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Intracellular angiotensin II elicits Ca^{2+} increases in A7r5 vascular smooth muscle cells

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Abstract

Recent studies show that angiotensin II can act within the cell, possibly via intracellular receptors pharmacologically different from typical plasma membrane angiotensin II receptors. The signal transduction of intracellular angiotensin II is unclear. Therefore, we investigated the effects of intracellular angiotensin II in cells devoid of physiological responses to extracellular angiotensin II (A7r5 vascular smooth muscle cells). Intracellular delivery of angiotensin II was obtained by using liposomes or cell permeabilisation. Intracellular angiotensin II stimulated Ca^{2+} influx, as measured by $^{45}Ca^{2+}$ uptake and single-cell fluorimetry. This effect was insensitive to extracellular or intracellular addition of losartan (angiotensin AT_1 receptor antagonist) or PD123319 ((*s*)-1-(4-[dimethylamino]-3-methylphenyl)methyl-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylate) (angiotensin AT_2 receptor antagonist). Intracellular angiotensin II stimulated inositol-1,4,5-trisphosphate ($Ins(1,4,5,)P_3$) production and increased the size of the $Ins(1,4,5,)P_3$ releasable $^{45}Ca^{2+}$ pool in permeabilised cells, independent of losartan and PD123319. Small G-proteins did not participate in this process, as assessed by using GDPβS. Intracellular delivery of angiotensin I was unable to elicit any of the effects elicited by intracellular angiotensin II. We conclude from our intracellular angiotensin application experiments that angiotensin II modulates Ca^{2+} homeostasis even in the absence of extracellular actions. Pharmacological properties suggest the involvement of putative angiotensin non-AT_1-/non-AT_2 receptors.

Keywords: Angiotensin II, intracellular; Liposome; Ca^{2+} influx; Ca^{2+} release; $Ins(1,4,5)P_3$; A7r5 cell

1. Introduction

Angiotensin II is an important effector peptide involved in the regulation of cardiovascular and renal function. Although classical physiology attributes its effects to circulating angiotensin II, angiotensin II can act locally as an autocrine hormone, producing patho-physiological effects at its production site (Dell'Italia et al., 1997; Van Kats et al., 1997). The effects of angiotensin II occur through interaction with specific plasma membrane receptors. To date, two such receptors have been identified, namely angiotensin AT_1 and AT_2 (Chiu et al., 1989; Griendling et al., 1997). Both of them belong to the family of seven transmembrane G protein-coupled receptors but are coupled to different signal transduction pathways. Stimulation of angiotensin AT_1 receptors activates phospholipase C and the formation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), which subsequently discharges Ca^{2+} from internal stores, activates mitogen-activated protein (MAP) kinase and stimulates cell growth, whereas angiotensin AT_2 receptors increase cGMP levels and inhibit cell growth (Unger et al., 1996; Hunyady et al., 1996; Horiuchi et al., 1999). The existence of additional sub-types of angiotensin

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II receptors is under investigation and studies suggest that angiotensin non-AT_1/non-AT_2 binding sites are involved in angiogenesis (Le Noble et al., 1996) and are present within the cytosolic fraction of placenta (Li et al., 1998).

Several studies have drawn attention to the effects of angiotensin II in the cell. Intracellular application of angiotensin II induces a $[Ca^{2+}]_i$ increase in vascular smooth muscle cells (Haller et al., 1996), whereas in heart muscle, it inhibits the functioning of gap-junctions (De Mello, 1996) and L-type Ca^{2+} currents (De Mello, 1998). Furthermore, the presence of specific intracellular angiotensin II-binding proteins has been reported in other preparations, such as liver (Kiron and Soffer, 1989), cardiovascular myocytes (Robertson and Khairallah, 1971; Sadoshima et al., 1993) and mesangial cells (Mercure et al., 1998). However, it is not clear whether these intracellular angiotensin II binding proteins represent internalised plasma membrane receptors or a genuine new class of angiotensin II receptors. We recently reported that intracellular angiotensin II induces contraction of rat aortic muscle by a mechanism independent of extracellular AT receptors (Brailoiu et al., 1999). The effects of intracellular angiotensin II in smooth muscle have been attributed to an interaction with specific intracellular AT receptors, because the effects were sensitive to specific AT_1 (Brailoiu et al., 1999; Haller et al., 1996) or partly sensitive to AT_2 receptor antagonists (Brailoiu et al., 1999).

The aim of the present work was to demonstrate that intracellularly administered angiotensin II induces cellular effects in a cell line that does not respond to extracellular angiotensin II. To this end, we used A7r5 vascular smooth muscle cells. Intracellular angiotensin II effects on $[Ca^{2+}]_i$ homeostasis were studied, since this parameter is of major importance in smooth muscle physiology. A7r5 cells lack functional responses typical for extracellular angiotensin II stimulation. However, after intracellular application, we found that angiotensin II is able to modulate $[Ca^{2+}]_i$ homeostasis at different levels. Part of this work has been communicated in abstract form (Filipeanu et al., 1998a).

2. Material and methods

2.1. Chemicals

All culture media were obtained from Gibco BRL (USA). Inositol 1,4,5-trisphosphate sodium salt (Ins-(1,4,5)P_3) was obtained from Boehringer (Germany). Losartan and PD123319 ((*s*)-1-(4-[dimethylamino]-3-methylphenyl)methyl-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylate) were kindly provided by Merck Sharp and Dohme (USA) and Park Davis (USA), respectively. Angiotensin II was supplied by the Academic Hospital Pharmacy of the University of Groningen. Fura-2 acetoxymethylester and angiotensin II-fluorescein were obtained from Molecular Probes (USA). $^{45}CaCl_2$ (specific activity: 19.3 Ci/g) and D-[inositol-1-^{3}H(*N*)]-inositol 1,4,5-trisphosphate (specific activity: 21.0 Ci/mmol) were obtained from Dupont-NEN (USA). [^{3}H] thymidine (specific activity: 24 Ci/mmol) was from Amersham Nederland (the Netherlands). GDPβS and all other compounds were obtained from Sigma (USA).

2.2. Cell culture

A7r5 cells (a stable cell line derived from foetal rat aorta) were kindly provided by Dr. H. De Smedt (K.U. Leuven, Belgium) and were cultured in Dulbeco's modified Eagle's medium (DMEM) including antibiotics and supplemented with 7 mM $NaHCO_3$, 10 mM HEPES at pH 7.2 and 10% foetal calf serum at 37°C in 95% air, 5% CO_2. Confluent monolayers in 75-cm^2 flasks (Costar) were subcultured by trypsinisation. The medium was changed twice a week.

