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Evidence for changes in the tachyphylactic property of recombinant angiotensin II AT_1 receptor expressed in CHO cells

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Abstract

The manifestation of tachyphylaxis to angiotensin II in Chinese hamster ovary (CHO) cells expressing the rat angiotensin II AT_1 receptor was investigated. The cells were transfected with a cDNA fragment containing the complete coding region of the angiotensin II AT_{1A} receptor gene, as well as 56 bp of its 3′- and 52 bp of its 5′-untranslated regions. These cells (CHO-AT_1) responded to angiotensin II by increases in intracellular Ca^{2+} concentration and inositol phosphate turnover, which were inhibited upon repeated administrations, characterizing the tachyphylaxis phenomenon. In contrast to smooth muscle cells, which are rendered tachyphylactic to angiotensin II but not to [2-lysine]angiotensin II ([Lys^2]angiotensin II), this analogue induced responses in CHO-AT_1 cells that were also inhibited upon repeated administrations. A smooth muscle cell line, which showed tachyphylaxis only to angiotensin II, became tachyphylactic also to [Lys^2]angiotensin II after transfection with the angiotensin II AT_1 receptor gene. Our findings suggest that posttranscriptional control directed by the 3′- or the 5′-untranslated regions in the angiotensin II AT_1 receptor gene may play a role in modulating the signal transduction pathways involved in the mechanism of angiotensin II tachyphylaxis.

Keywords: Angiotensin II; [Lys^2]angiotensin II; Angiotensin II AT_1 receptor, recombinant; Tachyphylaxis; CHO cell line; Smooth muscle cell line

1. Introduction

The interaction of angiotensin II with its AT_1 receptor in smooth muscle cells activates multiple signalling pathways that result in a complex sequence of temporally distinct responses (for reviews, see Thomas, 1999; Touyz and Schiffrin, 2000). Many of these responses are characterized by a specific desensitization that is partly due to internalization of the ligand–receptor complex, but also involves ligand-induced changes in receptor affinity. In most tissues, exposure to the agonist leads to receptor down-regulation (Cui et al., 2000; Anborgh et al., 2000; Richard et al., 1997), but in some smooth muscles, such as the guinea pig ileum and the rat aorta, distinct desensitization effects have been observed between prolonged and short-term exposures to angiotensin II. After exposures of several minutes to the ligand, a decrease in the responses is observed which was shown to be associated with protein kinase C inhibition (Shimuta et al., 1990). However, repeated administrations of angiotensin II of short duration (tachyphylactic protocol) elicit responses of decreasing amplitude, in a phenomenon known as tachyphylaxis. This behaviour is induced by angiotensin II but not by analogues lacking the guanidinium group of the arginine at position 2, such as [2-lysine]angiotensin II ([Lys^2]angiotensin II) (Miasiro et al., 1983). The tachyphylactic property was shown to be due to the inhibition of the inositol phosphate and Ca^{2+} responses, and seemed to involve a change of the receptor affinity rather than receptor internalization (Kanashiro et al., 1995).

Chinese hamster ovary (CHO) cells stably transfected with the coding sequence of the angiotensin AT_1 receptor gene (CHO-AT_1) respond to angiotensin II with activation of phospholipase C and consequent production of and increase in intracellular Ca^{2+} concentration (Ohnishi et al., 1992; Oppermann et al., 1996; Han et al., 1998) and also exhibit desensitization (Barker et al., 1995).

In this paper, we report the results of a study conducted to determine whether the structural requisites for angiotensin II to induce tachyphylaxis in CHO-AT_1 cells, as well as on a smooth muscle cell line transfected with exogenous

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angiotensin II AT_1 receptor cDNA, parallel those reported for isolated smooth muscle preparations.

2. Materials and methods

2.1. Cell culture and transfection

CHO cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. These cells were stably transfected with a cDNA fragment coding for the rat angiotensin II AT_{1A} receptor, containing 56 bp of the 3′- and 52 bp of the 5′-untranslated region, which was subcloned into the pTEJ8 vector as described by Han et al. (1998). Transfected cells were selected for the ability to grow in the presence of geneticin (0.8 mg/ml). After the cells were grown in the selective medium, they were frozen in liquid nitrogen, still in the presence of geneticin.

