Deusinglaan 1, 9713AV Groningen, The Netherlands

1 We recently demonstrated that intracellular application of Angiotensin II (Angiotensin II_{intr}) induces rat aorta contraction independent of plasma membrane Angiotensin II receptors. In this study we investigated the effects of Angiotensin II_{intr} on cell growth in A7r5 smooth muscle cells.

2 DNA-synthesis was increased dose-dependently by liposomes filled with Angiotensin II as measured by [^{3}H]-thymidine incorporation at high ($EC_{50} = 27 \pm 6$ pM) and low ($EC_{50} = 14 \pm 5$ nM) affinity binding sites with increases in E_{max} of 58 ± 4 and 37 ± 4% above quiescent cells, respectively. Cell growth was corroborated by an increase in cell number.

3 Extracellular Angiotensin II (10 pM – 10 μM) did not modify [^{3}H]-thymidine incorporation.

4 Growth effects of Angiotensin II_{intr} mediated *via* high affinity sites were inhibited by liposomes filled with 1 μM of the non-peptidergic antagonists losartan (AT_1-receptor) or PD123319 (AT_2-receptor) or with the peptidergic agonist CGP42112A (AT_2-receptor). E_{max} values were decreased to 30 ± 3, 29 ± 4 and 4 ± 2%, respectively, without changes in EC_{50}. The Angiotensin II_{intr} effect *via* low affinity sites was only antagonized by CGP42112A ($E_{max} = 11 \pm 3$%), while losartan and PD123319 increased E_{max} to 69 ± 4%. Intracellular applications were ineffective in the absence of Angiotensin II_{intr}.

5 Neither intracellular nor extracellular Angiotensin I (1 μM) were effective.

6 The Angiotensin II_{intr} induced growth response was blocked by selective inhibition of phosphatidyl inositol 3-kinase (PI-3K) by wortmannin (1 μM) and of the mitogen-activated protein kinase (MAPK/ERK) pathway by PD98059 (1 μM) to 61 ± 14 and 4 ± 8% of control, respectively.

7 These data demonstrate that Angiotensin II_{intr} induces cell growth through atypical AT-receptors *via* a PI-3K and MAPK/ERK -sensitive pathway.

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Keywords: Intracellular angiotensin II; growth; losartan; PD123319; CGP42112A; PI-3 kinase; MAP kinase; A7r5 cells

Abbreviations: Angiotensin II_{intr}, intracellular angiotensin II; CGP42112A, nicotinic acid-Tyr-(N-benzoylcarbonyl-Arg)-Lys-His-Pro-Ile-OH; DMEM, Dulbecco's Modified Eagle's Medium; ERK, extracellular signal-regulated kinase; FCS, foetal calf serum; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; losartan, (2-*n*- butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole); MAPK, mitogen-activated protein kinase; PI-3K, phosphatidyl inositol 3-kinase; PD98059, 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; PD123319, (s)-1-(4-[dimethylamino]-3-methylphenyl)methyl-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-c]pyridine-6-carboxylate

Introduction

It has been extensively documented that the renin-angiotensin system is a major factor in the regulation of cardiovascular homeostasis, including blood pressure, mineral balance and tissue remodelling (Weber, 1998). However, the beneficial effects of ACE inhibitors on tissue remodelling appear to be independent, at least in part, of their effects on blood pressure (Linz *et al*., 1995). In this respect, Angiotensin II can be generated either in the kidney and released in the circulation (circulating Angiotensin II) subsequently activating different plasma membrane receptors or it can be produced in different tissues to exert its effects at the place of production (local Angiotensin II; Danser & Schalekamp, 1996). To date, two different receptors have been cloned; namely the AT_1 and AT_2-subtype receptor (Griendling *et al*., 1996). These receptors are differently localized and have different functions, among which is modulation of cellular growth. The AT_1-receptor, which is prominent in adult tissues, stimulates cell growth (Matsukada & Ichikawa, 1997). In contrast, the AT_2-receptor, which is mostly abundant in foetal tissues, inhibits cell growth and promotes apoptosis (Xoriuchi *et al*., 1999).