2.3. Liposomes preparation and intracellular application

Liposomes were prepared as described previously (Brailoiu et al., 1993, 1999), using 10 mg phosphatidylcholine per ml of solution containing the substance to be incorporated. The number of lamellae was decreased by addition of diethyl ether in a ratio of 1:10 (v/v). Angiotensin II was dissolved at a concentration of 10^{-6} M in 140 mM KCl solution (pH 7.0). Control liposomes contained only 140 mM KCl. In order to remove non-incorporated solutes, liposomes were subjected to dialysis, two times during 120 min, against buffer solution in a ratio of 1:600 v/v (Sigma dialysis tubing, molecular weight cut-off: 12 400 Da). The buffer solution had the following composition (mM): 145 NaCl, 5 KCl, 0.5 $MgSO_4$, 0.5 $CaCl_2$, 10 glucose and 10 HEPES (pH adjusted to 7.4 with NaOH). Angiotensin II delivery into the cells was monitored by fluorescence microscopy as described previously (Kok et al., 1998). Cells were loaded with fluorescein–angiotensin II-filled liposomes according to the protocol as described below for $^{45}Ca^{2+}$ uptake by intact cells. The cells were washed three times with liposome-free buffer solution after loading for 5 min at room temperature and kept on ice until the photomicrographs were taken. The amount of angiotensin II delivered into the cells by this procedure was estimated by loading the cells with 10^{-6} M fluorescein–angiotensin II-filled liposomes and subsequent cell permeabilisation with saponin comparable to the method described for intracellular adenosine delivery (Brailoiu et al., 1993). Fluorescence was measured with an excitation wavelength 470 nm, an emission wavelength 520 nm, and a bandpass filter of 4 nm (Aminco Bowman LS Series 2). Liposome-encapsulated fluorescein–angiotensin II amounted to $12.4 \pm 0.9\%$ ($n = 4$) of the initial amount in the aqueous phase. Angiotensin II incorporated into the cells with an efficiency of $2.3 \pm 0.4\%$ ($n = 4$). The actual intracellular [angiotensin II] can be calculated

based on an estimation of the cell volume. The volume of A7r5 cells was estimated by determination of the maximal radius (r) of spherical cells and calculation of the volume (V) according to $V = 4/3\pi r^3$. Osmotic swelling of cells attached to the dish was obtained by sequential dilution of the culture medium by addition of water. The osmotic swelling process was monitored under microscopy and the maximal diameter reached was estimated with a standard micrometer. The maximal diameter was 13.4 ± 0.2 μm ($n = 64$), resulting in a volume of 1.26 ± 0.02 pl/cell. Therefore, with the protocol used to deliver liposomes filled with 10^{-6} M angiotensin II to the cells, the estimated intracellular [angiotensin II] is 18 ± 3 μM.

2.4. $^{45}Ca^{2+}$ uptake by intact cells

$^{45}Ca^{2+}$ uptake was measured essentially as described previously (Sipma et al., 1996). A7r5 cells were plated in six well plates 24 h prior to the experiment at a density of 10^5/well. Culture medium was replaced 1 h before the start of the experiment with a buffer solution of room temperature (22–24°C) containing (in mM): 145 NaCl, 5 KCl, 0.5 $MgSO_4$, 0.5 $CaCl_2$, 10 glucose and 10 HEPES (pH adjusted to 7.4 with NaOH). Uptake of $^{45}Ca^{2+}$ was measured at room temperature and was started by removing the solution and replacing it with the same buffer (1 ml) supplemented with 10 μCi $^{45}Ca^{2+}$ (specific activity 19.3 Ci/g) and indicated compounds. The liposomes were added at a ratio of 1:20 (v/v) in the buffer solution. Aspiration of the solution and addition of 1 ml ice-cold buffer in the absence of $CaCl_2$ stopped the uptake of $^{45}Ca^{2+}$ after 5 min. After this procedure, cells were washed three times with buffer without $CaCl_2$ but containing 2 mM EGTA. Cells were lysed in the presence of NaOH (1 ml, 1 M) and radioactivity was measured by liquid scintillation counting. Data are corrected for non-specific binding as determined by addition of buffer with $^{45}Ca^{2+}$ and immediate termination of uptake.

2.5. $[Ca^{2+}]_i$ measurements

Cells were loaded with the fluorescent Ca^{2+} indicator fura-2 acetoxymethylester (5 μM) for 45 min at 37°C. Ca^{2+} measurements were performed using an S100 Axiovert inverted microscope (Zeiss). The 340:380 ratio was acquired at room temperature at a frequency of 1 Hz using a cooled CCD camera (SensiCam) and Workbench 2.2. Imaging software (Axon Instruments). Liposomes were added at a ratio of 1:20 v/v, 5 min before agonist addition. Ratio values were transformed into $[Ca^{2+}]_i$ at the end of the experiment (Grynkiewicz et al., 1985).

2.6. $^{45}Ca^{2+}$-efflux measurements in permeabilised cells

The cells were plated 24–48 h before the experiment in six well plates (Costar) at a density of $1–1.5 \times 10^5$ cells/well. The experiments were carried out at room temperature (22–24°C) exactly as described previously (Missiaen et al., 1990; Van der Zee et al., 1995). In brief, the cells were equilibrated for 1 h with a modified buffer solution of the following composition (in mM): 135 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 11.6 HEPES and 11.5 glucose (pH adjusted to 7.4 with NaOH). Cells were permeabilised for 10 min using 40 μg/ml saponin in a solution containing (in mM): 100 KCl, 30 imidazole, 2 $MgCl_2$, 1 ATP and 1 EGTA (pH adjusted to 7.0 with KOH). Subsequently, the calcium stores were loaded with $^{45}Ca^{2+}$ by exposure for 5 min to 500 μl buffer solution containing 10.5 μCi/ml $^{45}CaCl_2$ (specific activity 19.3 Ci/g) with a final composition (in mM) of 100 KCl, 5 $MgCl_2$, 5 ATP, 5 NaN_3, 0.44 EGTA and 0.12 $CaCl_2$. The final $[Ca^{2+}]_{free}$ of this solution was calculated to be 150 nM. Efflux buffer solution containing (in mM) 100 KCl, 30 imidazole, 2 $MgCl_2$, 1 EGTA, and 5 NaN_3 (pH adjusted to 7.0 with KOH) was added (1 ml) and replaced every 2 min for 30 min. The $^{45}Ca^{2+}$ remaining in the cells at the end of the efflux procedure was extracted with 1 ml of 1 M NaOH. $^{45}Ca^{2+}$ release is expressed as the fractional loss per minute, representing the amount of $^{45}Ca^{2+}$ leaving the cell, normalised to the total amount of $^{45}Ca^{2+}$ in the cell.

2.7. Measurement of Ins(1,4,5)P_3

Mass measurement of Ins(1,4,5)P_3 was performed as described earlier (Simpa et al., 1996), using a standard curve of Ins(1,4,5)P_3 in ether-extracted trichloroacetic acid solution. The samples were assayed in 25 mM Tris/HCl (pH = 9), 1 mM EDTA, 1 mg bovine serum albumin, [^{3}H]Ins(1,4,5)P_3 (3.3 Ci/mmol, 2000 cpm/assay) and about 1 mg binding protein (isolated from fresh cattle liver) for 15 min. Bound and free radioactivity were separated by centrifugation. The radioactivity in the pellet was determined by scintillation counting.

