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Characterization of the angiotensin II AT_1 receptor subtype involved in DNA synthesis in cultured vascular smooth muscle cells

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1 This study was undertaken in cultured vascular smooth muscle cells to characterize the angiotensin II (AII) AT_1 receptor subtype involved in DNA synthesis because (i) the AII receptor involved in vascular proliferation has previously been characterized *in vitro* in rat aortic cells and identified as an AT_1 subtype and (ii) molecular cloning and biochemical studies have provided evidence for the existence of different AT_1 receptor subtypes.

2 In cultured rat aortic vascular smooth muscle (VSMC), exposure to AII (0.1 to 100 nM) resulted in a concentration-dependent increase in [^{3}H]-thymidine incorporation with an EC_{50} of 1.41 ± 0.51 nM. Maximal stimulation was observed in the presence of 100 nM AII and corresponded to 271 ± 40% of basal [^{3}H]-thymidine incorporation.

3 To characterize the AII AT_1 receptor subtype involved in this effect, cells were exposed to AII (3 nM) in the absence or presence of increasing concentrations of various AII receptor antagonists. The stimulatory effect of AII (3 nM) on [^{3}H]-thymidine incorporation in VSMC was antagonized by the non-selective AT_1/AT_2 receptor antagonist, [Sar^1,Ile^8]-AII (IC_{50} = 5.6 nM), by the AT_{1A}/AT_{1B} receptor antagonist, losartan (IC_{50} = 10.5 nM) and the AT_1 receptor antagonist, L-158809 (IC_{50} = 0.20 nM). The selective AT_2 receptor ligand, CGP 42112A, antagonized AII-induced [^{3}H]-thymidine incorporation with an IC_{50} of 6.3 ± 1.3 μM while the AT_2/AT_{1B} receptor antagonist, PD 123319, was found to be almost inactive ($IC_{50} > 10$ μM).

4 Under the same experimental conditions, angiotensin III (AIII) was found to be at least 50 times less potent than AII with an apparent EC_{50} of 81.6 ± 7.7 nM. At the highest concentration tested (10 μM), the effect of AIII corresponded to 327 ± 61% of basal [^{3}H]-thymidine incorporation.

5 These results confirm that AII can stimulate DNA synthesis in VSMC through an AT_1 receptor. Furthermore, the pharmacological characterization of this AT_1 receptor is compatible with the AT_{1A} receptor subtype recently described on cultured mesangial cells since (i) the AT_{1A}/AT_{1B} receptor antagonist losartan is active at nanomolar concentrations, (ii) micromolar concentrations of the AT_2/AT_{1B} receptor antagonist PD 123319 are ineffective at antagonizing the AII-induced [^{3}H]-thymidine incorporation and (iii) AII is at least 50 times more potent than AIII in stimulating DNA synthesis.

Keywords: Vascular smooth muscle cells; angiotensin II; DNA synthesis; [^{3}H]-thymidine; AT_1 receptor subtypes

Introduction

The role of angiotensin II (AII) in some forms of hypertension and the observation that angiotensin converting enzyme (ACE) inhibitors and AII receptor antagonists are more effective than β-blockers in reducing vascular hypertrophy in hypertensive rats (Wang & Prewitt, 1990; Osterrieder *et al.*, 1991) have led to the hypothesis that AII, in addition to its vasoconstrictor effects, may have mitogenic properties at the level of the vascular wall. In rats, AII infused at low doses induces vascular hypertrophy partly by a non pressor mechanism (Griffin *et al.*, 1991). The hypothesis of a trophic effect of AII has been strengthened by the observations that, *in vitro*, AII induces the expression of proto-oncogenes such as c-*fos* or c-*myc* (Kawahara *et al.*, 1988; Taubman *et al.*, 1989) involved in cell replication, as well as platelet-derived growth factor (PDGF)-A chain mRNA (Naftilan *et al.*, 1989). The effects of AII have been studied *in vitro* on vascular smooth muscle cells (VSMC) (Berk *et al.*, 1989; Owens, 1989) where both hypertrophic and hyperplasic responses have been observed, depending on the experimental conditions used (Dzau *et al.*, 1991, for discussion).