There is growing evidence for intracellular actions of Angiotensin II not related to activation of 'classical' plasma membrane receptors. We recently reported effects of intracellular Angiotensin II (Angiotensin II_{intr}) on rat aorta contraction, independent of activation of plasma membrane Angiotensin II receptors (Brailoiu *et al*., 1999). Intracellular Angiotensin II was reported to increase cytosolic [Ca^{2+}] in vascular smooth muscle cells (Haller *et al*., 1996; 1999), to inhibit gap conductance in heart muscle (De Mello, 1996) and to affect L-type Ca^{2+} channel in a specific manner (De Mello, 1998). Such changes in Ca^{2+} homeostasis are important for cell growth, therefore we addressed the following questions in

*Author for correspondence; E-mail: s.a.nelemans@farm.rug.nl

this investigation: (1) Is there a role for an Angiotensin II_{intr} receptor in vascular smooth muscle cell growth. (2) Is the receptor similar to the known subtypes based on its pharmacological profile. (3) Can we identify part of its signal transduction pathway leading to cell growth.

Methods

Cell culture

A7r5 vascular smooth muscle cells were grown in 75 cm^2 flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum (FCS), penicillin (50 $\mu g\ ml^{-1}$) and streptomycin (50 units ml^{-1}) at 37°C in a humidified atmosphere (5% CO_2). The cells were subcultured at 95% confluency by trypsinization. Cell number was established by counting dispersed cells in a Bürker counter (Schreek, Germany). Experiments were performed in 6 well plates (Costar, 9.6 $cm^2\ well^{-1}$) at a density of 10^5 cells $well^{-1}$, unless stated otherwise.

Determination of DNA synthesis

To obtain quiescent cells, the medium was replaced with DMEM containing 0.1% foetal calf serum 24 h after plating. The intracellular additions of Angiotensin II by liposomal delivery were performed 1 day after the switch to 0.1% FCS containing DMEM and lasted for another 24 h. Extracellular addition of various compounds happened 1 min prior to liposomal addition. [^{3}H]-thymidine (0.5 μCi, specific activity: 20 Ci $mmol^{-1}$) was added on each well during the final 3 h of incubation. The medium was withdrawn at the end of the incubation period and the cells were washed twice with ice-cold phosphate buffered saline (PBS). To remove non-genomic [^{3}H]-thymidine, the cells were incubated in the presence of 400 μl trichloroacetic acid for 1 h on ice. Finally, the cells were digested with 1 ml of 1 M NaOH and the incorporated radioactivity was measured in a β-scintillation counter. Quiescent cells incorporated 210 ± 11 d.p.m. $well^{-1}$ (mean $\pm$ s.e.mean, $n = 24$), whereas 10% FCS stimulated cells incorporated 1078 ± 71 d.p.m. $well^{-1}$ ($n = 24$).

Liposomes preparation

Liposomes containing Angiotensin II or Angiotensin I and control liposomes containing 140 mM KCl were prepared as described (Brailoiu *et al*., 1999) from egg phosphatidyl choline, using 10 mg ml^{-1} of solution to be incorporated. Dialysis against PBS solution was performed for 4 h in order to remove the non-incorporated compounds. To maintain sterile cell culture conditions the liposomes solution was filtrated (0.2 μm pore size). Liposomes were added to the medium above the cells in a ratio of 1 to 20 (v v^{-1}). If other compounds were delivered intracellularly, they were encapsulated together with Angiotensin II. The amount of Angiotensin II delivered intracellularly was determined using ^{125}I-angiotensin II filled liposomes. The incorporation into liposomes after the filtration step was $7.2 \pm 0.2\%$ ($n = 8$) of the initial amount of radioactive Angiotensin II added to the cells. Recovery of incorporated ^{125}I angiotensin II into the cells after incubation for 30 min amounted to $5.6 \pm 0.2\%$ ($n = 8$) of the initial amount of radioactive Angiotensin II added to the cells.

