Intracellular angiotensin II inhibits heterologous receptor stimulated \( \text{Ca}^{2+} \) entry.
**Abstract**

Recent studies show that angiotensin II (AngII) can act from within the cell, possibly via intracellular receptors pharmacologically different from typical plasma membrane AngII receptors. The role of this intracellular AngII (AngII<sub>i</sub>) is unclear. Besides direct effects of AngII<sub>i</sub> on cellular processes one could hypothesise a possible role of AngII<sub>i</sub> in modulation of cellular responses induced after heterologous receptor stimulation. We therefore examined if AngII<sub>i</sub> influences [Ca<sup>2+</sup>]<sub>i</sub> in A7r5 smooth muscle cells after serotonin (5HT) or UTP receptor stimulation. Application of AngII<sub>i</sub> using liposomes, markedly inhibited $^{45}$Ca<sup>2+</sup> influx after receptor stimulation with 5HT or UTP. This inhibition was reversible by intracellular administration of the AT<sub>1</sub>-antagonist losartan and not influenced by the AT<sub>2</sub>-antagonist PD123319. Similar results were obtained in single cell [Ca<sup>2+</sup>]<sub>i</sub> measurements, showing that AngII<sub>i</sub> predominantly influences Ca<sup>2+</sup> influx and not Ca<sup>2+</sup> release via AT<sub>1</sub>-like receptors. It is concluded that AngII<sub>i</sub> modulates signal transduction activated by heterologous receptor stimulation.

Abbreviations used: AngII<sub>i</sub>, intracellular Angiotensin II; 5HT, serotonin; Ins(1,4,5)P<sub>3</sub>, inositol 1,4,5-trisphosphate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetra-acetic acid; losartan, (2-<i>n</i>-butyl-4-chloro-5-hydroxymethyl-1-[(2’-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole); PD123319, (s)-1-(4-[dimethylamino]-3-methylphenyl)methyl-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylate

**Introduction**

Cellular effects of angiotensin II (AngII) observed under physiological and pathophysiological conditions are attributed to the interaction with specific AT<sub>1</sub>- and AT<sub>2</sub>- type AngII receptors in the plasma membrane [1]. These receptors are coupled to different signal transduction pathways. Stimulation of AT<sub>1</sub>-receptors activates phospholipase C, formation of Ins(1,4,5)P<sub>3</sub>, which subsequently discharges Ca<sup>2+</sup> from internal stores and activates MAP kinase, whereas AT<sub>2</sub>-receptors increase cGMP levels and inhibit cell growth [2, 3]. Intracellular AngII
Intracellular angiotensin II crosstalk with extracellular hormones

binding proteins have been reported in liver [4], storage granules of cardiac myocytes [5], mesangial cells [6], and within the cytosolic fraction of placenta [7]. Recent studies show that AngII can act from within the cell, possibly via intracellular receptors, which differ in their pharmacology from typical plasma membrane AngII receptors [8-11]. Intracellular application of AngII (AngIIi) inhibited cell communication through gap-junctions in heart muscle [8], induced [Ca\textsuperscript{2+}]\textsubscript{i} increases in vascular smooth muscle cells [9], initiated tyrosine phosphorylation in myocytes [10] and elicited contraction of rat aorta [11].

Crosstalk between different receptor systems is essential for physiological functioning. Second messengers induced after receptor stimulation often modulate other second messenger systems either activated by themselves or by other receptor types. Crosstalk have been described at the level of cAMP, Ins(1,4,5)P\textsubscript{3}, [Ca\textsuperscript{2+}]\textsubscript{i} and/or contraction after stimulation of heterologous receptors in smooth muscle [12-15]. With respect to AngII, only Ca\textsuperscript{2+} dependent transactivation of EGF receptors was reported after stimulation of the plasma membrane AT\textsubscript{1}-receptor [16].