Recently, the existence of AII receptor subtypes has been described based on differences in affinities for the non-peptide AII receptor antagonists, losartan, PD 123319 or PD 123177, or the peptide CGP 42112A (Bumpus *et al.*, 1991; Bottari *et al.*, 1993 for review). According to the current nomenclature (Bumpus *et al.*, 1991), the designation AT_1 has been proposed for the receptor subtype sensitive to losartan, and AT_2 for the subtype sensitive to PD 123177 (Blankley *et al.*, 1991) and CGP 42112A (Whitebread *et al.*, 1989). Functional studies have revealed that the AT_1 receptor subtype is responsible for the well described effects of AII on vasoconstriction, aldosterone and adrenaline release as well as water intake (Criscione *et al.*, 1990; Wong *et al.*, 1990; Herblin *et al.*, 1991). In cultured VSMC, there is now good evidence that the trophic effects of AII are mediated through the stimulation of an AT_1 receptor subtype (Chiu *et al.*, 1991; Bunkenburg *et al.*, 1992). On the other hand, the pathophysiological role of the AT_2 receptor subtype is not yet well understood (Criscione *et al.*, 1990; Dudley *et al.*, 1990; Herblin *et al.*, 1991; Timmermans *et al.*, 1991; Bottari *et al.*, 1993).

More recently, development of the expression cloning technique has made it possible to identify at least three different cDNAs coding for type 1 AII receptors, a rat renal and vascular smooth muscle cell receptor now termed AT_{1A}, a rat adrenal receptor AT_{1B} (Inagami *et al.*, 1992) and an AT_{1C} receptor subtype abundant in cultured VSMC and mesangial cells (Hahn *et al.*, 1993). Thus far, one class of AT_1 receptor gene has been identified for bovines and man (Sasaki *et al.*, 1991; Furuta *et al.*, 1992; Takayanagi *et al.*, 1992) with a high degree of homology to the AT_{1A} sequence (Mauzy *et al.*, 1992). The question of the existence of AT_1 receptor subtypes

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in human tissues is still under debate (Yoshida et al., 1992; Mauzy et al., 1992), while a cDNA coding for an AT_1 different from the AT_{1A} subtype has been recently isolated from a human placental cDNA library (Kuroda et al., 1993). Therefore, it seems likely that subtypes of AT_1 receptors can also be expressed in man.

Although the different AT_1 receptor subtypes identified so far have been expressed in COS-7 cells, their pharmacological profiles have been difficult to distinguish, essentially because of the limited number of agonists and antagonists used for comparison in these experiments (Murphy et al., 1992). On the other hand, in a recent study aimed at characterizing the AII receptor subtypes present in cultured rat renal mesangial cells (Ernsberger et al., 1992; Zhou et al., 1993), two different AT_1 receptor subtypes also termed AT_{1A} and AT_{1B} have been described on the basis of differences in their pharmacological profile. Of particular interest was the fact that the AT_2 receptor ligand, PD 123319, exhibited a nanomolar affinity for a site labelled with [^{125}I]-AII, sensitive to losartan but showing no affinity for the peptide-selective AT_2 receptor ligand, CGP 42112A (Ernsberger et al., 1992). Moreover, this receptor subtype appeared to be coupled to adenylate cyclase through a G_i protein, further supporting the hypothesis of an AT_1 receptor subtype, different from the AT_{1A} and tentatively classified as an AT_{1B} receptor (Zhou et al., 1993). It should be emphasized that this pharmacologically based AT_{1A}/AT_{1B} distinction is in no way similar to the $AT_{1A}/AT_{1B}/AT_{1C}$ classification derived from the molecular cloning results.

Therefore, the objectives of the present work were to establish experimental conditions under which AII is able to induce DNA synthesis, and to characterize the AT_1 receptor subtype involved in this effect in the light of the recent AT_{1A}/AT_{1B} pharmacological characterization proposed in cultured renal mesangial cells (Ernsberger et al., 1992).

Methods

Cell culture

Rat thoracic aortic smooth muscle cells were isolated and cultured by a modification of the method of Chamley-Campbell et al. (1979). Briefly, 11 week old male Wistar Kyoto rats (Charles River, France) were killed by cervical dislocation. Thoracic aortae were excised and placed in Hanks balanced salt solution (HBSS, Gibco, France). Aortic strips were cleaned of fatty tissue, opened longitudinally and pre-digested in collagenase (87.5 u ml^{-1}, Intermed, France). Endothelial cells were removed by scraping the inner surface of the vessels with forceps. The adventitia was carefully removed with forceps and medial cells were dissociated by collagenase (87.5 u ml^{-1}) and elastase (10 μM, Biosys, France) during gentle stirring. The resulting pellet was resuspended in Minimum Essential Medium (Gibco) containing 10% foetal calf serum (Gibco), penicillin (100 u ml^{-1}, Gibco) and streptomycin (100 μg ml^{-1}, Gibco). Cells were seeded in 48 multiwell plates (Costar) at 1×10^4 cells/well for proliferation studies, and in 25 cm^2 plastic culture flasks (Costar) for subsequent subcultures. Cells were harvested at one week intervals with trypsin-EDTA (0.05% trypsin, 0.02% EDTA, Gibco) and were used between passages 4 and 10.