Measurement of inositol (1,4,5) trisphosphate (Ins(1,4,5)P_3)

Mass measurements of Ins(1,4,5)P_3 were performed as described earlier (Sipma *et al*., 1995), using an isotope dilution ligand binding assay. In brief, samples were assayed in 25 mM Tris/HCl (pH = 9.0), 1 mM EDTA, 1 mg bovine serum albumin, D-[inositol-1-^{3}H(N)]-Ins(1,4,5)P_3 (21.0 Ci $mmol^{-1}$, 2000 c.p.m. $assay^{-1}$) and 1 mg binding protein isolated from beef liver. Bound and free radioactivity was separated by centrifugation. The radioactivity in the pellet was determined by scintillation counting.

Measurement of intracellular Ca^{2+}

Intracellular [Ca^{2+}] was measured using Fura-2 fluorometry as described (Filipeanu *et al*., 1997). Cells were loaded with 5 μM Fura-2 acetoxymethyl ester at 22°C, for 45 min in the dark. Fluorescence was measured at 37°C.

Chemicals

All cell culture media were purchased from Gibco BRL, phosphatidyl choline type X-E, Angiotensin I, Angiotensin II, and wortmannin from Sigma Chemical Co, CGP 422112A (nicotinic acid-Tyr-(N-benzoylcarbonyl-Arg)-Lys-His-Pro-Ile-OH) from RBI, and PD98059 (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one) from Calbiochem. Fura 2-AM was obtained from Molecular Probes, losartan (2-*n*- butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole) from Merck, Sharpe and Dohme, PD123319 ((s)-1-(4-[dimethylamino]-3-methylphenyl)methyl-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-c]pyridine-6-carboxylate) from Park-Davis, [6-^{3}H]-thymidine from Amersham Int, D-[inositol-1-^{3}H(N)]-Ins(1,4,5)P_3 from NEN Life Science Products, and all other agents from Merck.

Data analysis

Data are given as mean $\pm$ s.e.mean. The results of the growth experiments are expressed as percentage of the radioactivity incorporated by control quiescent cells. Independent measurements were performed in at least two different passages. Measurements were normalized against liposomes filled with 10^{-7} M Angiotensin II, present in every experimental protocol. Statistical significance was tested by one-way ANOVA followed by Bonferroni test. A value of $P < 0.05$ was considered statistically significant. Concentration response curves were fitted and the corresponding parameters calculated using Multifit (Dr J.H. Proost, Department of Pharmacokinetics and Drug Delivery, University Centre for Pharmacy, University of Groningen). Curve fitting was based on the following sigmoidal model: $Y = V_1 + V_2 \times X \hat{} V_3 / (X \hat{} V_3 + V_4 \hat{} V_3) + V_5 \times X \hat{} V_6 / (X \hat{} V_6 + V_7 \hat{} V_6)$. Fitting for a single binding site was performed after omission of the term containing the parameters V5, V6 and V7. To determine if the data were fitted significantly better with one or two binding sites the variance was calculated using the *F*-test.

Results

Intracellular Angiotensin II stimulates cell growth in quiescent A7r5 cells

Addition of Angiotensin II filled liposomes increases DNA synthesis in a dose-dependent fashion, as measured by [^{3}H]-thymidine incorporation into A7r5 cells (Figure 1). The first observation above the background was obtained with liposomes containing 10 pM Angiotensin II, whereas the maximum effect was reached with liposomes containing 0.1 μM Angiotensin II (doubling of [^{3}H]-thymidine incorporation compared to quiescent cells). Data analysis showed that the increases in [^{3}H]-thymidine incorporation were better described using a model with two binding site kinetics. Parameters of the dose-response curve are given in Table 1. The simplest model with $V_6 = 1$ was used for further analysis since varying the Hill-coefficient (V_6) from 1 to 9 for the second binding site did not significantly alter the fitting results. Neither 'empty' control liposomes, filled only with 140 mM KCl, nor liposomes filled with the parent peptide Angiotensin I (1 μM) affected [^{3}H]-thymidine incorporation (102.7$\pm$3.8%, $n=36$ and 102.6$\pm$0.8%, $n=12$ of control cells, respectively). The growth stimulating effect of liposomes containing 0.1 μM Angiotensin II was corroborated in experiments showing actual increases in cell number (Table 2).