There are no data published on a possible role of intracellular AngII in the modulation of cellular responses induced after stimulation of different plasma membrane receptors (heterologous receptor stimulation). Therefore, we investigated the effects of intracellular application of AngII on [Ca\textsuperscript{2+}]\textsubscript{i} in A7r5 smooth muscle cells after serotonin (5HT) and UTP receptor stimulation.

**Methods**

*Cell culture.* A7r5 vascular smooth muscle cells (kindly provided by Dr. H. De Smedt, K. U. Leuven, Belgium) were grown in 75 cm\textsuperscript{2} flasks in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (50 μg ml\textsuperscript{-1}) and streptomycin (50 units ml\textsuperscript{-1}) at 37\textdegree C in a humidified atmosphere (5% CO\textsubscript{2}). The cells were subcultured at 95% confluency by trypsinization. \textsuperscript{45}Ca\textsuperscript{2+} experiments were performed in 6 well plates (Costar, 9.6 cm\textsuperscript{2} well\textsuperscript{-1}) at a density of 10\textsuperscript{5} cells well\textsuperscript{-1}. For fluorescence
experiments the cells were plated 24-72 hours before the start of the experiment in LabTek II (type 155382) chambers.

**Chemicals.** All culture media were obtained from Gibco BRL (U.S.A.). Inositol 1,4,5-trisphosphate sodium salt was obtained from Boehringer (Germany). Fura2-AM and AngII-fluorescein were obtained from Molecular Probes (U.S.A.). $^{45}\text{CaCl}_2$ (specific activity: 19.3 Ci g$^{-1}$) and D-[2-3H]inositol 1,4,5-trisphosphate (specific activity: 3.3 Ci mmol$^{-1}$) were obtained from Dupont-NEN (U.S.A.). AngII was supplied by the Academic Hospital Pharmacy of the University of Groningen. Losartan (2-$n$- butyl-4-chloro-5-hydroxymethyl-1-[(2’-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole) was provided by Merck Sharp & Dohme (U.S.A.) and PD123319 ((s)-1-(4-[dimethylamino]-3-methylphenyl)methyl-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylate) by Park Davis Company (U.S.A.). All other compounds were obtained from Sigma (U.S.A.).

**Liposomes preparation and intracellular application.** Liposomes were prepared as described previously [17, 18], 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). AngII 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, 2 times during 120 min, against buffer solution in a ratio of 1/600 v/v. (Sigma dialysis tubing, molecular weight cut-off: 12400 dalton). 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). The amount of Ang II delivered into the cells by this procedure was estimated by loading the cells with $10^{-6}$ M fluorescein-AngII filled liposomes and subsequent cell permeabilization with saponin comparable to the method described for intracellular adenosine delivery [17]. Fluorescence was measured with excitation wavelength 470 nm,
emission wavelength 520 nm, and a bandpass filter of 4 nm (Aminco Bowman LS Series 2). Liposome-incapsulated fluorescein-AngII amounted to 12.4 ± 0.9 % (n=4) of the initial amount in the aqueous phase. Angiotensin II incorporated into the cells with an efficiency of 2.3 ± 0.4 % (n=4). The actual [AngII] can be calculated if an estimation of the cell volume is established. The cellular 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*π*r³. Cells were slightly attached to the culture dish and the culture medium was sequentially diluted with an equal volume of water. The osmotic swelling process was followed under microscopy and the maximal diameter reached was estimated with a standard micrometer in view. The maximal diameter was 13.4 ± 0.2 μm (n=64), resulting in a volume of 1.26 ± 0.02 pL cell⁻¹. Therefore, if the protocol is used to deliver liposomes filled with 10⁻⁶ M AngII to the cells, this will result in an estimated intracellular [AngII] of 18 ± 3 μmol/L.