Quiescence was induced by replacing culture medium in multiwell plates with Dulbecco's Modified Eagle's Medium mix HAM's F12 (Gibco), supplemented with insulin (1 μM, Sigma), transferrin (5 μg ml^{-1}, Gibco) and ascorbic acid (100 μM, Sigma). This medium has been reported to maintain cells in a nondividing state which resembles that of cells in the normal arterial wall (Libby & O'Brien, 1983). Smooth muscle cells were identified by indirect immunofluorescent staining with a monoclonal anti-α-smooth muscle actin antibody (Sigma).

Proliferation studies

DNA synthesis in VSMC was assessed in cells maintained in quiescent medium for 72 h.

After a 20 h pre-incubation of cells with various agonists, [^{3}H]-thymidine (0.5 μCi/well, 5.0 Ci mmol^{-1}, Amersham, U.K.) was added to the culture medium for a 4 h incubation period in the continuing presence of agonists. When studied, antagonists, or vehicles were incorporated 1 h before agonists.

At the end of the incubation, cells were washed twice with 0.5 ml cold phosphate-buffered-saline (PBS, Gibco). [^{3}H]-DNA was precipitated with ice cold trichloracetic acid (10%), solubilized in sodium hydroxide (0.5 M) at 37°C and quantified by liquid scintillation counting.

Drugs

The following drugs were used: angiotensin II (human, Sigma), angiotensin III (Sigma), [Sar^1,Ile^8]-angiotensin II ([Sar^1,Ile^8]-AII) (Sigma); losartan, DuP 753 (2-n-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl) biphenyl-4yl) methyl] imidazole, potassium salt) was kindly provided by Dupont de Nemours (U.S.A.); PD 123319 (1-[[4-(dimethylamine)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6- carboxylic acid) kindly provided by Parke-Davis laboratories; L-158809 ((5,7-dimethyl-2-ethyl-3[[2'-(1H-tetrazol-5-yl)[1,1'-biphenyl]-4-yl] methyl]-3H-imidazo [4,5-b] pyridine, monohydrate, Merck) was synthesized by the Department of Chemistry, Synthélabo Recherche (Bagneux, France). CGP 42112A (nicotinic acid-Tyr-(N$^\alpha$-benzyl-oxycarbonyl-Arg)Lys-His-Pro-Ile-OH, Ciba-Geigy), recombinant human PDGF-AA and PDGF-BB were obtained from Bachem Laboratories (Switzerland).

To prevent adherence of AII related peptides to plastic wares and degradation by peptidases in culture medium, stock solutions and subsequent dilutions of AII, AIII, [Sar^1, Ile^8]-AII and CGP 42112A were prepared in distilled water containing 1% bovine serum albumin (BSA, Sigma) and 5 mg ml^{-1} bacitracin (Sigma). Final concentrations of BSA and bacitracin in multiwell plates were 0.1% and 0.5 mg ml^{-1}, respectively.

Other drug solutions were prepared in dimethylsulphoxide (DMSO) at 1 mM and subsequent dilutions were made in distilled water. DMSO concentrations in multiwell plates never exceeded 0.1%, which is the highest concentration tolerated by cells without damage.

Statistics

In the figures, results are expressed as mean ± s.e.mean. Student's t test for unpaired data was used to compare the effects of AII and PDGF-AA or -BB with respective control values, and probabilities below the 0.05 level were considered significant. The concentration-response curves and the interactions between antagonists and agonists were analysed by the Allfit programme according to the extra sum of squares principle (De Lean et al., 1978). The EC_{50} values obtained by fitting the logistic equations are expressed as parameter estimates ± approximate standard errors.

Results

Time course of AII-induced [^{3}H]-thymidine incorporation

The kinetics of [^{3}H]-thymidine incorporation in response to AII (3 nM) were examined over a 36 h period at 4–6 h intervals. Addition of AII to cultured cells induced a time-dependent increase in [^{3}H]-thymidine incorporation (Figure 1). The rate of incorporation was maximal between the 20th

and 24th hour of incubation as shown by the steeper slope of the response curve during this period of time.