A spontaneously transformed smooth muscle cell line was obtained from rabbit aorta as described by Buonassisi and Colburn (1983). Briefly, after the removal of adventitia and intima, small rings of the aorta were treated with collagenase (0.1%) and viokinase (0.1%) in Hank's buffered salt solution (HBSS) and incubated at 37 °C in an atmosphere of 5% CO_2 for 90 min. The aortic rings were then distributed into tissue culture flasks (Falcon, 25 cm^2) containing HBSS, and the medium was changed every 3 days. Proliferating smooth muscle cells in log growth phase were obtained at 3–5 days, confluent cells at 8–10 days. Cells were harvested every 3 days with 0.25% viokase and subcultured by diluting the Ham's F12 medium 1:2, during 1 year. The established smooth muscle cell line was maintained at 37 °C in a 5% CO_2 atmosphere in Ham's F-12 medium supplemented with 10% fetal bovine serum plus 0.5 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The smooth muscle nature of these cells was ascertained by immunocytochemical localization performed with a smooth muscle-specific α-actin antibody (ASM-1, Progen, Heidelberg, Germany) labeled with a fluorescence marker (Bendhack et al., 1992). Staining of the cells with this antibody revealed that all cells in the preparation were labeled. It was further determined that these cells were free from contamination with endothelial cells or fibroblasts by immunocytochemical staining with antibodies against von Willebrand's factor coupled to a fluorescent marker.

The smooth muscle cells containing the endogenous angiotensin II receptor were also transfected with exogenous angiotensin II AT_1 receptor cDNA, as described above for the CHO cells.

2.2. $^{45}Ca^{2+}$ uptake

Confluent cells in culture dishes were washed three times with Tyrode solution (137 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, 0.49 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 5.6 mM glucose, pH 7.4) and equilibrated for 1 h at 37 °C. The cells were then incubated for 20 s with the desired $^{45}Ca^{2+}$-labeled solutions in the presence of the agonists, after which the cells were washed and counted in the scintillation liquid, in a Packard β counter, model Tri Carb 2100 TR, as previously described (Shimuta et al., 1990).

2.3. Inositol phosphate production

Confluent cells ($1-2 \times 10^6$ cells) expressing angiotensin II AT_1 receptor were cultivated for 18–24 h in inositol-free medium (199 Dulbecco supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin) in 3.5-cm^2 culture plates containing 5 μCi [2-^{3}H]myoinositol. The cells were washed twice with Tyrode solution and then stimulated with 1 μM [Lys^2]angiotensin II or 0.1 μM angiotensin II for 30 min, in the presence of 10 mM LiCl as previously described (Han et al., 1998). The [^{3}H]inositol phosphate was eluted with 1.0 M ammonium formate in 0.1 M formic acid and the eluate was counted, in the scintilation liquid, in a Packard β counter. A concentration–response curve, generated by iterative nonlinear regression analysis (GraphPad Prism Software, San Diego, CA) was used to determine ED_{50} and maximum effect values.

2.4. Cytosolic Ca^{2+} measurements

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurements were made using confluent CHO-AT_1 cells cultured on coverslips as described by Shimuta et al. (1993). In brief, transfected cells were incubated at 37 °C for 2 h in a solution containing 2 μM fura-2-acetoxy-methyl ester (fura-2/AM). After washing the cells with Tyrode solution, the desired ligand solution was added. The fura-2 fluorescence from CHO-AT_1 cells excited at 505 nm, as well as the background fluorescence were recorded at either 380 or 340 nm and the 340:380 nm fluorescence ratio was determined at 0.5-s intervals. At the end of each experiment, the maximum and minimum ratios for calcium-bound and free fura-2 were obtained by adding digitonin and EGTA at final concentrations of 50 μM and 12 mM, respectively. Mean basal $[Ca^{2+}]_i$ and responses to agonists were obtained in confluent cultures when $\geq 80-90\%$ of the total coverslip surface was covered with CHO-AT_1 cells, and calculated as described by Grynkiewicz et al. (1985).

2.5. Induction of tachyphylaxis

To study the induction of tachyphylaxis by [Lys^2]angiotensin II or angiotensin II, CHO-AT_1 cells were submitted either to a single treatment with the ligand or to a "tachyphylactic protocol": three 90-s exposures to the ligand followed by washing with Tyrode solution and an additional 3.5-min resting period (during which the cell's inositol

phosphate or Ca^{2+} levels returned to basal values). After washing with Tyrode solution, the [^{3}H]inositol phosphate or Ca^{2+} responses were measured. In the experiments in which the effect of acid washing was studied, the Tyrode solution was replaced by glycine buffer solution (50 mM glycine, 150 mM NaCl, pH 3.0).

2.6. Materials

The peptides angiotensin II and its analogue [Lys^2]angiotensin II were synthesized in our laboratory. [2-^{3}H]myoinositol (20 Ci/mmol) and $^{45}Ca^{2+}$ (615 mCi/mmol) were purchased from Amersham International (Little Chalfont, Buckinghamshire, UK) and Dowex AG1-X8 resin (100–200 mesh in formate form) from Bio-Rad (Richmond, CA). Cell culture media, fetal calf serum, Ham's F-12 medium, Hanks' medium, antibiotics, geneticin and tissue culture flasks were purchased from Gibco BRL (Gaithersburg, MD) and Qiagen columns from Qiagen (Chatsworth, CA). Fura-2/AM was from Molecular Probes (Leiden, Netherlands). All other chemicals were obtained from Sigma (St. Louis, MO).