The growth stimulating effect of Angiotensin II filled liposomes (0.1 μM Angiotensin II) was unchanged by extracellular addition (1 μM) of the nonpeptidergic AT_1-type receptor antagonist losartan, the nonpeptidergic AT_2-type receptor antagonist PD123319, or the peptidergic AT_2-type receptor agonist CGP42112A (97.1$\pm$4.0%, 103.9$\pm$2.8%, and 91.7$\pm$5.6% of control cells, $n=18$, respectively).

In contrast to intracellular delivered Angiotensin II, extracellular application of Angiotensin II (10 pM to 10 μM) did not change [^{3}H]-thymidine incorporation in quiescent A7r5 cells. A similar amount of radioactivity as in control cells was incorporated after 0.1 μM extracellular Angiotensin II compared to control cells (98.1$\pm$3.2%, $n=12$). A7r5 cells apparently lack functional AT -receptors which is also evident from the inability of extracellular Angiotensin II (10 μM) to change basal Ins(1,4,5)P_3 formation (2.3$\pm$0.6 *vs* 2.1$\pm$0.8 pmol 10^5 cells^{-1}, $n=12$) or basal intracellular Ca^{2+} concentration (57$\pm$6 *vs* 58$\pm$7 nM, $n=12$), as reported before (Filipeanu *et al.*, 1998a,b).

Furthermore, extracellular application of Angiotensin I (1 μM, 102$\pm$2.4%, $n=12$), losartan (1 μM, 95.6$\pm$4.7%, $n=24$), PD123319 (1 μM, 98.7$\pm$5.8%, $n=16$) or CGP42112A (1 μM, 92.9$\pm$4.8%, $n=18$) did not affect [^{3}H]-thymidine incorporation.

Pharmacology of intracellular Angiotensin II

We next attempted to characterize pharmacologically the effects of Angiotensin II_{intr}. Addition of liposomes filled with losartan (1 μM), PD123319 (1 μM) together with various concentrations of Angiotensin II reduced [^{3}H]-thymidine incorporation (Figure 1). Abolition of the growth stimulating effect of Angiotensin II was obtained by liposomes filled with the AT_2-type receptor agonist CGP42112A (1 μM, Figure 1). All three agents substantially reduced stimulation of the high affinity binding site. Non-competitive inhibition in this receptor site is likely involved in view of the decreasing E_{max} value without changes in EC_{50}. The following rank order of antagonist potencies was obtained: CGP42112A > PD123319 = losartan (Figure 2, Table 3). In contrast, at the low affinity binding site these compounds elicited opposite effects. Again strong inhibition was observed for CGP42112A, but losartan and PD123319 significantly increased E_{max} as compared to control. It is noticeable that maximal values obtained at this site are comparable to the control value at the high affinity binding site (Figure 2, Table 3). To verify the nature of the antagonism by the compounds studied additional experiments were performed at other concentrations of the antagonists across a limited range of Angiotensin II concentrations (Table 3). Although one should be cautious not to over interpret the fitting results of the limited data, it is clear that no evidence of a parallel shift of the log dose-response relationship was observed for both binding sites. The lowest antagonist concentration used (30 nM losartan) was insufficient to induce inhibition. At high antagonist concentrations (e.g. 30 μM PD123319) further increases were not observed in the E_{max} of the low affinity binding site. This was also concluded from the experiment in which losartan and PD123319 (both 1 μM) were given simultaneously, and from an experiment using losartan (10 μM) or PD123319 (10 μM) at a single dose of Angiotensin II (100 nM), showing maximal [^{3}H]-thymidine incorporation was maintained (98.2$\pm$4.1%, $n=6$ and 109.0$\pm$6.5%, $n=6$ for losartan- and PD123319 filled liposomes, respectively).