Effects of AII and AIII on VSMC [^{3}H]-thymidine incorporation

Exposure of rat thoracic aortic cells to AII (0.1 nM–0.1 μM) 20 h before the [^{3}H]-thymidine pulse increased [^{3}H]-thymidine incorporation in a concentration-dependent manner, with an EC_{50} value of 1.41 ± 0.51 nM ($n = 6$) (Figure 2). The effect of AII was maximal at a concentration of 100 nM and averaged 271 ± 40% of basal [^{3}H]-thymidine incorporation. Under the same experimental conditions, AIII (1 nM–10 μM) increased [^{3}H]-thymidine incorporation in a concentration-dependent manner. However, the maximum effect of AIII was not reached even at the highest concentration tested (10 μM). At 10 μM, the effect of AIII corresponded to 327 ± 61% of basal [^{3}H]-thymidine incorporation. The apparent EC_{50} value obtained by fitting this concentration-response curve was 81.6 ± 7.7 nM ($n = 6$).

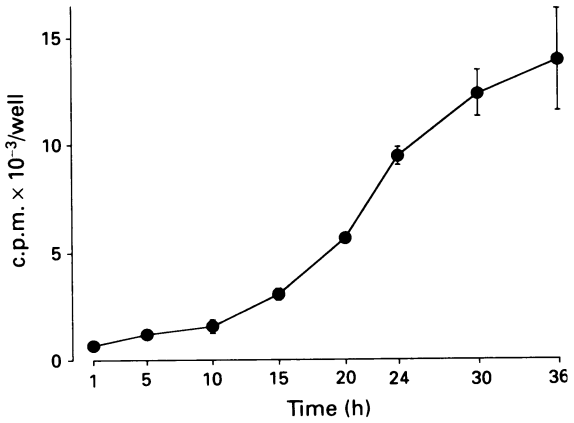

Figure 1 Time course of angiotensin II (AII)-induced [^{3}H]-thymidine incorporation. AII (3 nM) and [^{3}H]-thymidine were added to vascular smooth muscle cells at the time 0. The medium was removed at the times indicated and labelled DNA was extracted in trichloracetic acid-insoluble, NaOH-soluble material. Each point is the mean of eight determinations performed on two different cultures.

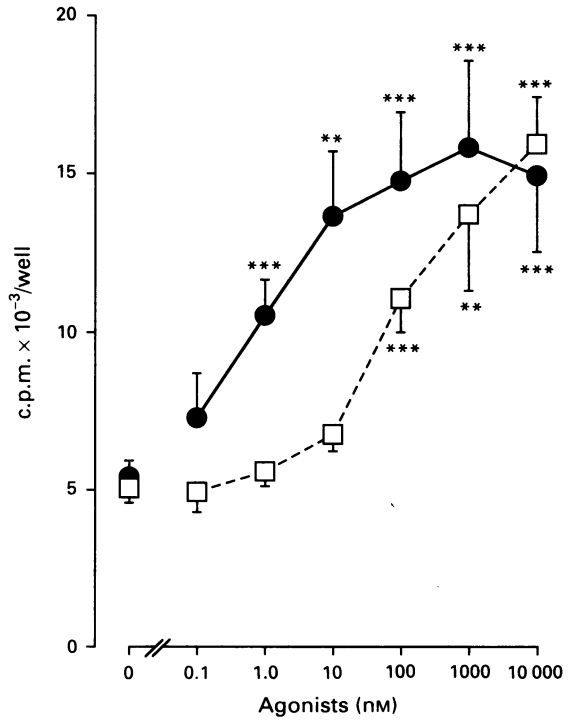

Figure 2 Effects of angiotensin II (AII) and AIII on vascular smooth muscle cells (VSMC) [^{3}H]-thymidine incorporation. VSMC were incubated for 20 h with AII (0.1 nM–1 μM, ●) or AIII (0.1nM–100 μM, □) before [^{3}H]-thymidine was added for 4 h. Results are expressed in counts per minute (c.p.m. × 10^{-3}) per well. Data are mean ± s.e.mean of 6 concentration-response curves carried out on cells cultured from passages 4 to 10. $^{**}P < 0.01$; $^{***}P < 0.001$ when compared with control value.

Effects of PDGF-AA and PDGF-BB on the [^{3}H]-thymidine incorporation in cultured VSMC

Exposure of cultured VSMC to PDGF-AA and PDGF-BB (10–100 ng ml^{-1}) significantly increased [^{3}H]-thymidine incorporation in a concentration-dependent manner, but maximum effects were not reached, even at the highest concentrations tested (100 ng ml^{-1}). The apparent EC_{50} values obtained by fitting these concentration-response curves were 16.6 ± 0.8 (PDGF-AA, $n = 4$–6) and 25.3 ± 4.7 ng ml^{-1} (PDGF-BB, $n = 5$) (Figure 3).

Effects of AII receptor antagonists on the AII-induced [^{3}H]-thymidine incorporation

The effects of antagonists were tested against the [^{3}H]-thymidine incorporation produced by AII (3 nM). The addition of the octapeptide [Sar^1,Ile^8]-AII (0.3–100 nM), a potent but non selective AII receptor antagonist, antagonized the AII-induced [^{3}H]-thymidine incorporation in a concentration-dependent manner with an $IC_{50} = 5.6 \pm 1.2$ nM (Figure 4).