The expression plasmids for the pTEJ8 vector containing the angiotensin II AT_1 receptor cDNA were kindly provided by Professor Thue W. Schwartz from the Laboratory for Molecular Pharmacology, Copenhagen, Denmark.

3. Results

3.1. Responses of CHO-AT_1 cells to [Lys^2]angiotensin II and to angiotensin II

The activation of CHO-AT_1 cells by 30-min treatments with the peptides was assessed by measuring the stimulation of phospholipase C by means of concentration–effect curves for total [^{3}H]inositol phosphate formation. [Lys^2]angiotensin II induced effective responses with an ED_{50} value of 16 ± 1.5 nM (n = 3), whereas the ED_{50} value for the angiotensin II-induced effect was 1.1 ± 0.1 nM (n = 5). The maximum effects induced by the two peptides were not significantly different: the increases over the basal value (568 ± 28 cpm/10^6 cells, taken as 100%) were 122 ± 8% for [Lys^2]angiotensin II and 120 ± 7% for angiotensin II.

Since angiotensin II, besides stimulating phosphoinositide hydrolysis, also promotes Ca^{2+} release from intracellular stores, as well as Ca^{2+} entry into smooth muscle cells (Timmermans et al., 1993), both the Ca^{2+} uptake and the increases of $[Ca^{2+}]_i$ in response to 1 μM [Lys^2]angiotensin II and to 0.1 μM angiotensin II were determined in CHO-AT_1 cells. It was observed that after 20-s pulses with $^{45}Ca^{2+}$ the two peptides induced similar increases in Ca^{2+} uptake over the basal value (148 ± 8 cpm/10^6 cells, taken as 100%): 80 ± 3% (n = 4) for [Lys^2]angiotensin II and 111 ± 3% (n = 4) for angiotensin II. [Lys^2]angiotensin II (1 μM) or angiotensin II (0.1 μM) also induced abrupt elevations of $[Ca^{2+}]_i$, reaching 290 ± 25% (n = 3) or 250 ± 23% (n = 3), respectively, in relation to the basal value for $[Ca^{2+}]_i$, which was 110 ± 10 nM, considered as 100%. This was followed by a return to near the basal value within 60 s (Fig. 1).

3.2. Effect of repeated administrations of [Lys^2]angiotensin II or angiotensin II to CHO-AT_1 cells

When CHO-AT_1 cells were submitted to repeated treatments with [Lys^2]angiotensin II (tachyphylactic protocol) the responses to the third stimulation were decreased in relation to the first (Figs. 1 and 2). This tachyphylactic response amounted to 50% in the [^{3}H]inositol phosphate turnover, 94% in the $^{45}Ca^{2+}$ uptake and 83% in the $[Ca^{2+}]_i$ responses. For the case of angiotensin II, the tachyphylaxis amounted to 67% in the [^{3}H]inositol phosphate turnover, 97% in the $^{45}Ca^{2+}$ uptake and 92% in the $[Ca^{2+}]_i$ responses.

3.3. Reversal of tachyphylaxis in CHO-AT_1 cells

It was previously shown that angiotensin II-induced tachyphylaxis in smooth muscles is reversed after a 30-min resting period (Paiva et al., 1977). To determine whether this also occurs in CHO-AT_1 cells, the tachyphylactic protocol was used to induce tachyphylaxis to [Lys^2] angiotensin II or to angiotensin II, which reduced the Ca^{2+}