2.8. Measurement angiotensin-converting enzyme activity

To determine angiotensin-converting enzyme activity, cells were plated 48 h before the experiment in 25-cm^2 flasks (Costar) at a density of 10^5 cells/flask. Cells were trypsinized at confluence, centrifuged at $2500 \times g$ and the pellet was resuspended in 0.2 ml phosphate-buffered saline solution and homogenized by sonification. Cell homogenates were assayed as described before (Roks et al., 1999). The lower detection limit of the assay was 2 pmol/mg protein/min.

2.9. Statistics

All experiments were performed in series with $n \geq 4$ on different days using different cell passages. The results are expressed as means ± S.D. Statistical differences were

tested either by analysis of variance (ANOVA) followed by Bonferroni post-test or by unpaired Student's *t*-test considering $P \leq 0.05$ significantly different.

3. Results

3.1. Intracellular delivery of angiotensin II by liposomes

We used liposomes to administer angiotensin II intracellularly. Various compounds can be delivered by liposomes, while plasma membrane integrity is maintained (Brailoiu et al., 1993; Brailoiu and Van der Kloot, 1996; Filipeanu et al., 1998b). Cells are not metabolically compromised by liposome treatment, since methylene blue is still excluded and incubation with control liposomes (24 h) did not affect cell growth (data not shown). Intracellular delivery of angiotensin II was also followed by fluorescence microscopy of the cells incubated with liposomes containing fluorescein–angiotensin II. These experiments confirmed the intracellular delivery of fluorescein–angiotensin II. At a concentration of 10^{-6} M fluorescein–angiotensin II, the fluorescence pattern showed a relatively uniform cytosolic distribution in comparison to control liposomes filled with 140 mM KCl (Fig. 1, middle and left panel). At much higher concentrations (3×10^{-5} M-filled liposomes), fluorescein–angiotensin II appeared also in more vesicular-like structures, indicating the uptake of fluorescence by internal organelles (Fig. 1, right panel). No fluorescence was observed in the nucleus at any concentration. It should be noted that the [angiotensin II] used to generate all functional data (10^{-6} M angiotensin II-filled liposomes) ensured a relatively uniform intracellular distribution.

3.2. $^{45}Ca^{2+}$ uptake in intact cells

To examine the effects of intracellular angiotensin II on Ca^{2+} homeostasis in A7r5 cells, we first used $^{45}Ca^{2+}$ flux as a parameter. Addition of extracellular angiotensin II did not modify the basal $^{45}Ca^{2+}$ uptake ($2.2 \pm 3.8\%$ vs. control). In contrast, addition of 10^{-6} M angiotensin II-containing liposomes induced a moderate increase in $^{45}Ca^{2+}$ uptake by $32.2 \pm 11.2\%$ ($n = 16$), which was not sensitive to the intracellular administration of the angiotensin AT_1 receptor antagonist losartan (10^{-6} M) or to the angiotensin AT_2 receptor antagonist PD123319 (10^{-6} M; Fig. 2). Control liposomes did not change basal $^{45}Ca^{2+}$ uptake. Extracellular addition of losartan or PD123319 was ineffective in reversing the intracellular angiotensin II effect. Angiotensin I (10^{-6} M)-filled liposomes did not modify basal $^{45}Ca^{2+}$ uptake (data not shown).

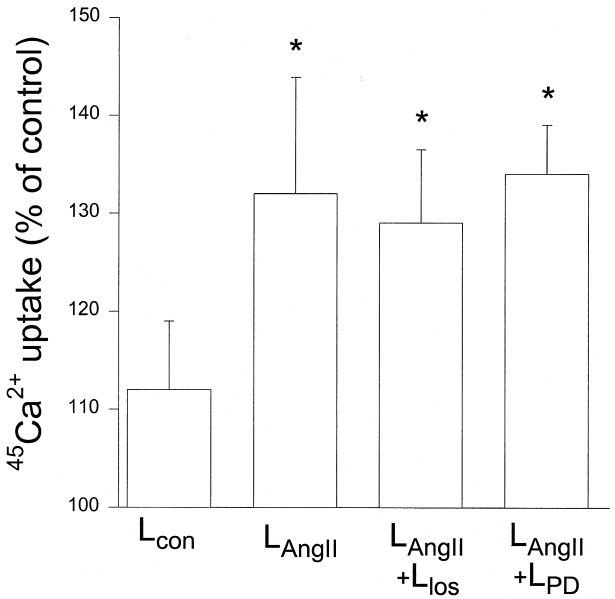

Fig. 2. Effects of intracellular angiotensin II on $^{45}Ca^{2+}$ uptake by intact A7r5 cells. Uptake was measured for control liposomes filled with 140 mM KCl (L_{con}, $n = 18$) or liposomes filled with angiotensin II alone (10^{-6} M, L_{AngII}, $n = 18$) or in the presence of losartan (10^{-6} M, $L_{angII} + L_{los}$, $n = 12$) or PD123319 (10^{-6} M, $L_{angII} + L_{PD}$, $n = 12$). Net uptake was measured for 5 min. Data are presented as means ± S.D. The basal 100% level corresponds to 57 ± 3 dpm ($n = 48$). Significance indications: $^{*}P < 0.05$ vs. L_{con}.

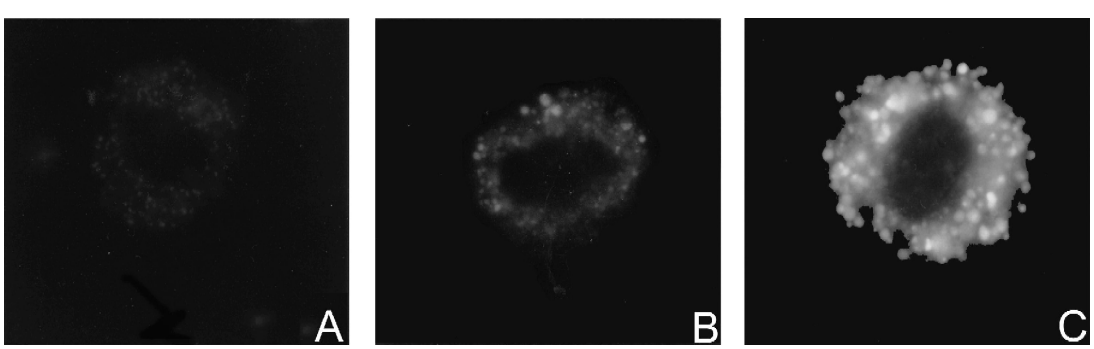

Fig. 1. Intracellular delivery of fluorescein–angiotensin II in A7r5 cells. Cells were incubated with: (A) control liposomes containing KCl (140 mM), (B) liposomes containing fluorescein–angiotensin II (10^{-6} M) or (C) liposomes containing fluorescein–angiotensin II (3×10^{-5} M). Representative photomicrographs of 3–6 coverslips are shown.