Exposure to the selective AT_1 receptor antagonist, losartan (Chiu *et al.*, 1990) (0.1 nM–1 μM) inhibited in a concentration-dependent manner the [^{3}H]-thymidine incorporation induced by AII with an IC_{50} value of 10.5 ± 1.8 nM (Figure 4). Under the same experimental conditions, exposure to L-158809, a potent and selective AT_1 receptor antagonist (Mantlo *et al.*, 1991) (0.01–3 nM) also antagonized the AII-induced [^{3}H]-thymidine incorporation with an IC_{50} value of 0.20 ± 0.02 nM.

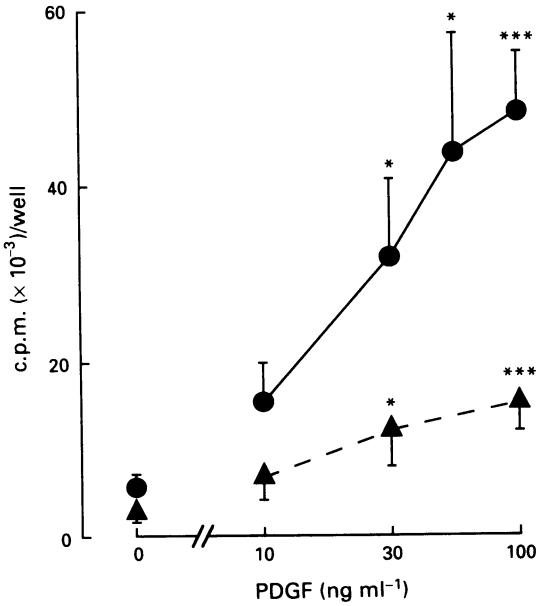

Figure 3 Effect of platelet-derived growth factor (PDGF)-AA and PDGF-BB on vascular smooth muscle cells [^{3}H]-thymidine incorporation. Cells were exposed to either PDGF-AA (●) or PDGF-BB (▲) for 24 h. Results are expressed in c.p.m. (× 10^{-3})/well. Each point represents the mean ± s.e.mean of 4 to 6 (PDGF-AA) or 5 (PDGF-BB) experiments. $^{*}P < 0.05$; $^{***}P < 0.001$ when compared with control value.

In order to establish the type of competition involved in this interaction, a complete concentration-response curve to AII was constructed in the absence and in the presence of 0.01 μM losartan. Under these experimental conditions, losartan significantly shifted to the right the entire concentration-response curve to AII ($EC_{50} = 2.4 \pm 0.6$ nM in control experiments; $EC_{50} = 6.3 \pm 1.0$ nM in the presence of losartan, 0.01 μM) (Allfit comparison $P < 0.05$ when comparing both curves). A pK_B value of 8.2 for losartan was calculated from the concentration-ratios obtained in these experiments. The maximum effect of AII was not depressed, suggesting competitive antagonism (Figure 5).

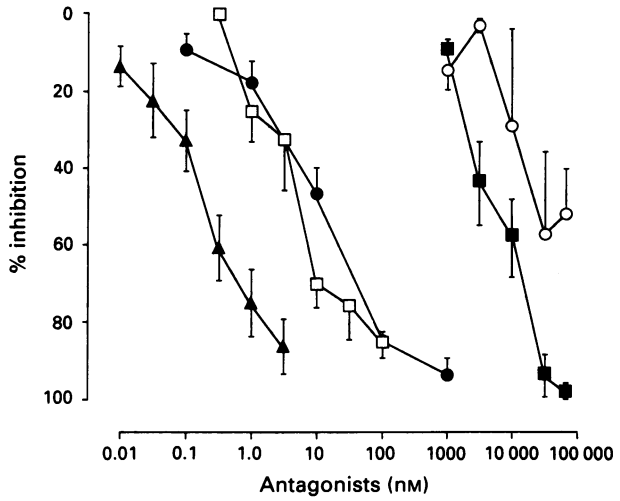

Figure 4 Effects of antagonists on angiotensin II (AII)-induced [^{3}H]-thymidine incorporation in vascular smooth muscle cells (VSMC). [Sar^1,Ile^8] AII (0.3–100 nM, □), losartan (0.1nM–1 μM, ●), L-158809 (0.1–3 nM, ▲), CGP 42112A (1–50 μM, ■) and PD 123319 (1–50 μM, ○) were added to quiescent VSMC 1 h before AII (3 nM). Results are expressed as percentage inhibition of AII-induced [^{3}H]-thymidine incorporation and are mean ± s.e.mean of 6 to 8 determinations (except for CGP 42112A, $n = 3$).