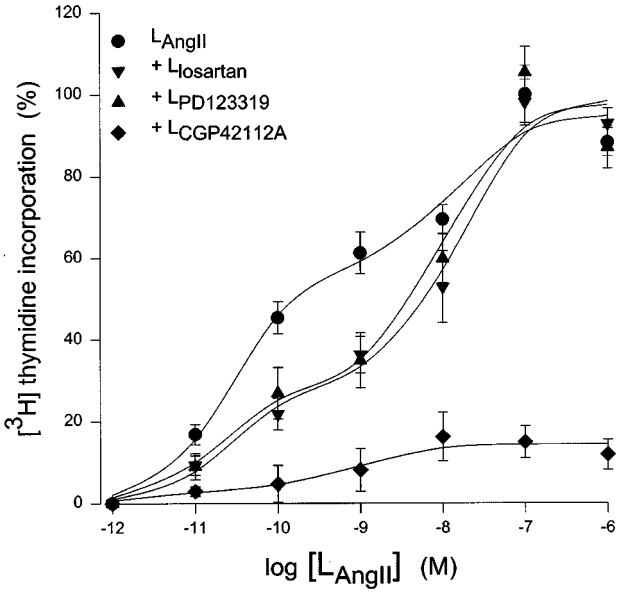

Figure 1 Effects of Angiotensin II filled liposomes on A7r5 cell growth. Effect curves of Angiotensin II filled liposomes alone ($n=12$) and in the presence of liposomes encapsulating losartan, PD123319 or CGP42112A (all 1 μM, $n=12$). All results were reported as increases (%) above [^{3}H]-thymidine incorporation in quiescent cells. The maximal value (100% at $L_{Angiotensin\ II} = 0.1\ \mu M$) corresponds to 448$\pm$56 d.p.m. well^{-1}, $n=24$). Lines were fitted according to the two binding site model given in Table 1.

Table 1 Dose-response parameters of Angiotensin II_{intr} induced [^{3}H]-thymidine incorporation

		Control ($L_{Angiotensin\ II}$)	
Binding sites		*1*	*2**
E_{max}	(V_2)	93.5 ± 2.5 (%)	58.3 ± 3.7 (%)
Hill-coefficient	(V_3)	0.46 ± 0.04	1.02 ± 0.16
EC_{50}	(V_4)	$2.2 \pm 0.5 \times 10^{-10}$ (M)	$2.7 \pm 0.6 \times 10^{-11}$ (M)
E_{max}	(V_5)	–	36.9 ± 3.8 (%)
Hill-coefficient	(V_6)	–	1 (fixed)
EC_{50}	(V_7)	–	$1.4 \pm 0.5 \times 10^{-8}$ (M)

Dose-effect curves of liposomes filled with Angiotensin II ($L_{Angiotensin\ II}$) in the range of 1 pM to 1 μM were fitted using the equation: $Y = V_1 + V_2 \times X^{\wedge\diamond} V_3/(X^{\wedge} V_3 + V_4^{\wedge} V_3) + V_5 \times X^{\wedge} V_6/(X^{\wedge} V_6 + V_7^{\wedge} V_6)$. The last term was omitted for the single site fit. Effects are expressed as increases (%) above [$_3$H]-thymidine incorporation in quiescent cells ($V_1 = 0$) and presented as mean $\pm$ s.e.mean, $n = 12$ for each concentration. Significance level: **F*-value = 23.8, $P < 0.0001$ *vs* single site model.

Table 2 Effect of intracellular applied Angiotensin II on cell number

	Cell number flask^{-1}
Control	$81 \pm 11 \times 10^3$
$L_{control}$	$75 \pm 6 \times 10^3$
$L_{Angiotensin\ II}$	$114 \pm 12 \times 10^3$*

A7r5 cells were plated in 25 cm^2 flasks at a density of 2.10^3 cells cm^{-2}. After 24 h the medium containing 10% FCS was replaced for 24 h with medium containing 0.1% FCS. Then either intracellular Angiotensin II ($L_{Angiotensin\ II}$) using liposomes filled with Angiotensin II (0.1 μM) or control liposomes ($L_{control}$) containing 140 mM KCl were applied. The cell number was counted 24 h after application of the liposomes (mean $\pm$ s.e.mean, $n = 4$, determination in triplicate). Significance level: *$P < 0.05$ *vs* $L_{control}$.