3.3. $[Ca^{2+}]_i$ measurements

Similar results were obtained by measuring $[Ca^{2+}]_i$ with fura-2 fluorescence. Basal $[Ca^{2+}]_i$ amounted to 57 ± 4 nM ($n = 54$). Addition of extracellular angiotensin II (10^{-6} M) did not change this value (58 ± 6 nM, $n = 12$). In contrast, angiotensin II-filled liposomes induced a slowly developing Ca^{2+} increase (Fig. 3A). A dose-dependent effect was observed, starting from 10^{-9} M angiotensin II and reaching a maximal increase of 34 ± 6 nM ($n = 8$) at 10^{-6} M angiotensin II (Fig. 3B). The EC_{50} value was obtained with liposomes filled with three 10^{-8} M angiotensin II. Taken into account the delivery efficiency and the determined cell volume, this value can be extrapolated to an actual effective intracellular [angiotensin] of approximately 200 nM. Angiotensin II-filled liposomes failed to

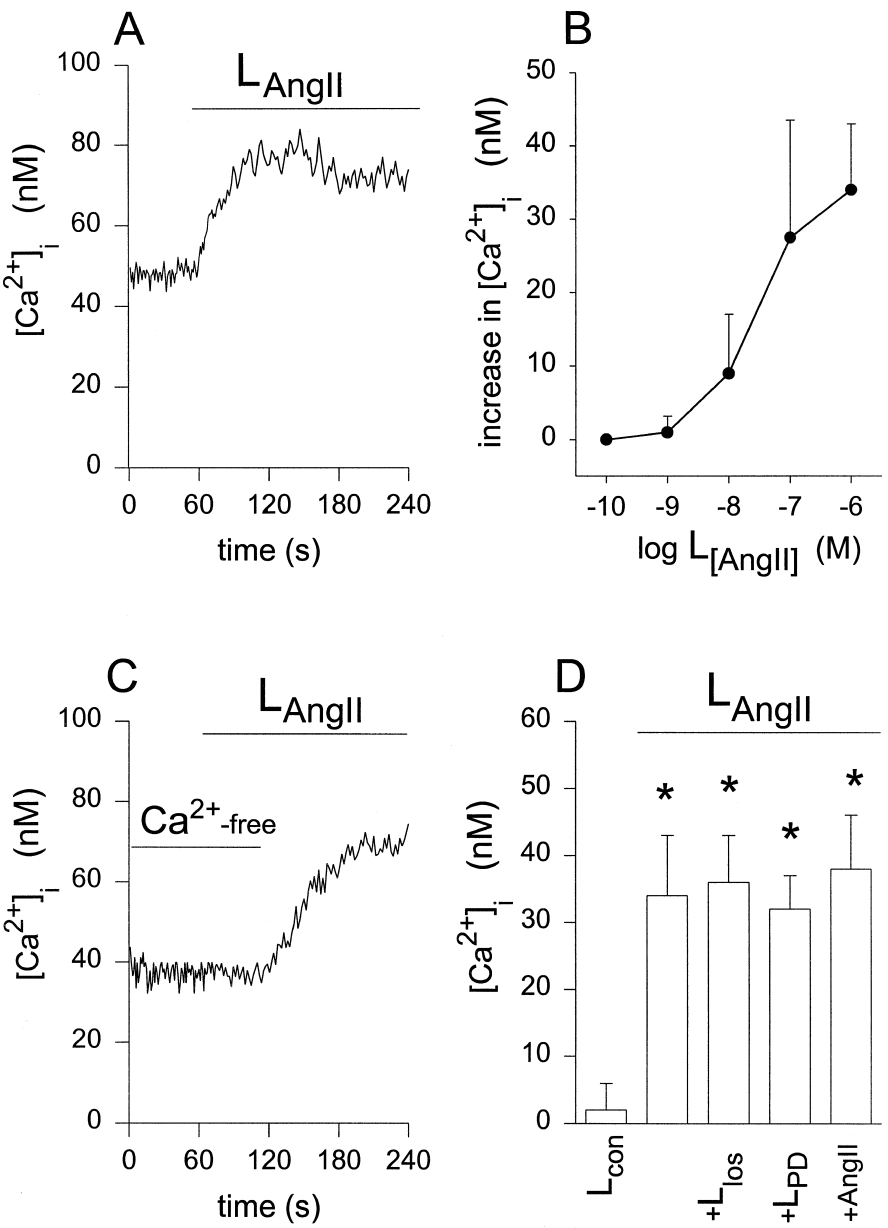

Fig. 3. Effect of intracellular angiotensin II on $[Ca^{2+}]_i$ in intact A7r5 cells. Panel A: typical trace showing the effects of angiotensin II-containing liposomes (10^{-6} M, L_{AngII}) in normal Ca^{2+} containing medium. Panel B: Concentration dependence of Ca^{2+} increases above control values using liposomes filled with different concentrations of angiotensin II. Data are presented as means ± S.D. ($n = 6$–8). Panel C: typical trace showing the effect of angiotensin II-containing liposomes (10^{-6} M, L_{AngII}) in Ca^{2+}-free extracellular medium followed by restoration of normal Ca^{2+} concentration. Panel D: statistics showing the effects on $[Ca^{2+}]_i$ of intracellular angiotensin II, intracellular angiotensin II antagonists and extracellular angiotensin II. Control liposomes were filled with KCl (140 mM, L_{con}). Liposomes were filled either with angiotensin II (10^{-6} M, L_{AngII}), angiotensin II together with losartan (10^{-6} M, $L_{AngII} + L_{los}$), angiotensin II together with PD123319 (10^{-6} M, $L_{AngII} + L_{PD}$) or angiotensin II–containing liposomes were combined with extracellular angiotensin II (10^{-6} M, L_{AngII} + AngII). Data are presented as means ± S.D. ($n = 12$ for each condition). Significance indications: $^{*}P < 0.05$ vs. L_{con}.

Table 1
Effects of intracellular angiotensin II application on Ins(1,4,5)P_3 formation in intact A7r5 cells in comparison to extracellular stimulation
Cells were stimulated with control liposomes filled with 140 mM KCl, with liposomes filled with angiotensin II (10^{-6} M) or with extracellular angiotensin II (10^{-6} M). Ins(1,4,5)P_3 formation was measured 1 min after stimulation. Serotonin (5-HT, 10^{-5} M, 1 min) was used as positive control for G-protein-coupled receptor stimulation. Data are presented as means ± S.D. (number of experiments).

Treatment	Ins(1,4,5)P_3 formation (pmol 10^5 cells^{-1})
Basal	0.23 ± 0.05 (12)
Control liposomes (L_{con})	0.21 ± 0.07 (6)
Angiotensin II-filled liposomes	0.37 ± 0.05 (6)[a,b]
Extracellular angiotensin II	0.22 ± 0.04 (6)
Extracellular 5-HT	0.52 ± 0.08 (6)[a]

[a]Significance indications: $P < 0.05$ vs. basal.
[b]Significance indications: $P < 0.05$ vs. control liposomes.

increase $[Ca^{2+}]_i$ in Ca^{2+}-free medium, but induced a normal response after restoration of the normal extracellular Ca^{2+} concentration (Fig. 3C). The voltage-dependent Ca^{2+} channel blocker verapamil (10^{-5} M) did not modify the angiotensin II-induced Ca^{2+} influx (30 ± 5 nM, $n = 4$). Inclusion of angiotensin AT_1 and angiotensin AT_2 receptor antagonists, losartan and PD123319, into the liposomes did not alter the effects of angiotensin II-filled liposomes (Fig. 3D). Extracellular angiotensin II administration prior to intracellular angiotensin II application also did not modify the intracellular angiotensin II effect (Fig. 3D).