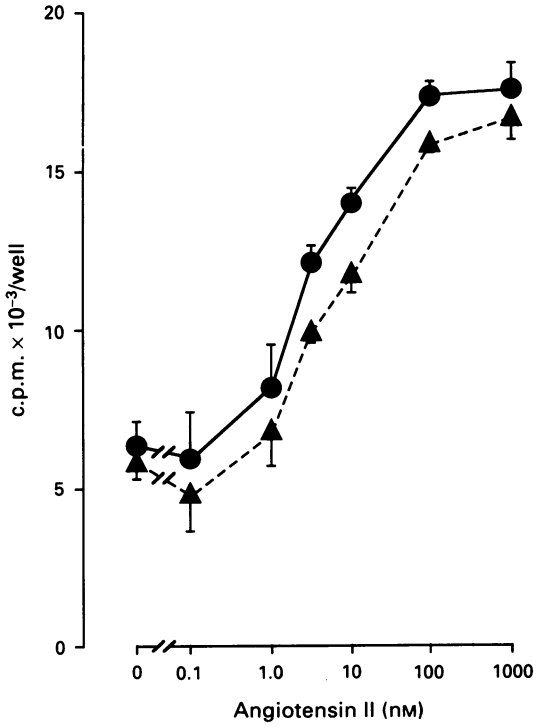

Figure 5 Antagonism by losartan of the angiotensin II (AII)-induced [^{3}H]-thymidine incorporation in vascular smooth muscle cells (VSMC). The effects of AII (0.1nM–1 μM) were determined in cultured VSMC in the absence (●) or in the presence of losartan (0.01 μM) (▲). Results are expressed in c.p.m. ($\times 10^{-3}$)/well and are the mean ± s.e.mean of 3 experimental values. The two concentration-response curves were compared according to the extra sum of squares principle (De Lean *et al.*, 1978) and were found significantly different at the $P < 0.05$ level. A pK_B value of 8.2 for losartan was calculated from the concentration-ratios.

In contrast to the potency of AT_1 selective antagonists at antagonizing the AII-induced response, the selective AT_2 receptor ligand, CGP 42112A (Criscione *et al.*, 1990) inhibited the AII-induced [^{3}H]-thymidine incorporation only at micromolar concentrations ($IC_{50} = 6.3 \pm 1.3$ μM). Under the same experimental conditions, the AT_2 receptor antagonist, PD 123319 (Dudley *et al.*, 1990) was found to be almost inactive ($IC_{50} > 10$ μM) (Figure 4).

Effects of adrenoceptor antagonists on the AII-induced [^{3}H]-thymidine incorporation

In order to test the selectivity of the AII-induced effect on [^{3}H]-thymidine incorporation, the effects of the α-adrenoceptor antagonist, phenoxybenzamine (0.1 nM–1 μM) and the β-adrenoceptor antagonist, propranolol (0.1 nM–1 μM) were evaluated. Neither of these antagonists modified the AII-induced [^{3}H]-thymidine incorporation under these experimental conditions (data not shown).

Discussion

In cultured VSMC, AII at nanomolar concentrations induces a significant and concentration-dependent increase in [^{3}H]-thymidine incorporation with a maximum effect of AII (100 nM) corresponding to 271% of basal [^{3}H]-thymidine incorporation. Results concerning the ability of AII to induce DNA synthesis are conflicting (Campbell-Boswell & Robertson, 1981; Geisterfer *et al.*, 1988). Significant increases of [^{3}H]-thymidine incorporation have been reported (177% in the presence of 500 nM AII; Paquet *et al.*, 1990), as well as a modest increase of 20–30% (Geisterfer *et al.*, 1988) or no effect at all even at the high concentration of 100 nM (Bobik *et al.*, 1990). These discrepancies can be partially explained by differences in the experimental protocols used, among which the duration of incubation with AII as well as the labelling period with [^{3}H]-thymidine appear to be determinant factors to observe an increase in DNA synthesis. Under our experimental conditions, an incubation period of VSMC of less than 15 h duration in the presence of AII, did not induce any significant increase in DNA synthesis, while the most efficient period of [^{3}H]-thymidine incorporation was observed between the 20th and 24th hour of incubation with AII. Therefore it is likely that the reported lack of effect of AII in inducing an increase in DNA synthesis (Bobik *et al.*, 1990) could be explained by a too short duration of incubation, since AII was applied for only 16 h with 1 h [^{3}H]-thymidine labelling. Moreover, the observation that PDGF but not AII was able to increase DNA synthesis under these experimental conditions (Bobik *et al.*, 1990), is consistent with the ability of PDGF to shorten the lag time before the start of DNA synthesis, indicating an acceleration of the induction of competence as previously reported (Kimura *et al.*, 1992). On the other hand, in studies reporting a significant although weak effect of AII on DNA synthesis (Geisterfer *et al.*, 1988), it should be emphasized that the experimental procedure did not mention the presence of any antipeptidase, such as bacitracin, in the culture medium, therefore raising the question of a possible degradation of AII during prolonged exposure to VSMC.