**45Ca²⁺ uptake by intact cells.** These experiments were performed as described previously (19). In brief, culture medium was replaced 1 hr before the start of the experiment with a buffer solution containing (mM): 145 NaCl, 5 KCl, 0.5 MgSO₄, 0.5 CaCl₂, 10 glucose and 10 HEPES (pH adjusted to 7.4 with NaOH). Uptake of ⁴⁵Ca²⁺ was measured at room temperature (22-24 °C) and started by removing the solution and replacing it by the same buffer (1 ml) supplemented with 10 μCi ⁴⁵Ca²⁺ (specific activity 19.3 Ci g⁻¹) 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 1ml ice-cold buffer stopped uptake of ⁴⁵Ca²⁺ in the absence of CaCl₂ after 5 min. Thereafter, cells were washed 3 times with buffer without CaCl₂ and 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 were corrected for non-specific binding as determined by adding buffer with ⁴⁵Ca²⁺ and immediately terminating uptake.
[Ca\textsuperscript{2+}]\textsubscript{i} measurements. Cells were loaded with 5 μM fura2-AM for 45 min at 37°C. Calcium measurements were performed using a S100 Axiovert inverted microscope (Zeiss). The 340/380 ratio was acquired at room temperature with at a frequency by 1 Hz using a cooled CCD camera (SensiCam) and Imaging Workbench 2.2. software (Axon Instruments). Liposomes were added at a ratio of 1/20 vol/vol, 5 min before agonist addition. Ratio values were transformed in [Ca\textsuperscript{2+}]\textsubscript{i} at the end of the experiment [20].

Measurement of Ins (1,4,5)P\textsubscript{3}. Mass measurement of Ins (1,4,5)P\textsubscript{3} was performed using a ligand binding assay as described earlier in detail [15].

Statistics. All experiments were performed in series with n ≥ 4. The results are expressed as mean ± S.D. Statistical differences were tested either by ANOVA analysis followed by Bonferroni post-test or by unpaired Student’s t-test considering p ≤ 0.05 significantly different.

Results
A7r5 cells represent an attractive model to investigate the effects of intracellular AngII. Since these cells do not respond to extracellular addition of AngII (10\textsuperscript{-6} M) in terms of [Ca\textsuperscript{2+}]\textsubscript{i}, or Ins(1,4,5)P\textsubscript{3} levels (Table 1) one can study the effects of AngII, on signal transduction processes without interference of cellular signalling activated by plasma membrane AngII receptors.

Table 1 Effects of extracellular AngII on [Ca\textsuperscript{2+}]\textsubscript{i} and Ins(1,4,5)P\textsubscript{3} levels in A7r5 cells

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<th>[Ca\textsuperscript{2+}]\textsubscript{i} (nM)</th>
<th>Ins(1,4,5)P\textsubscript{3} (pmol/10\textsuperscript{5} cells)</th>
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<td>Basal</td>
<td>57 ± 6</td>
<td>0.23 ± 006</td>
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<tr>
<td>AngII</td>
<td>58 ± 6</td>
<td>0.22 ± 0.04</td>
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Peak [Ca\textsuperscript{2+}]\textsubscript{i} and Ins(1,4,5)P\textsubscript{3} were measured 30 s after stimulation with extracellular AngII (10\textsuperscript{-6} M). n ≥ 6 in each case.
We used liposomes to administer AngII intracellularly. Various compounds can be delivered by liposomes, while plasma membrane integrity is maintained (17, 18, 21). Cells are also not metabolically compromised by liposome treatment, since methylene blue is still excluded and incubation with control liposomes (24 hr) did not affect cell growth (data not shown). Moreover, pre-treatment with control liposomes (filled with 140 mM KCl) did not affect Ca$^{2+}$ increases induced by either 5HT or UTP (Fig. 1).