3.4. Ins(1,4,5)P_3 production

The involvement of phospholipase C activation after stimulation with intracellular angiotensin II was examined by measuring Ins(1,4,5)P_3 mass production (Table 1). Extracellular serotonin (5-HT) stimulated Ins(1,4,5)P_3 production substantially (2.2-fold increase), indicating the presence of a functional G-protein/phospholipase C system in these cells. Stimulation with angiotensin II-filled liposomes resulted in a 1.7-fold stimulation of Ins(1,4,5)P_3 formation. As expected, in view of the absence of functional plasma membrane angiotensin AT_1 receptors, no net increase was observed after stimulation with extracellular angiotensin II.

3.5. $^{45}Ca^{2+}$ fluxes in permeabilised cells

To further investigate signal transduction pathways influenced by intracellular angiotensin II, we used perme-

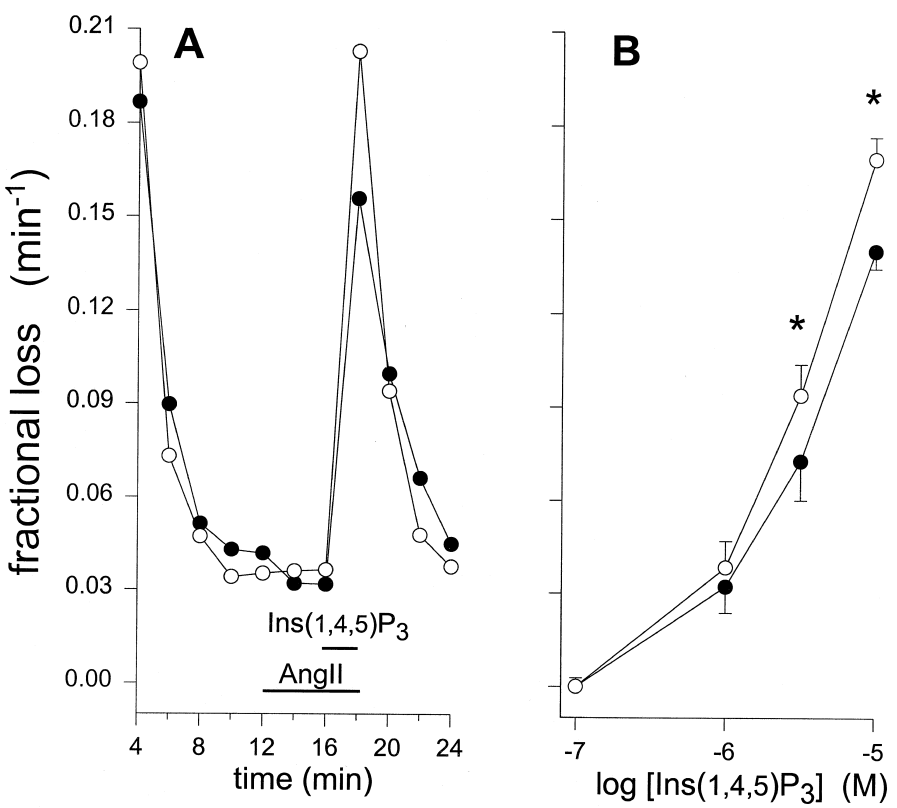

Fig. 4. Effects of angiotensin II on Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release in permeabilised A7r5 cells. (A) Typical experiment in permeabilised cells with preloaded non-mitochondrial $^{45}Ca^{2+}$ stores. Control conditions (●) or (○) in the presence of angiotensin II (10^{-6} M) added at $t = 12$. Horizontal bars represent the presence of Ins(1,4,5)P_3 (10^{-5} M) and angiotensin II. Note that angiotensin II did not release $^{45}Ca^{2+}$ by itself, but potentiated Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release. (B) Stimulatory effect of angiotensin II (10^{-6} M) depends on [Ins(1,4,5)P_3]. Data are presented as means ± S.D. ([Ins(1,4,5)P_3] below 10^{-5} M: $n = 4$, at 10^{-5} M: $n = 36$ in the absence and $n = 12$ in the presence of angiotensin II). Significance indications: * $P < 0.05$ vs. Ins(1,4,5)P_3 alone.

abilised A7r5 cells after pre-loading the calcium stores with $^{45}Ca^{2+}$. The size of the Ins(1,4,5)P_3-sensitive store is represented by the fractional loss of $^{45}Ca^{2+}$ in response to 10^{-5} M Ins(1,4,5)P_3 from $t = 16$ to $t = 18$ min after the start of the efflux measurement, amounted to 0.14 ± 0.01 min^{-1} ($n = 36$; Fig. 4A). The basal fractional loss was not changed by addition of 10^{-6} M angiotensin II at time $t = 12$ min. However, this pre-treatment potentiated the subsequent $^{45}Ca^{2+}$ release induced by Ins(1,4,5)P_3 by $21 \pm 3\%$ ($P < 0.05$; Fig. 4A). The stimulatory effect of angiotensin II on Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release was dependent on the Ins(1,4,5)P_3 concentration, becoming apparent above 3×10^{-6} M Ins(1,4,5)P_3 (Fig. 4B).

Furthermore, this potentiation was dependent on the angiotensin II concentration with an EC_{50} value of approximately 2 nM (Fig. 5A). The effect of angiotensin II was not sensitive to the angiotensin AT_1 receptor antagonist losartan or to the angiotensin AT_2 receptor antagonist PD123319 (Fig. 5B). As in the case of $^{45}Ca^{2+}$ flux in intact cells, the related peptide angiotensin I (10^{-6} M) modified neither Ins(1,4,5)P_3-mediated $^{45}Ca^{2+}$ release ($n = 4$, data not shown) nor basal $^{45}Ca^{2+}$ release ($99 \pm 2\%$ vs. control, $n = 4$). Cell permeabilisation with saponin is expected to destroy any plasma membrane receptor signal transduction. Accordingly, 5-HT (10^{-5} M) was unable to induce $^{45}Ca^{2+}$ release in permeabilised cells ($101 \pm 2\%$ vs. control, $n = 4$).