It is important to note that an increase in DNA synthesis does not always reflect hyperplasia but can also suggest polyploidy, without true mitosis. There is now increasing evidence to indicate that AII acts preferentially as an hypertrophic agent in VSMC derived from normotensive rats but

can induce hyperplasia in some situations such as culture of VSMC derived from SHR rats (Millar *et al.*, 1990; Bunkenburg *et al.*, 1992) or in the presence of low concentrations of serum. Therefore, AII could play a role as a 'partial' growth factor leading to enhanced cellular protein and DNA synthesis but lacking the properties to induce a real mitosis.

The mechanism by which AII induces DNA synthesis in cultured VSMC remains to be defined. Under our experimental conditions, PDGF-AA and PDGF-BB both induce an increase in [^{3}H]-thymidine incorporation, the maximum effect of PDGF-BB being higher than that of PDGF-AA. Since AII induces the expression of proto-oncogenes such as c-*fos*, c-*jun* or c-*myc* involved in cell replication as well as PDGF-A chain mRNA in cultured vascular smooth muscle cells (Kawahara *et al.*, 1988; Taubman *et al.*, 1989; Naftilan *et al.*, 1989), it is possible that the mitogenic effect of AII could be mediated through an increase in synthesis and/or release of PDGF-AA. Such an autocrine effect of AII has in fact been described (Itoh *et al.*, 1990). In contrast, it has been reported that the increase in [^{3}H]-thymidine incorporation induced by interleukin-1α but not by AII in cultured VSMC can be antagonized by an antibody specific for PDGF-AA (Ikeda *et al.*, 1990), although it was not clear whether the cells used in these studies were responsive by themselves to PDGF-AA. In fact, an AII-induced increase in DNA synthesis could be limited by the ability of cells to respond to PDGF-AA. On the other hand, the secretion of transforming growth factor β1 (TGF-β1) and/or basic fibroblast growth factor (bFGF) may be critical for determining the magnitude of the trophic responses to AII (Dzau *et al.*, 1991; Itoh *et al.*, 1993). Whether the AII-induced trophic effect is the result of a balance between proliferative (bFGF) and antiproliferative (TGF-β1) autocrine production needs further investigation.

Recently, the development of non peptide AII receptor antagonists has enabled the characterization of two AII receptor subtypes, AT_1 and AT_2. In cultured VSMC, most of the trophic effects of AII have been attributed to the AT_1 subtype (Chiu *et al.*, 1991; Bunkenburg *et al.*, 1992). Under our experimental conditions, AII-induced DNA synthesis is antagonized by nanomolar concentrations of the selective AT_1 receptor antagonists, losartan and L-158809 and micromolar concentrations of CGP 42112A while PD 123319 was almost inactive. Losartan (0.01 μM) significantly shifts to the right the concentration-response curve of angiotensin II without decreasing the maximal effect of AII, supporting the view that it acts as a competitive antagonist of the AII-induced [^{3}H]-thymidine incorporation. The pK_B value of 8.2 for losartan agrees well with the pA_2 value of 8.4 reported previously in rabbit isolated aorta contracted by AII (Chiu *et al.*, 1990). Taken together, these results are compatible with the involvement of an AT_1 receptor located on VSMC, as previously described, mediating the AII-induced [^{3}H]-thymidine incorporation indicative of DNA synthesis. These results do not rule out the possibility that VSMC in culture could express AT_2 receptors but suggest that this receptor subtype does not significantly contribute to the increase in DNA synthesis in response to AII in this experimental model.