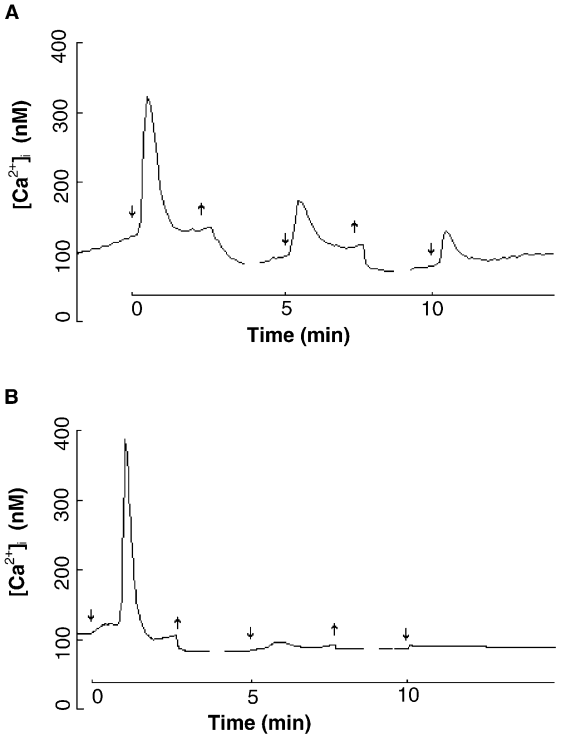

Fig. 1. Representative traces showing the effect of repeated (three 90-s incubation at 5-min intervals) stimulations with 1 μM [Lys^2]angiotensin II (A) or 0.1 μM angiotensin II (B) on the intracellular calcium concentration ($[Ca^{2+}]_i$) in CHO-AT_1 cells pretreated with fura-2/AM. Downward and upward arrows indicate addition and removal of ligands, respectively.

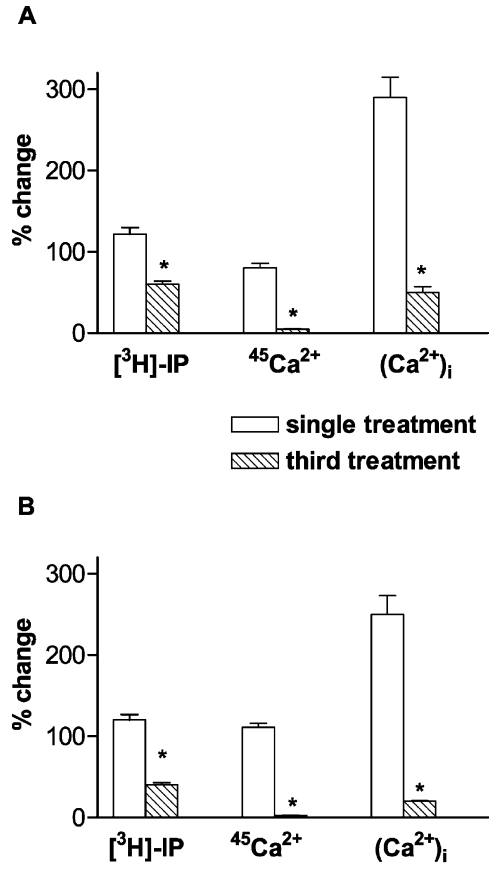

Fig. 2. Effect of repeated treatments with [Lys^2]angiotensin II (A) or angiotensin II (B) on the [^{3}H]inositol phosphate ([^{3}H]-IP) production, calcium uptake ($^{45}Ca^{2+}$) and intracellular Ca^{2+} ($[Ca^{2+}]_i$) responses of CHO-AT$_1$ cells. Responses to the agonists were recorded after the cells were exposed either to a single treatment or to the tachyphylactic protocol (three 90-s treatments at 5-min intervals) with 1 μM [Lys^2]angiotensin II or 0.1 μM angiotensin II. The values represent the means ± S.D. obtained from four experiments performed in triplicates. [a]Significantly different ($P < 0.05$) from the response to the single treatment.

responses by 64% or 73%, respectively. After a 30-min resting period, single challenges with either peptide showed that the [Lys^2]angiotensin II-induced $^{45}Ca^{2+}$ uptake was recovered (8 ± 2% tachyphylaxis), whereas the CHO-AT$_1$ cells still presented blunted responses to angiotensin II (71 ± 7% tachyphylaxis).

3.4. Effect of pH

In smooth muscle cells, the pH of the medium affects the manifestation of angiotensin II tachyphylaxis, which is attenuated by increases in the pH (Paiva et al., 1974). Therefore, we have studied the effect of pH on the changes induced by [Lys^2]angiotensin II or angiotensin II in $^{45}Ca^{2+}$ uptake in CHO-AT$_1$ cells. We found that the tachyphylaxis induced by 1 μM [Lys^2]angiotensin II amounted to 65% at pH 7.0, 63% at pH 7.4 and 66% at pH 8.0. The tachyphylactic response to 0.1 μM angiotensin II also was not significantly affected by the pH of the medium in the pH range 7.0–8.0.

3.5. Effect of acid washings

The binding of angiotensin II to its receptor is followed by a sequestration of the ligand–receptor complex by the cell membrane, as a first step of an internalization process (Griendling et al., 1987). If the cells are exposed to a mildly acid medium before the complex is sequestered, the ligand–receptor complex is dissociated, without drastic alterations of the cell membrane (Griendling et al., 1987; Oppermann et al., 1996; Ascoli, 1982). Tachyphylaxis in smooth muscle cells was shown to occur during this early phase of the process, before the sequestration step (Kanashiro et al., 1995). Accordingly, we have verified the effect of acid washes within the first 15 s of the treatment with the ligands on the development of tachyphylaxis in CHO-AT$_1$ cells. The cells were submitted to repeated treatments with 1 μM [Lys^2]angiotensin II or 0.1 μM angiotensin II for 90 s at 5-min intervals and $^{45}Ca^{2+}$ uptake was determined in the responses to the first or third treatments, after washing the cells with either Tyrode or pH 3.0 glycine buffer.