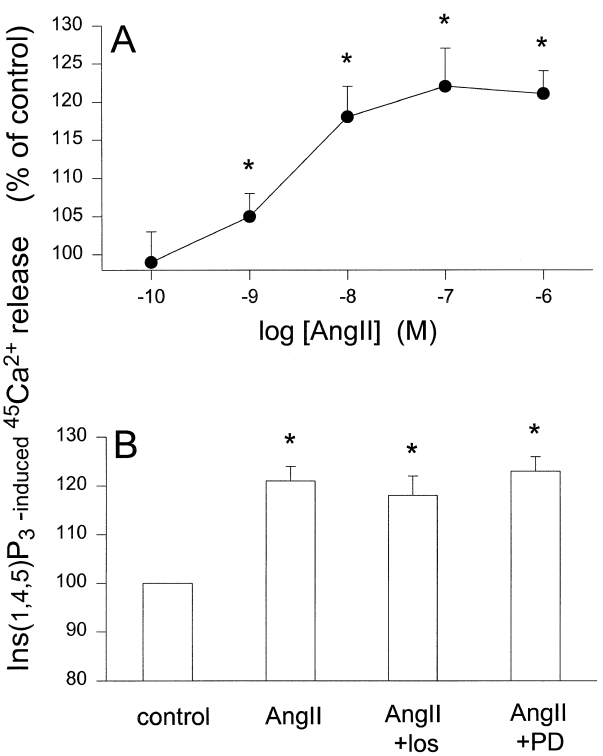

Fig. 5. Pharmacological properties of the angiotensin II effect on Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release in permeabilised cells. (A) Concentration dependence of angiotensin II on 10^{-5} M Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release. The experiment was performed as described in the legend of Fig. 4. Data are presented as means ± S.D. ($n = 12$ at 10^{-6} M and $n = 4$ at lower concentrations). Significance indications: $^{*}P < 0.05$ vs. Ins(1,4,5)P_3 alone. (B) Effects of the angiotensin AT_1 receptor antagonist losartan (10^{-6} M, $n = 4$) and the angiotensin AT_2 receptor antagonist PD123319 (10^{-6} M, $n = 4$) on angiotensin II (10^{-6} M)-stimulated $^{45}Ca^{2+}$ release induced by Ins(1,4,5)P_3 (10^{-5} M). Number of control experiments: $n = 36$ in the absence and $n = 12$ in the presence of angiotensin II. Data are presented as means ± S.D. Significance indications: $^{*}P < 0.05$ vs. Ins(1,4,5)P_3 alone.

Table 2
Effect of GDPβS on Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release in permeabilised A7r5 cells
$^{45}Ca^{2+}$ release was initiated by 10^{-5} M Ins(1,4,5)P_3 as described in the legend of Fig. 4. Angiotensin II (10^{-6} M) and GDPβS (5×10^{-6} M) were added 4 min prior to this stimulus. Data are presented as means ± S.D. (four experiments).

Treatment	Effect (%)
Control	100
+ GDPβS	55 ± 12^{a}
+ Angiotensin II	122 ± 4^{a}
+ GDPβS + angiotensin II	$78 \pm 18^{b,c}$

[a] Significance indications: $P < 0.05$ vs. control.
[b] Significance indications: $P < 0.05$ vs. GDPβS.
[c] Significance indications: $P < 0.05$ vs. angiotensin II.

Small G proteins were previously reported to modulate Ca^{2+} mobilisation (Xu et al., 1996a,b; Loomis-Husselbee et al., 1998). To investigate whether such proteins are involved in the observed angiotensin II effects, we tested the responses in the presence of GDPβS. Although this compound greatly attenuated the effects of Ins(1,4,5)P_3, the potentiating effect of angiotensin II was still present (Table 2).

3.6. Intracellular angiotensin-converting enzyme activity

Intracellular angiotensin-converting enzyme activity was determined in exponentially growing A7r5 cell cultures. No activity above the lower detection limit was observed ($n = 3$).

4. Discussion

Although intracellular effects of angiotensin II have been shown previously (Brailoiu et al., 1999; Haller et al., 1996; De Mello 1996, 1998), this is the first report demonstrating its effects in a cell line devoid of extracellular angiotensin II effects. Because of the presence of plasma membrane angiotensin II receptors in previous studies, one could argue that the effects observed were possibly mediated by internalisation of plasma membrane receptors occupied by angiotensin II. A7r5 vascular smooth muscle cells lack functional responses to extracellular angiotensin II, such as increases in Ins(1,4,5)P_3 formation and $[Ca^{2+}]_i$, which are typical for extracellular angiotensin II stimula-

tion (Griendling et al., 1997; Horiuchi et al., 1999). To administer intracellular angiotensin II, we used either liposomes or delivered angiotensin II directly into the cytosol of permeabilised cells. With the liposome technique, the integrity of the plasma membrane is maintained (Brailoiu et al., 1993; Brailoiu and Van der Kloot, 1996) and fluorescence microscopy confirmed the intracellular delivery of angiotensin II. The intracellular actions of angiotensin II were not modified by extracellular application of angiotensin II and/or its antagonists. Together, these data preclude that the intracellular actions of angiotensin II were mediated by an internalised angiotensin AT receptor. Therefore, the first conclusion drawn from our study is that intracellular angiotensin II acts independently of extracellular angiotensin II receptor stimulation.

Our results obtained for both $^{45}Ca^{2+}$ uptake and $[Ca^{2+}]_i$ measurements unequivocally demonstrate that intracellular angiotensin II stimulates Ca^{2+} influx via non-voltage-dependent Ca^{2+} channels in A7r5 cells. A similar observation was made in adult rat aorta, showing that the intracellular angiotensin II-induced contraction was entirely dependent on Ca^{2+} influx from the extracellular space (Brailoiu et al., 1999). The action of intracellular angiotensin II in permeabilised cells has not been studied before. Angiotensin II did not release Ca^{2+} from intracellular stores, but potentiated the effects of Ins(1,4,5)P_3 in a losartan- and PD123319-insensitive manner. The observed increase in Ins(1,4,5)P_3-induced Ca^{2+} release by intracellular angiotensin II was of a similar magnitude to that observed for various other compounds in permeabilised A7r5 cells (Missiaen et al., 1997) and other cell types (Van der Zee et al., 1995; Loomis-Husselbee et al., 1998). The involvement of G-proteins was noticed in Ins(1,4,5)P_3-induced Ca^{2+} channel activation in pancreatic acinar cells (Xu et al., 1996a,b) and in Ins(2,4,5)P_3-activated Ca^{2+} mobilisation in L1210 cells (Loomis-Husselbee et al., 1998). Although we observed a large inhibition of Ins(1,4,5)P_3-activated Ca^{2+} mobilisation by GDPβS, as observed in acinar cells (Xu et al., 1996b), the stimulation of Ca^{2+} release by angiotensin II was unaffected. This indicates that G-proteins are not involved in the modulation of Ins(1,4,5)P_3-activated Ca^{2+} mobilisation by intracellular angiotensin II. In saponin-permeabilised A7r5 cells, Ca^{2+} was no longer released upon stimulation with 5-HT, suggesting the absence of functional plasma membrane receptors under this condition. The effective concentration range for the effects of intracellularly delivered angiotensin II observed in the present study was almost similar to that obtained for intracellular angiotensin II-induced rat aorta contraction (Brailoiu et al., 1999) and intracellular angiotensin II induced-growth of A7r5 cells (Filipeanu et al., 2001). From the observed EC_{50} values in the present study, one can extrapolate the effective intracellular [angiotensin II] to be approximately between 2 and 200 nM, depending on the data obtained for Ins(1,4,5)P_3-induced Ca^{2+} release or the data for liposomal angiotensin II-induced Ca^{2+} increases. Reported values of intracellular [angiotensin II] are in the range of low pM to low nM in cardiac tissue and cardiomyocytes (De Mello and Danser, 2000; Sadoshima et al., 1993). Therefore, it is not unlikely that intracellularly effective concentrations can accumulate in the cell. Whether physiological stimuli can induce these particular cellular concentrations or whether specific intracellular compartments are involved remains to be established.