Recently, molecular biological approaches have provided evidence for the existence of AT_1 receptor subtypes (Inagami *et al.*, 1992). In rat, VSMC and adrenal cells express different AT_1 mRNA coding for different receptors named AT_{1A} and AT_{1B} respectively. The tissue distribution of these AT_{1A} and AT_{1B} receptor subtypes is heterogeneous, suggesting possible differences in the functions mediated through these receptors, although little is known about their pharmacology. It should be pointed out that the currently available AII receptor antagonists do not discriminate between these AT_1 subtypes. Recently, by means of binding and functional techniques in cultured rat renal mesangial cells, it has been possible to characterize two AII AT_1 receptors, both coupled to a G-protein and exhibiting differences in their affinity for known AT_1 or AT_2 receptor antagonists (Ernsberger *et al.*, 1992). The proposed 'AT_{1A}' receptor subtype shows a nanomolar affinity for losartan, a micromolar affinity for PD 133319 and is more sensitive to AII than to AIII. The proposed 'AT_{1B}' receptor subtype exhibits a micromolar affinity for losartan, a high nanomolar affinity for PD 123319 and AIII, but is nevertheless distinct from an AT_2 receptor since (i) it is coupled to a G protein (Bottari *et al.*, 1991), (ii) it shows a micromolar affinity for CGP 42212A as opposed to the nanomolar affinity of this ligand for the AT_2 receptor (Whitebread *et al.*, 1989) and (iii) losartan is active at micromolar concentrations compared to its millimolar affinity for the AT_2 receptor (Timmermans *et al.*, 1991).

In light of the pharmacologically described AT_1 receptor subtypes present in cultured rat mesangial cells (Ernsberger *et al.*, 1992), the profile of the AT_1 receptor involved in DNA synthesis modulation in VSMC (present results) is clearly not compatible with an AT_{1B} subtype since DNA synthesis in cultured VSMC (i) is antagonized by nanomolar concentrations of losartan, but (ii) is not antagonized even by micromolar concentrations of PD 123319. Moreover the observation that DNA synthesis in VSMC is more sensitive to AII than to AIII is compatible with the involvement of an AT_{1A} subtype. However, it should be emphasized that the classification proposed by Ernsberger *et al.* (1992) has not been confirmed and is not in agreement with the current nomenclature (Bumpus *et al.*, 1991; Inagami *et al.*, 1992). Evolution in this domain is very rapid, and re-evaluation of the classification would certainly be of interest.

In this context, it is interesting to note that in addition to the 'cloned receptor' proposed classification (AT_{1A}/AT_{1B}, Inagami *et al.*, 1992), a novel AT_{1C} receptor subtype has been identified in rats (Hahn *et al.*, 1993). The AT_{1C} receptor seems to be abundant in cultured VSMC and mesangial cells and weakly expressed in brain and kidney. The nucleotide sequence of the AT_{1C} receptor presents a higher degree of homology (90%) with the AT_{1A} receptor sequence than with the AT_{1B} receptor sequence previously described (Inagami *et al.*, 1992). These findings raise the interesting possibility that the pharmacologically described 'AT_{1A}' receptor in cultured mesangial cells (Ernsberger *et al.*, 1992) could be identical to the AT_{1C} receptor subtype obtained by molecular biology techniques in the same cell type (Hahn *et al.*, 1993). Furthermore, considering the presence of an AT_{1C} receptor subtype in cultured rat VSMC and mesangial cells (Hahn *et al.*, 1993) but not in rat aortae and the high degree of homology (90%) between the AT_{1C} and AT_{1A} nucleotide sequence, it is tempting to speculate whether the AT_{1A} receptor subtype identified by molecular cloning techniques in rat vascular tissues could be modified by tissue-culture conditions into the AT_{1C} receptor subtype which has been identified to a high level in cultured cells. Interestingly, a recombinant rat AT_{1A} receptor has been expressed in Chinese hamster ovary cells and confers to these cells the ability to synthesize DNA in response to AII (Teutsch *et al.*, 1992). Furthermore in these transfected cells, AII induces a real proliferation (increase in cell number) in the absence of any other growth factor indicating in these conditions that AII can act as a growth factor and not simply as a cofactor.

Although similarities exist between the AT_{1A} receptor (Ernsberger *et al.*, 1992) and the AT_{1C} receptor (Hahn *et al.*, 1993), clearly more studies are necessary to correlate the $AT_{1A}/AT_{1B}/AT_{1C}$ classification based on molecular cloning and the pharmacologically described AT_{1A}/AT_{1B} receptors present in cultured mesangial cells.

In conclusion, our results provide evidence that AII induces DNA synthesis through an AT_1 receptor which shares the same pharmacological properties as the AT_{1A} subtype pharmacologically described in cultured mesangial cells and could possibly be identical to the AT_{1C} receptor subtype sequence identified in cultured VSMC or mesangial cells. These results support the hypothesis of a role of AII as a hypertrophic agent in cardiovascular pathological situations. The ability of AII receptor antagonists to prevent the 'growth factor-like' effect of AII both *in vitro* and *in vivo*

suggests that these molecules could be useful not only in the treatment of hypertension but also in the prevention of the myointimal proliferation observed in some pathological situations.

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