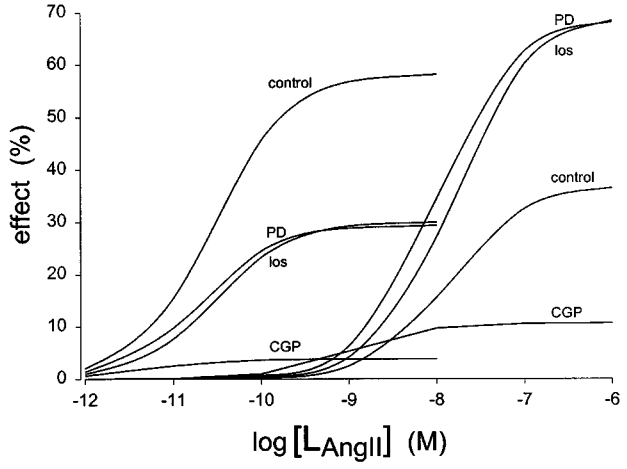

Figure 2 Contribution of the different binding sites to growth induced by intracellular Angiotensin II in the absence or presence of antagonists. Dose-response curves were plotted using the data of Figure 1 according to the model as given in Table 1. Angiotensin II filled liposomes were in the absence (control) or in the presence of liposomes encapsulating losartan (los), PD123319 (PD) or CGP42112A (CGP). Values obtained for E_{max} and EC_{50} are presented in Table 3.

In the absence of Angiotensin II filled liposomes, losartan-, PD123319-, and CGP4112A-filled liposomes did not modify basal [^{3}H]-thymidine incorporation into quiescent cells ($n = 12$; $99.1 \pm 2.9\%$, $101.5 \pm 2.4\%$, $98.3 \pm 5.5\%$ of control, respectively).

Signal transduction of intracellular Angiotensin II effects

Extracellular signals often use multiple pathways to modify cell growth. In order to gain insight in the mechanism involved in growth stimulation by Angiotensin II_{intr} two of these pathways were tested. Intracellular Angiotensin II induced cell growth was totally abolished by inhibition of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway by co-incubation of the cells with extracellular PD98059 (1 μM, Figure 3). Growth stimulation was also reduced, but to a lesser extent by inhibition of the phosphatidyl inositol 3-kinase (PI-3K) pathway with wortmannin (1 μM, Figure 3).

Discussion

In the present study, we demonstrate that Angiotensin II_{intr} stimulates cell growth in quiescent A7r5 cells. Although 'classical' plasma membrane Angiotensin II receptors are involved in growth and apoptotic processes underlying cardiovascular remodelling (Dzau & Horiuchi, 1998) our results suggest additional targets for Angiotensin II at sites which have not previously been recognized. The growth response was characterized by the presence of two distinct binding sites for Angiotensin II_{intr}. The high affinity site (picomolar range) was sensitive to intracellular delivered antagonists in the rank order of potencies, CGP42112A > PD123319 = losartan. In contrast, only the peptidergic antagonist CGP42112A could inhibit the low affinity site (nanomolar range). The other compounds even increased the maximal effect to the value obtained by stimulation of the high affinity site, indicating that both sites are possibly closely involved in the growth response. Also important is the observation that all three compounds are ineffective in the absence of Angiotensin II_{intr}, showing that occupation of the receptor site by Angiotensin II is needed for their action. Although the nature and physiological significance of these distinct sites needs further investigation, it is unlikely that 'classical' AT_1- or AT_2-receptors (De Gasparo *et al*., 1998) mediate the Angiotensin II_{intr} effect on [^{3}H]-thymidine incorporation in view of the different potencies and the lack of competitive inhibition observed, and the growth inhibitory action of both CGP42112A and PD123319. These compounds were described as agonist and antagonist of the anti-proliferative AT_2-receptor subtype,

Table 3 Effect of $L_{Angiotensin\ II}$ on [^{3}H]-thymidine incorporation in the presence of various agents on dose-response parameters