We have shown that both stimulation of Ca^{2+} influx and potentiation of Ins(1,4,5)P_3-mediated $^{45}Ca^{2+}$ release are not affected by the typical angiotensin AT_1 receptor and angiotensin AT_2 receptor antagonists, losartan and PD123319. Together, these results obtained with different techniques demonstrate that modulation of $[Ca^{2+}]_i$ by intracellular angiotensin II occurs at different subcellular levels. Angiotensin II acts via angiotensin non-AT_1-/non-AT_2 type receptors to promote basal Ca^{2+} influx and to increase the Ins(1,4,5)P_3 releasable Ca^{2+} pool. The involvement of angiotensin non-AT_1/non-AT_2 type receptors in the intracellular effects of angiotensin II has been suggested before. Intracellular angiotensin II inhibited the L-type Ca^{2+} current via a angiotensin non-AT_1/non-AT_2 mechanism in rat cardiac myocytes, but stimulated this current in hamster cardiac myocytes (De Mello, 1998). In some aspects, the intracellular effects of angiotensin II described in this study are different from those seen in other cell types. In adult vascular smooth muscle cells, intracellular angiotensin II induced Ca^{2+} influx as well, but its effect was completely abolished by an angiotensin AT_1 receptor antagonist (Haller et al., 1996). An angiotensin AT_1 receptor-like mechanism was also reported for the effect of intracellular angiotensin II on cell-to-cell communication in cardiomyocytes (De Mello, 1996). In rat aorta rings, both intracellular angiotensin II and angiotensin I mediated smooth muscle contraction through mechanisms sensitive to both angiotensin AT_1 receptor or angiotensin AT_2 receptor antagonists (Brailoiu et al., 1999). Angiotensin I was not a substitute for intracellular angiotensin II in A7r5 cells. This was also observed for the intracellular angiotensin-induced growth of these cells (Filipeanu et al., 2001). These studies, including the present observations, indicate the heterogeneity of intracellular angiotensin II receptors with respect to modulation of Ca^{2+} homeostasis among different cell types.

The physiological function of intracellular angiotensin II receptors is still unclear. A possible (patho-) physiological role for intracellular angiotensin II is supported by the presence of specific intracellular angiotensin II binding proteins (Robertson and Khairallah, 1971; Kiron and Soffer, 1989; Li et al., 1998). The only data concerning the functionality of the angiotensin non-AT_1/non-AT_2 type receptor is the involvement of this receptor in angiogenesis (Le Noble et al., 1996). The presence of intracellular angiotensin II in cardiovascular tissue is established (Van Kats et al., 1997), supporting the concept of angiotensin

synthesis at cardiac sites, possibly after internalisation of plasma-derived renin into the cells (De Mello and Danser, 2000). In addition to the heart, in cardiomyocytes (Sadoshima et al., 1993) and in various other cell types (Erdmann et al., 1996; Hermann and Ring, 1994; Mercure et al., 1998), the subcellular localisation or measurable levels of intracellular angiotensin II, angiotensin I, or other angiotensin metabolites have been reported. Endogenous angiotensin II might be a physiological substrate of putative intracellular angiotensin receptors. Internalisation of angiotensin II together with the receptor complex after stimulation of the plasma membrane angiotensin AT_1 receptor might contribute to intracellular angiotensin II levels (Anderson et al., 1993; Hein et al., 1997). The absence of measurable functional responses to extracellularly applied angiotensin II on $[Ca^{2+}]_i$ and the persistence of an intracellular angiotensin II effect after cell permeabilisation make this supposition unlikely in A7r5 cells. The absence of plasma membrane angiotensin II receptors and the presence of putative angiotensin II intracellular receptors together with the absence of angiotensin-converting enzyme activity in A7r5 cells make these cells a valuable model for studying the intracrine angiotensin system.

In summary, we showed that angiotensin II is an important modulator of cell function even in the absence of extracellular actions. Intracellular application of angiotensin II stimulates Ca^{2+} influx and Ins(1,4,5)P_3 formation and increases Ins(1,4,5)P_3-inducible Ca^{2+} release. The pharmacological properties suggest the involvement of putative intracellular angiotensin non-AT_1/non-AT_2 receptors.

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References

Anderson, K.M., Murahashi, T., Dostal, D.E., Peach, M.J., 1993. Morphological and biochemical analysis of angiotensin II internalisation in cultured rat aortic smooth muscle cells. Am. J. Physiol. 264, C179–C188.

Brailoiu, E., Van der Kloot, W.V., 1996. Bromoacetylcholine and acetylcholinesterase introduced via liposomes into motor nerve endings block increases in quantal size. Pfluegers Arch.-Eur. J. Physiol. 432, 413–418.

Brailoiu, E., Serban, D.N., Slatineanu, S., Filipeanu, C.M., Petrescu, B.C., Branisteanu, D.D., 1993. Effects of liposome-entrapped adenosine in the isolated rat aorta. Eur. J. Pharmacol. 250, 489–492.

Brailoiu, E., Filipeanu, C.M., Tica, A., Toma, C.P., De Zeeuw, D., Nelemans, S.A., 1999. Contractile effects by intracellular angiotensin II via receptors with a distinct pharmacological profile in rat aorta. Br. J. Pharmacol. 126, 1133–1138.

Chiu, A.T., Herblin, W.F., McCall, D.E., Ardecky, R.J., Carini, D.J., Duncia, J.V., Pease, L.J., Wong, P.C., Wexler, R.R., Johnson, A.L. et al., 1989. Identification of angiotensin II receptor subtypes. Biochem. Biophys. Res. Commun. 165, 196–203.

Dell'Italia, L.J., Meng, Q.C., Balcells, E., Wei, C.C., Palmer, R., Hageman, G.R., Durand, J., Hankes, G.H., Oparil, S., 1997. Compartmentalisation of angiotensin II generation in the dog heart. Evidence for independent mechanisms in intravascular and interstitial spaces. J. Clin. Invest. 100, 253–258.

De Mello, W.C., 1996. Renin–angiotensin system and cell communication in the falling heart. Hypertension 27, 1267–1272.

De Mello, W.C., 1998. Intracellular angiotensin regulates the inward calcium current in cardiac myocytes. Hypertension 32, 976–982.

De Mello, W.C., Danser, A.H.J., 2000. Angiotensin II and the heart. On the intracrine renin–angiotensin system. Hypertension 35, 1183–1188.