Influx of $^{45}$Ca$^{2+}$ was increased about 2-fold after stimulation with 5HT or UTP for 5 min (Fig. 1). This increase was partially inhibited by liposomes containing 10$^{-6}$ M AngII, whereas control liposomes were ineffective. The effect of AngII filled liposomes was unaffected by the extracellular addition of the AT$_1$-receptor antagonist losartan (10$^{-6}$ M) or the AT$_2$-receptor antagonist PD123319 (10$^{-6}$ M, n=6, data not shown). In contrast, inclusion of losartan into liposomes together with AngII reversed the inhibition of $^{45}$Ca$^{2+}$ influx by AngII. Liposomes

**Fig. 1. Effects of intracellular AngII on $^{45}$Ca$^{2+}$ uptake induced by 5HT or UTP.** Panel A: $^{45}$Ca$^{2+}$ uptake induced by extracellular 5HT (10$^{-5}$ M) for 5 min (first bar), in the presence of liposomes filled with 140 mM KCl (L$\text{con}$) or filled with either 10$^{-6}$ M Ang II (+L$\text{AngII}$) alone or together with 10$^{-6}$ M losartan (+L$\text{AngII} + \text{Llos}$) or 10$^{-6}$ M PD123319 (+L$\text{AngII} + \text{LPD}$). **Panel B:** idem for extracellular stimulation with UTP (10$^{-3}$ M). Data are presented as mean ± S.D. (n=6) and expressed as % of uptake in the absence of extracellular 5HT or UTP amounting 85 dpm/10$^5$ cells. * P<0.05 vs control stimulation (5HT or UTP alone).
containing both AngII and PD123319 were ineffective in reversal of the AngII effect.

The effect of AngII on 5HT and UTP induced changes in $[\text{Ca}^{2+}]_i$ homeostasis was confirmed by single cell $[\text{Ca}^{2+}]_i$ measurements using fura2 fluorescence. Pre-incubation with Ang II containing liposomes reduced the 5HT and UTP induced $[\text{Ca}^{2+}]_i$ increases similarly as in the $^{45}\text{Ca}^{2+}$ uptake experiments to approximately 30% (Fig. 2).

![Graphs showing effects of intracellular AngII on $[\text{Ca}^{2+}]_i$ induced by 5HT or UTP.](image)

**Fig. 2.** Effects of intracellular AngII on $[\text{Ca}^{2+}]_i$ induced by 5HT or UTP.

**Upper panels:** Changes in $[\text{Ca}^{2+}]_i$ as measured by single cell fura2 fluorescence induced by extracellular 5HT (A, $10^{-5}$ M) or UTP (B, $10^{-3}$ M) in the absence (a) or presence (b) of liposomes filled with $10^{-6}$ M AngII.

**Lower panels:** Maximal $[\text{Ca}^{2+}]_i$ increases induced by $10^{-5}$ M 5HT (left panel) or $10^{-3}$ M UTP (right panel) in the absence (first bar) or the presence of liposomes (5 min pre-incubation) filled with 140 mM KCl ($L_{\text{con}}$) or with $10^{-6}$ M AngII ($L_{\text{AngII}}$). Data are presented as mean ± S.D. (n=6). * P<0.05 vs control.
Control liposomes filled with 140 mM KCl were ineffective. The inhibitory effect was reversed by losartan containing liposomes, but not by PD123319 containing liposomes (Table 2). These results show that intracellular AngII exerts its effect through an AT₁-like receptor, probably via inhibition of Ca²⁺ influx.

Table 2 Inhibition of 5HT induced [Ca²⁺]ᵢ increases by intracellular Ang II. Effects of antagonists

<table>
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<th>[Ca²⁺]ᵢ increases</th>
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<tr>
<td>L control + 5HT</td>
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<tr>
<td>L AngII + 5HT</td>
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<tr>
<td>L(AngII + losartan) + 5HT</td>
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<td>L(AngII + PD123319) + 5HT</td>
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Increases in peak [Ca²⁺]ᵢ were measured after stimulation with 5HT (10⁻⁵ M) and liposomes filled with AngII (10⁻⁶ M) either in the absence or presence of losartan (10⁻⁶ M) or PD123319 (10⁻⁶ M). n ≥ 6 in each case. a) different from L control + 5HT (P<0.05). b) different from L AngII + 5HT (P<0.05).