	E_{max} (h) (%)	E_{max} (l) (%)	EC_{50} (h) (M)	EC_{50} (l) (M)	Datapoints (n)
$L_{Angiotensin\ II}+L_{los7.5}$	68±8	42±10	$3\pm2\ 10^{-11}$	$2\pm2\ 10^{-8}$	24
$L_{Angiotensin\ II}+L_{los6}$	30±3*	69±4*	$3\pm1\ 10^{-11}$	$1.5\pm0.3\ 10^{-8}$	84
$L_{Angiotensin\ II}+L_{PD6}$	29±4*	69±4*	$2\pm1\ 10^{-11}$	$1.0\pm0.2\ 10^{-8}$	84
$L_{Angiotensin\ II}+L_{PD4.5}$	40±8*	61±10*	$5\pm4\ 10^{-11}$	$2\pm1\ 10^{-8}$	24
$L_{Angiotensin\ II}+L_{los6}+L_{PD6}$	17±2*	65±3*	$1.5\pm0.9\ 10^{-11}$	$3.2\pm0.7\ 10^{-8}$	84
$L_{Angiotensin\ II}+L_{CGP6}$	4±2*	11±3*	$0.5\pm1.2\ 10^{-11}$*	$0.1\pm0.1\ 10^{-8}$	84

Liposomes were filled with Angiotensin II ($L_{Angiotensin\ II}$) in the range of 1 pM to 1 μM and added in the presence of losartan (L_{los}), PD123319 (L_{PD}) or CGP42112A (L_{CGP}) at concentrations as indicated by its anti-log. Effects are expressed as increases (%) above [^{3}H]-thymidine incorporation in quiescent cells. Parameters were obtained after fitting the two binding site model as given in Table 1, with the constraints $V_1=0$, $V_3=V_6=1$ and presented for the high (h)- and low (l) affinity sites as mean±s.e.mean. Datapoints were obtained from either seven or four different concentrations with $n=12$ or 6 each. Significance level: $^*P<0.05$ *vs* $L_{Angiotensin\ II}$ (Table 1).

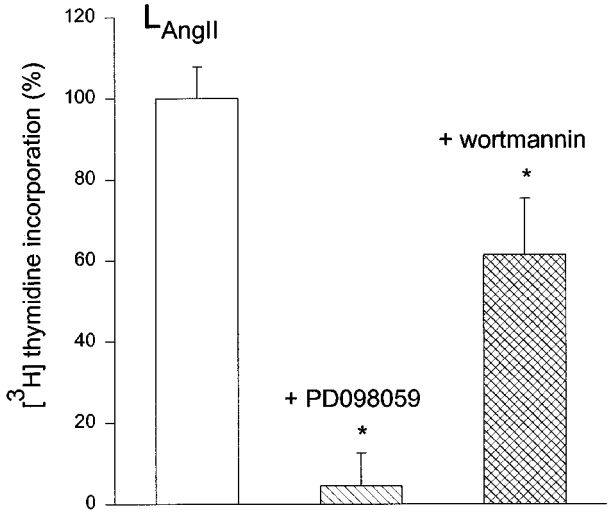

Figure 3 Involvement of MAPK and PI-3K in Angiotensin II induced cell growth. Cell growth induced by Angiotensin filled liposomes ($L_{Angiotensin\ II}$, 0.1 μM) was inhibited by simultaneous extracellular treatment with PD98059 (1 μM, $n=16$) or wortmannin (1 μM, $n=16$).

respectively (Timmermans *et al*., 1992; De Gasparo *et al*., 1998). Antagonist activity of CGP42112A on AT_1- or AT_2-receptors has only been reported for extracellular Angiotensin II mediated phospholipase A2 activation (Lokuta *et al*., 1994). The presence of an unusual type of receptor in A7r5 cells becomes also apparent from the lack of the growth response by extracellular addition of Angiotensin II and the ineffectiveness of extracellular addition of AT_1-or AT_2-receptor antagonists. No functional plasma membrane AT-receptors seems to be present in A7r5 cells, also in view of the absence of other cellular responses to extracellular Angiotensin II like Ins(1,4,5)P_3 formation and cytosolic [Ca^{2+}] elevation. Therefore, the growth response elicited by Angiotensin II_{intr} is likely to be mediated by atypical AT-receptors with distinct pharmacology from AT_1- or AT_2-receptors.

Liposomes filled with the parent peptide Angiotensin I did not affect DNA synthesis. In contractility studies of adult rat aorta, we observed that both Angiotensin I and Angiotensin II filled liposomes induced contraction (Brailoiu *et al*., 1999). Either the contractile and growth responses are not intimately related or different pharmacological profiles of Angiotensin II_{intr}-receptors are present in different cell types. Differences in pharmacological receptor profiles among different cell types is supported by recent observations that Angiotensin II_{intr} inhibited inward Ca^{2+} current in rat cardiac myocytes, but stimulated this current in hamster cardiac myocytes (De Mello, 1998).