The results, presented in Fig. 3, show that tachyphylaxis to both [Lys^2]angiotensin II and angiotensin II was pre-

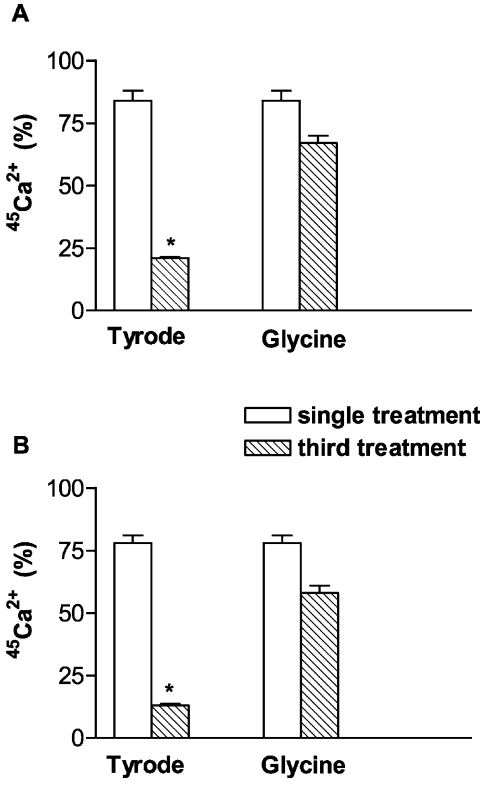

Fig. 3. Efffect of acid washing on the induction of tachyphylaxis by [Lys^2]angiotensin II (A) or angiotensin II (B) in CHO-AT$_1$ cells. The cells were exposed either to a single treatment or to the tachyphylactic protocol (three 15-s treatments at 5-min intervals) with 1 μM [Lys^2]angiotensin II or 0.1 μM angiotensin II, followed by washing with either Tyrode or glycine buffer (pH 3.0), and the $^{45}Ca^{2+}$ uptake responses were measured. The data are means ± S.D. of four experiments performed in triplicate. [a]Significantly different ($P < 0.05$) from the response to the single treatment.

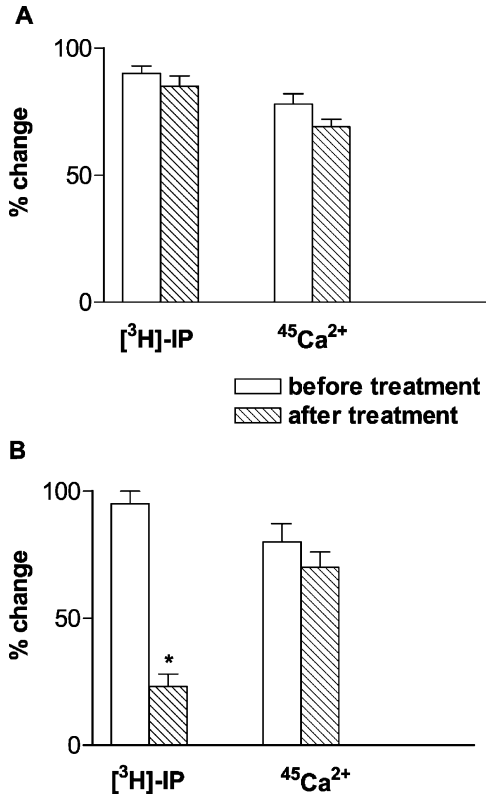

Fig. 4. Responses to ATP before and after induction of tachyphylaxis by [Lys^2]angiotensin II (A) or angiotensin II (B). CHO-AT$_1$ cells were treated with the tachyphylactic protocol (two 90-s treatments at 5-min intervals) with 1 μM [Lys^2]angiotensin II or 0.1 μM angiotensin II. The [^{3}H]inositol phosphate ([^{3}H]-IP) formation and $^{45}Ca^{2+}$ uptake induced by 10 μM ATP were recorded before and after the tachyphylactic protocol, and the percent changes in the responses are shown. The data are means ± S.D. of four experiments performed in triplicate. [a]Significantly different ($P < 0.05$) from the response to the single treatment.

vented when glycine buffer but not Tyrode solution was used in the washings.

3.6. Homologous and heterologous cross-tachyphylaxis

CHO-AT$_1$ cells rendered tachyphylactic to [Lys^2]angiotensin II showed inhibited responses to angiotensin II (76 ± 5%) and, conversely, responses to [Lys^2]angiotensin II were greatly reduced (90 ± 8%) in cells in which tachyphylaxis was induced by previous treatments with angiotensin II.