Erdmann, B., Fuxe, K., Ganten, D., 1996. Subcellular localisation of angiotensin II immunoreactivity in the rat cerebellar cortex. Hypertension 28, 818–824.

Filipeanu, C.M., Henning, R.H., De Zeeuw, D., Nelemans, S.A., 1998a. Functional evidence for a role of intracellular angiotensin II in A7r5 cells. Br. J. Pharmacol. 123, 141 P. (abstract).

Filipeanu, C.M., Brailoiu, E., Petrescu, G., Nelemans, S.A., 1998b. Extracellular and intracellular arachidonic acid-induced contractions in rat aorta. Eur. J. Pharmacol. 349, 67–73.

Filipeanu, C.M., Henning, R.H., De Zeeuw, D., Nelemans, A., 2001. Intracellular angiotensin II and cell growth of vascular smooth muscle cells. Br. J. Pharmacol. 132, 1590–1596.

Griendling, K.K., Ushio-Fukai, M., Lassegue, B., Alexander, R.W., 1997. Angiotensin II signaling in vascular smooth muscle. New concepts. Hypertension 29, 366–373.

Grynkiewicz, G., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3449.

Haller, H., Lindschau, C., Erdmann, B., Quass, P., Luft, F.C., 1996. Effects of intracellular angiotensin II in vascular smooth muscle cells. Circ. Res. 79, 765–772.

Hein, L., Meinel, L., Pratt, R.E., Dzau, V.J., Kobilka, B.K., 1997. Intracellular trafficking of angiotensin II and its AT1 and AT2 receptors: evidence for selective sorting of receptor and ligand. Mol. Endocrinol. 11, 1266–1277.

Hermann, K., Ring, J., 1994. Human leukocytes contain angiotensin I, angiotensin II and angiotensin metabolites. Int. Arch. Allergy Immunol. 103, 152–159.

Horiuchi, M., Akishita, M., Dzau, V.J., 1999. Recent progress in angiotensin II type 2 receptor research in the cardiovascular system. Hypertension 33, 613–621.

Hunyady, L., Balla, T., Catt, K.J., 1996. The ligand binding site of the angiotensin AT1 receptor. Trends Pharmacol. Sci. 17, 135–140.

Kiron, M.A., Soffer, R.L., 1989. Purification and properties of a soluble angiotensin II-binding protein from rabbit liver. J. Biol. Chem. 264, 4138–4142.

Kok, J.W., Babia, T., Filipeanu, C.M., Nelemans, A., Egea, G., Hoekstra, D., 1998. PDMP blocks brefeldin A-induced retrograde membrane transport from golgi to ER: evidence for involvement of calcium homeostasis and dissociation from sphingolipid metabolism. J. Cell Biol. 142, 25–38.

Le Noble, F.A., Kessels-Van Wylick, L.C., Hacking, W.J., Slaaf, D.W., Oude Egbrink, M.G., Struijker-Boudier, H.A., 1996. The role of angiotensin II and prostaglandins in arcade formation in a developing microvascular network. J. Vasc. Res. 33, 480–488.

Li, X., Shams, M., Zhu, J., Khalig, A., Wilkes, M., Whittle, M., Barnes, N., Ahmed, A., 1998. Cellular localisation of AT1 receptor mRNA and protein in normal placenta and its reduced expression in intra-

uterine growth. angiotensin II stimulates the release of vasorelaxants. J. Clin. Invest. 101, 442–454.

Loomis-Husselbee, J.W., Walker, C.D., Bottomly, J.R., Cullen, P.J., Irvine, R.F., Dawson, A.P., 1998. Modulation of Ins(2,4,5)P3-stimulated Ca^{2+} mobilisation by Ins(1,3,4,5)P4: enhancement by activated G-proteins, and evidence for the involvement of a GAP1 protein, a putative Ins(1,3,4,5)P4 receptor. Biochem. J. 331, 947–952.

Mercure, C., Ramla, D., Garcia, R., Thibault, G., Deschepper, C.F., Reudelhuber, T.L., 1998. Evidence for intracellular generation of angiotensin II in rat juxtaglomerular cells. FEBS Lett. 422, 395–399.

Missiaen, L., Declerck, I., Droogmans, G., Plessers, L., De Smedt, H., Raeymaekers, L., Casteels, R., 1990. Agonist-dependent Ca^{2+} and Mn^{2+} entry dependent on state of filling of Ca^{2+} stores in aortic smooth muscle cells of the rat. J. Physiol. (London) 427, 171–186.

Missiaen, L., Parys, J.B., De Smedt, H., Sienaert, I., Sipma, H., Vanlingen, S., Maes, K., Casteels, R., 1997. Effect of adenine nucleotides on *myo*-inositol-1,4,5-trisphosphate-induced calcium release. Biochem. J. 325, 661–666.

Robertson Jr., A.L., Khairallah, P.A., 1971. Angiotensin II: rapid localisation in nuclei of smooth and cardiac muscle. Science 172, 1138–1139.

Roks, A.J., Van Geel, P.P., Pinto, Y.M., Buikema, H., Henning, R.H., De Zeeuw, D., Van Gilst, W.H., 1999. Angiotensin-(1–7) is a modulator of the human renin–angiotensin system. Hypertension 34, 296–301.

Sadoshima, J., Xu, Y., Slayter, H.S., Izumo, S., 1993. Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. Cell 75, 977–984.

Sipma, H., Van der Zee, L., Van den Akker, J., Den Hertog, A., Nelemans, A., 1996. The effect of the PKC inhibitor GF109203X on the release of Ca^{2+} from internal stores and Ca^{2+} entry in DDT_1 MF-2 cells. Br. J. Pharmacol. 119, 730–736.

Unger, T., Chung, O., Csikos, T., Culman, J., Gallinat, S., Gohke, P., Hohle, S., Meffert, S., Stoll, M., Stroth, U., Zhu, Y.Z., 1996. Angiotensin receptors. J. Hypertens., Suppl. 14, S95–S103.

Van der Zee, L., Sipma, H., Nelemans, A., Den Hertog, A., 1995. The role of inositol 1,3,4,5-tetrakisphosphate in internal Ca^{2+} mobilisation following histamine H1 receptor stimulation in DDT_1 MF-2 cells. Eur. J. Pharmacol. 289, 463–469.

Van Kats, J.P., De Lannoy, L.M., Danser, J.A.H., Van Meegen, J.R., Verdouw, P.D., Schalekamp, M.A., 1997. Angiotensin II type 1 (AT1) receptor-mediated accumulation of angiotensin II in tissues and its intracellular half-life in vivo. Hypertension 30, 42–49.

Xu, X., Zeng, W., Muallem, S., 1996a. Regulation of the inositol 1,4,5-trisphosphate - activated Ca^{2+} channel by activation of G proteins. J. Biol. Chem. 271, 11737–11744.

Xu, X., Zeng, W., Diaz, J., Muallem, S., 1996b. Spatial compartmentalisation of Ca^{2+} signalling complexes in pancreatic acini. J. Biol. Chem. 271, 24684–24690.