The presence of various atypical angiotensin receptors was reported previously (Noble *et al*., 1993; 1996; Smith, 1995; Regitz-Zagrosek *et al*., 1996; Li *et al*., 1998; Moriuchi *et al*., 1998). The pharmacological profile of one of those atypical angiotensin receptors resembles the profile obtained in A7r5 cells. Although observed in another species, this receptor mediates microvascular network formation in chick embryo, has a low affinity for losartan and PD123319 and is antagonized by CGP42112A (Noble *et al*., 1993; 1996). Further studies are necessary to elucidate if the receptors activated by Angiotensin II_{intr}, as observed by us, are related to one of those atypical receptors, and to establish their existence and binding profiles in other tissues.

Extracellular Angiotensin II induces several effects commonly evoked by growth factor receptor stimulation, such as tyrosine phosphorylation or activation of the Ras/ERK pathway ultimately leading to protein synthesis and cell cycle progression (Berk, 1999; Eguchi *et al*., 1999; Inagami *et al*., 1999). Stimulation of plasma membrane AT_1 -receptors activate the MAPK cascade in vascular smooth muscle cells other than A7r5 cells (Ge & Anand-Srivastava, 1998; Li *et al*., 1998; Moriuchi *et al*., 1998) and this pathway is inhibited by PD98059 (Servant *et al*., 1996; Ushio-Fukai *et al*., 1998). Our experiments showed that inhibition of this pathway by PD98059 effectively blocked the growth response to Angiotensin II_{intr} administration. Activation of the MAPK cascade can be achieved *via* the PI-3K pathway, but a redundant pathway stimulates MAPK when large numbers of receptors are activated (Duckworth & Cantley, 1997). Interestingly, wortmannin only partially inhibited our Angiotensin II_{intr} effect, a finding also reported for extracellular Angiotensin II induced growth (Berk, 1999) and for other stimuli or cell types activated (Balla *et al*. 1998; Gutkind, 1998). This indicates that a strong signal is evoked by the Angiotensin II_{intr} mediated stimulation, comparable to activation of large number of 'classical' plasma membrane receptors.

The obvious physiological candidate to stimulate the Angiotensin II_{intr}-receptor is Angiotensin II. Intracellular trafficking of Angiotensin II might be important for directing Angiotensin II to certain cellular locations to fully express its biological response. Several studies have demonstrated that

Angiotensin II is internalized into the cells *via* an AT_1-but not AT_2-mediated process (Anderson *et al*., 1993; Hein *et al*., 1997). Intracellular pools of Angiotensin II were noticed in cardiomyocytes (Sadoshima *et al*., 1993) and recently angiotensin peptides, ACE-activity and AT_1-receptors were detected in a renal endosomal fraction (Imig *et al*., 1999). The functional targets for Angiotensin II_{intr} are still unclear, but nuclear binding-proteins were reported for Angiotensin II (Booz *et al*., 1992; Tang *et al*., 1992; Jimenez *et al*., 1994). Interaction of Angiotensin II_{intr} with proteins at the cytosolic side of the plasma membrane also occurs in view of the results of Angiotensin II_{intr} on Ca^{2+} channels and gap junctions (De Mello 1996; 1998; Haller *et al*., 1996; 1999). This is possibly only a secondary related phenomenon, since the MAP-kinase pathway shown to be activated by Angiotensin II_{intr} in the present paper, modulates the opening of L-type Ca^{2+} channels in cardiomyocytes (Murata *et al*., 1999).

In conclusion, these data demonstrate that intracellular delivered Angiotensin II induces cell growth in A7r5 cells. Atypical AT-receptors are involved in view of the ineffectiveness of extracellular addition and the rank order of antagonist potencies obtained by intracellular application. The Angiotensin II_{intr} induced growth response is mediated *via* a PI-3K and MAPK/ERK – sensitive pathway. Angiotensin II_{intr} actions, inaccessible for common treatment, might open new views in the understanding and treatment of cardio-vascular related diseases.

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