The specificity of the tachyphylactic state was tested by measuring the response of tachyphylactic cells to ATP, which acts on different receptors but whose response is at least partly coupled to the phospholipase C signalling pathway in CHO cells (Strassheim and Williams, 2000). Fig. 4 shows that CHO-AT$_1$ cells rendered tachyphylactic to [Lys^2]angiotensin II responded normally to 10 μM ATP. However, when tachyphylaxis was induced by angiotensin II the effect of ATP on [^{3}H]inositol phosphate production was inhibited, but not its effect on $^{45}Ca^{2+}$ uptake.

3.7. Tachyphylaxis to [Lys^2]angiotensin II and angiotensin II in aortic smooth muscle cells expressing exogenous angiotensin II AT1 receptor

In order to verify if the tachyphylaxis to [Lys^2]angiotensin II observed in CHO-AT$_1$ cells might be a property of that cell line, the experiments were also performed in spontaneously transformed aortic smooth muscle cells (Buonassisi and Colburn, 1983). These cells express the angiotensin II AT$_1$ receptor, as indicated by their responses to both ligands, and could be rendered tachyphylactic to angiotensin II but not to [Lys^2]angiotensin II (Table 1), in agreement with previous results obtained with isolated smooth muscle cells (Kanashiro et al., 1995). However, when these cells were transfected with exogenous angiotensin II AT$_1$ cDNA, their behaviour became similar to that of the CHO-AT$_1$ cells, presenting tachyphylaxis to both angiotensin II and [Lys^2]angiotensin II (Table 1).

4. Discussion

The responses of CHO-AT$_1$ cells to [Lys^2]angiotensin II and to angiotensin II were similar to those previously described for smooth muscle tissues and cells, with increases in the inositol phosphate turnover, Ca^{2+} influx and $[Ca^{2+}]_i$ levels (Miasiro et al., 1983; Kanashiro et al.,

Table 1
Tachyphylaxis in a smooth muscle cell line transfected with cDNA for the angiotensin AT$_1$ receptor

Treatments	[Lys^2]angiotensin II responses (percent change over basal)				Angiotensin II responses (percent change over basal)			
	Control		Transfected		Control		Transfected	
	[^{3}H]-IP	$^{45}Ca^{2+}$	[^{3}H]-IP	$^{45}Ca^{2+}$	[^{3}H]-IP	$^{45}Ca^{2+}$	[^{3}H]-IP	$^{45}Ca^{2+}$ uptake
Single treatment	60 ± 2	47 ± 2	57 ± 4	44 ± 2	58 ± 1	56 ± 2	77 ± 3	53 ± 3
Third treatment	54 ± 2	44 ± 2	9 ± 0.2[a]	1 ± 0.1[a]	5 ± 0.2[a]	5 ± 0.1[a]	4 ± 0.3[a]	1 ± 0.1[a]
Tachyphylaxis (percent)	10	6	84	98	91	91	95	98

Smooth muscle cells, transfected or not (controls), with cDNA for the AT$_1$ receptor were submitted to the tachyphylactic protocol with 1 μM [Lys^2]angiotensin II or 0.1 μM angiotensin II, and the [^{3}H]inositol phosphate ([^{3}H]-IP) formation and $^{45}Ca^{2+}$ uptake were measured. Tachyphylaxis was estimated as the percent inhibition of the third response relative to that of the single treatment. The values are the means ± S.D. obtained from four experiments performed in triplicates.
[a] Significantly different ($P < 0.05$) from the response to single treatment.

1995). With regard to the tachyphylactic behaviour, however, important differences were observed. Thus, in CHO-AT_1 cells, in contrast to the behaviour of smooth muscle cells, angiotensin II-induced tachyphylaxis showed no pH-dependence, and its reversal was slower.

A more striking difference was the finding that [Lys^2]angiotensin II was able to induce tachyphylaxis in CHO-AT_1 cells, which also involved both the inositol phosphate turnover and Ca^{2+} uptake responses, and showed no pH dependence in the range 7.0–8.0. This contrasts with the previous findings that, in smooth muscle preparations (Miasiro et al., 1983) or cultured smooth muscle cells (Shimuta et al., 1993), angiotensin II analogues lacking the guanido group in position 2 of the peptide chain were unable to cause tachyphylaxis. This difference in behaviour might be due to the fact that the tachyphylactic property may be regulated by an untranslated region of the angiotensin II AT_1 receptor gene not contained in the cDNA fragment used for transfecting the CHO cells. This is supported by the finding that a smooth muscle cell line, which showed tachyphylaxis to angiotensin II and not to [Lys^2]angiotensin II, also became tachyphylactic to both peptides after being transfected with the cDNA fragment used in this study.