To clarify which part of Ca²⁺ signalling is affected by intracellular AngII we further investigated this effect on 5HT induced changes in [Ca²⁺]ᵢ. Using a Ca²⁺ chelation/readmission protocol, Ca²⁺ release and Ca²⁺ influx could be discriminated.

Stimulation with 5HT predominantly elevates [Ca²⁺]ᵢ via Ca²⁺ influx, which is largely mediated via voltage dependent Ca²⁺ channel, as demonstrated by the almost complete inhibition by 10⁻⁶ M verapamil (Fig. 3). Intracellular application of AngII inhibits this 5HT induced Ca²⁺ influx to a similar extent as verapamil, possibly indicating that inhibition of this channel by AngII occurs, whereas it does not affect Ca²⁺ release.
Discussion

Effects of AngII can be mediated via mechanisms atypical for plasma membrane AngII receptors in various cell types [8-11]. This implies that patho-physiological actions of AngII might occur intracellularly and therefore not accessible to classical pharmacological agents working at the plasma membrane. Many studies described crosstalk between components of different receptor classes and at different levels downstream receptor activation [22-24]. However, the novelty of the present work is to show interactions between an agonist (AngII) and
heterologous receptor stimulation not initiated by AngII at the plasma membrane level, but by intracellular receptor stimulation.

In this study we have used A7r5 cells which cannot be stimulated by extracellular AngII as concluded from the results on Ins(1,4,5)P3 formation and [Ca\textsuperscript{2+}]\textsubscript{i}. Therefore interference of the results obtained by AngII\textsubscript{i} on Ca\textsuperscript{2+} homeostasis with possible effects induced by plasma membrane AT-receptors receptor is excluded. AngII\textsubscript{i} potently reduced the Ca\textsuperscript{2+} signal evoked by 5HT and UTP stimulation. In view of the experiments performed with the antagonist losartan and PD123319 it is suggested that this effect of AngII\textsubscript{i} be mediated by an AT\textsubscript{1}-like receptor, but the ineffectiveness of AngI indicate that it might be different from common plasma membrane AT\textsubscript{1} receptors [25].

Reductions of Ca\textsuperscript{2+} increases induced by AngII\textsubscript{i} are the not the consequence of inhibition of Ca\textsuperscript{2+} release from intracellular stores, but can be totally attributed to inhibition of Ca\textsuperscript{2+} influx. The parallel effect of AngII\textsubscript{i} and verapamil indicates that the L-type Ca\textsuperscript{2+} channel might be the target for this effect. This novel finding of the AngII\textsubscript{i} induced inhibition of heterologous receptor activated Ca\textsuperscript{2+} influx can be supported by the observations made in rat cardiomyocytes showing reduction in amplitude of inward voltage-dependent Ca\textsuperscript{2+} current after dialysing AngII into the cell [26].

Inhibition of heterologous receptor activated Ca\textsuperscript{2+} influx might be of physiological relevance in the process of fine-tuning of multiple receptor signalling pathways. Also overstimulation due to exposure to different mediators can be avoided by such a mechanism, particular beneficial under patho-physiological conditions in which Ca\textsuperscript{2+} overload is implicated like e.g. ischemia. For such a negative protective feedback mechanism availability of AngII\textsubscript{i} is essential. It is likely that AngII\textsubscript{i} can fulfil this role, since intracellular pools of AngII were observed in cardiomyocytes [5] and renal endosomes [27] under physiological conditions.

In conclusion, AngII\textsubscript{i} inhibited Ca\textsuperscript{2+} entry induced after heterologous receptor stimulation with either 5HT or UTP via an AT\textsubscript{1}-like receptor in A7r5 smooth muscle cells.
Chapter IV

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REFERENCES