The participation of the 3′- and 5′-untranslated regions of the angiotensin II AT_1 receptor in determining cellular responses to the agonist (Thekkumkara et al., 1998), including down-regulation (Xu and Murphy, 2000), has been demonstrated.

Similarly to what was observed for angiotensin II in cultured smooth muscle cells, early washing of the CHO-AT_1 cells with a pH 3.0 buffer solution, which removes the peptide ligand before it is sequestered by the cell membrane, prevented tachyphylaxis to both angiotensin II or [Lys^2]angiotensin II. Washing with Tyrode solution, under the same experimental conditions, did not avoid tachyphylaxis. This indicates that the maintenance of the ligand–receptor complex, at least during the sequestration step, is necessary for promoting the changes in the receptor that lead to the tachyphylactic state. Another difference between smooth muscle and CHO-AT_1 cells is the manifestation of cross-tachyphylaxis between angiotensin II and ATP, which does not occur in smooth muscle cells (Moore and Khairallah, 1976; Oshiro et al., 1984). Interestingly, the inositol phosphate turnover, but not the calcium influx response to ATP was depressed in CHO-AT_1 cells rendered tachyphylactic to angiotensin II. This may be due to the fact that the activation of phospholipase C (and subsequent inositol phosphate release) and the activation of voltage-dependent calcium channels leading to calcium influx are independently coupled to receptor activation (Ohnishi et al., 1992). Apparently, different calcium influx pathways are activated by the two receptors, whereas a common step in the phosphoinositide signaling pathway may be shared by the two systems.

The lack of cross-tachyphylaxis between [Lys^2]angiotensin II and ATP may be related to the proposed differences between this analogue and angiotensin II regarding their receptor binding modes (Shimuta et al., 1990), and further investigation of this property may bring more information about that hypothesis.

Our results with the smooth muscle cell line provides evidence that transfection of a cDNA fragment corresponding to a truncated angiotensin II AT_1 receptor mRNA containing the complete coding region of the receptor gene, as well as 56 bp of its 3′- and 52 bp of its 5′-untranslated regions, may change the functional expression of the receptor in cell lines. In support of this hypothesis, Thekkumkara et al. (1998) demonstrated a role for the angiotensin II AT_1 receptor's 3′-untranslated region in determining cellular responses to agonist. On the other hand, Xu and Murphy (2000) showed that the 5′-untranslated region is involved in cyclic AMP-mediated down-regulation of the angiotensin II AT_1 receptor gene expression, whereas deletions of the 3′-untranslated region had no effect. Our results suggest that posttranscriptional control by these untranslated regions may play a role in modulating the coupling of the angiotensin II AT_1 receptor to the signal transduction pathways involved in the mechanism of angiotensin II tachyphylaxis.

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References

Anborgh, P.H., Seachrist, J.L., Dale, L.B., Ferguson, S.S., 2000. Receptor/beta-arrestin complex formation and the differential trafficking and resensitization of beta2-adrenergic and angiotensin II type 1A receptors. Mol. Endocrinol. 14, 2040–2053.

Ascoli, M., 1982. Internalization and degradation of the receptor-bound human choriogonadotropin in Leydig tumor cells. J. Biol. Chem. 257, 13306–13311.

Barker, S., Kapas, S., Fluck, R.J., Clark, A.J.L., 1995. Effects of the selective protein kinase C inhibitor Ro 31-7549 on human angiotensin II receptor desensitisation and intracellular calcium release. FEBS Lett. 369, 263–266.

Bendhack, L.M., Sharma, R.V., Bhalla, R.C., 1992. Altered signal transduction in vascular smooth muscle cells of spontaneously hypertensive rats. Hypertension 19, 42–48.

Buonassisi, V., Colburn, P., 1983. Antibodies to the heparan sulfate proteoglycans synthesized by endothelial cell cultures. Biochim. Biophys. Acta 760, 1–12.

Cui, T., Nakagami, H., Iwai, M., Takeda, Y., Shiuchi, T., Tamura, K., Daviet, L., Horiuchi, M., 2000. ATRAP, novel AT_1 receptor associated protein, enhances internalization of AT_1 receptor and inhibits vascular smooth muscle cell growth. Biochem. Biophys. Res. Commun. 279, 938–941.

Griendling, K.K., Delafontaine, P., Rittenhouse, S.E., Gimbrone Jr., M.A., Alexander, R.W. 1987. Correlation of receptor sequestration with sustained diacylglycerol accumulation in angiotensin II-stimulated cultured vascular smooth muscle cells. J. Biol. Chem. 262, 14555–14562.

Grynkiewicz, G., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+}

indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.

Han, H.M.C.B., Shimuta, S.I., Kanashiro, C.A., Oliveira, L., Han, S.W., Paiva, A.C.M., 1998. Residues Val^{254}, His^{256}, and Phe^{259} of the angiotensin AT_1 receptor are not involved in ligand binding but participate in signal transduction. Mol. Endocrinol. 12, 810–814.

Kanashiro, C.A., Paiva, T.B., Paiva, A.C.M., Prioste, R.N., Aboulafia, J., Shimuta, S.I., 1995. Angiotensin II tachyphylaxis in the guinea pig ileum and its prevention: a pharmacological and biochemical study. J. Pharmacol. Exp. Ther. 275, 1543–1550.

Miasiro, N., Oshiro, M.E.M., Paiva, T.B., Paiva, A.C.M., 1983. Role of the two N-terminal residues of angiotensin II in the production of tachyphylaxis. Eur. J. Pharmacol. 87, 397–406.

Moore, A., Khairallah, P.A., 1976. Further studies on angiotensin tachyphyalxis. J. Pharmacol. Exp. Ther. 197, 575–581.

Ohnishi, J., Ishido, M., Shibata, T., Inagami, T., Murakami, K., Miyazaki, H., 1992. The rat angiotensin II AT_{1A} receptor couples with three different signal transduction pathways. Biochem. Biophys. Res. Commun. 186, 1094–1101.

Oppermann, M., Freedman, N.J., Alexander, R.W., Lefkowitz, R.J., 1996. Phosphorylation of the type 1A angiotensin II receptor by G protein-coupled receptor kinases and protein kinase C. J. Biol. Chem. 271, 13266–13272.

Oshiro, M.E.M., Miasiro, N., Paiva, T.B., Paiva, A.C.M., 1984. Angiotensin tachyphylaxis in the isolated rabbit aorta. Blood Vessels 21, 72–79.

Paiva, T.B., Juliano, L., Nouailhetas, V.L.A., Paiva, A.C.M., 1974. The effect of pH on tachyphylaxis to angiotensin peptides in the isolated guinea pig ileum and rat uterus. Eur. J. Pharmacol. 25, 191–196.

Paiva, T.B., Mendes, G.B., Paiva, A.C.M., 1977. Specific desensitization (tachyphylaxis) of the guinea-pig ileum to angiotensin II. Am. J. Physiol. 232, H223–H230.

Richard, D.E., Laporte, S.A., Bernier, S.G., Leduc, R., Guillemette, G., 1997. Desensitization of AT_1 receptor-mediated cellular responses requires long term receptor down-regulation in bovine adrenal glomerulosa cells. Endrocrinology 138, 3828–3835.

Shimuta, S.I., Kanashiro, C.A., Oshiro, M.E.M., Paiva, T.B., Paiva, A.C.M., 1990. Angiotensin II desensitization and Ca^{2+} and Na^{+} fluxes in cultured intestinal smooth muscle cells. J. Pharmacol. Exp. Ther. 253, 1215–1221.

Shimuta, S.I., Kanashiro, C.A., Ferreira, A.T., Oshiro, M.E.M., Paiva, T.B., Paiva, A.C.M., 1993. Role of Na^{+} and protein kinase C in angiotensin desensibilization and tachyphylaxis in the guinea-pig ileum. Naunyn-Schimiedeberg's Arch. Pharmacol. 347, 425–431.

Strassheim, D., Williams, C.L., 2000. P2Y2 purimergic and M3 muscarinic acetylcholine receptors activate different phospholipase C-beta isoforms that are uniquely susceptible to protein kinase C-dependent phosphorylation and inactivation. J. Biol. Chem. 275 (50), 39767–39772.

Thekkumkara, T., Thomas, W.G., Motel, T.J., Baker, K.M., 1998. Functional role for the angiotensin II receptor (AT_{1A}) 3′-untranslated region in determining cellular responses to agonist: evidence for recognition by RNA binding proteins. Biochem. J. 329, 255–264.

Thomas, W.G., 1999. Regulation of angiotensin II type 1 (AT_1) receptor function. Regul. Pept. 79, 9–23.

Timmermans, P.B.M.W.M., Wong, P.C., Chiu, A.T., Herblin, W.F., Benfield, P., Carini, D.J., Lee, R.J., Wexler, R.R., Saye, J.A.M., Smith, R.D., 1993. Angiotensin II receptors and angiotensin II receptor antagonists. Pharmacol. Rev. 45, 205–251.

Touyz, R.M., Schiffrin, E.L., 2000. Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. Pharmacol. Rev. 52, 639–672.

Xu, K., Murphy, T.J., 2000. Reconstitution of angiotensin receptor mRNA down-regulation in vascular smooth muscle. J. Biol. Chem. 275, 7604–7611.