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Pharmacological characterization of a specific binding site for angiotensin IV in cultured porcine aortic endothelial cells

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Abstract

This study demonstrated the existence of a specific binding site for angiotensin IV in porcine aortic endothelial cells. Non-equilibrium kinetic analyses at 37°C allowed the calculation of a kinetic K_d of 0.44 nM. Pseudo-equilibrium saturation binding studies at 37°C for 90 min indicated the presence of a single high-affinity site ($K_d = 3.87 \pm 0.60$ nM), saturable and abundant ($B_{max} = 9.64 \pm 1.44$ pmol/mg protein). Competitive binding studies demonstrated the following rank order of effectiveness: angiotensin IV > angiotensin III > angiotensin II > angiotensin I > angiotensin II-(1–7), while 2-n-butyl-4-chloro-5-hydroxymethyl-1 [(2′-(1H-tetrazol-5-yl) biphenyl-4-yl) methyl] imidazol (DuP 753: losartan), 1-(4-amino-3-methyl-phenyl) methyl-5-diphenylisoethyl-4,5,6,7-tetrahydro-1H-imidazo [4,5-C] pyridine-6-carboxylic acid (PD 123177) or nicotinic acid-Tyr-(N^{α}-benzyl-oxycarbonyl-Arg) Lys-His-Pro-Ile-OH (CGP 42112A) were inactive at the concentration of 100 μM. This binding site is, therefore, distinct from angiotensin II receptors, AT_1 and AT_2. Addition of the divalent cations Mg^{2+}, Mn^{2+} or Ca^{2+} to the incubation buffer resulted in 90–95% inhibition of the [^{125}I]angiotensin IV-specific binding to porcine aortic endothelial cells. Furthermore, the chelator, EGTA, at 5 mM increased the number of binding sites ($B_{max} = 17.8 \pm 2.5$ pmol/mg protein), with no change in affinity ($K_d = 5.7 \pm 1.3$ nM). Exposure of porcine aortic endothelial cell membranes to the non-hydrolyzable GTP analog, GTPγS, had no effect on [^{125}I]angiotensin IV binding. The presence of a high concentration of binding sites for angiotensin IV in porcine aortic endothelial cells suggests that this peptide may play an important role in the modulation of the cardiovascular system.

Keywords: Angiotensin IV binding; Aortic endothelial cell; (Porcine)

1. Introduction

The angiotensin family of peptides, specifically angiotensin II and angiotensin III, are known to be involved in the regulation of cardiovascular function and body water homeostasis (Johnson, 1990). It is currently thought that angiotensin II and angiotensin III are the biologically active forms of angiotensin, mediating their effects through the activation of angiotensin AT_1 receptors (Wong et al., 1990), while the role of angiotensin AT_2 receptors is not yet fully clarified (see Nahmias and Strosberg, 1995, for a review). Recent studies have suggested a possible physiological role for smaller fragments of angiotensin II, such as angiotensin II-(1–7), not directly related to the well-described functions of angiotensin II through its AT_1 receptors (Ferrario et al., 1991). The C-terminal 3–8 hexapeptide fragment of angiotensin II, with the amino-acid sequence, Val-Tyr-Ile-His-Pro-Phe (angiotensin II-(3–8) or angiotensin IV), was first shown to possess biological activity on its own and to interact with a unique binding site in guinea pig hippocampus (Harding et al., 1992), while specific angiotensin IV binding sites were found in the brain and cardiovascular tissues from various species (Swanson et al., 1992). These observations have been extended and high-affinity angiotensin IV binding sites have now been described in bovine aortic or coronary venular endothelial cells (Hall et al., 1992), in several regions of the brain (Miller-Wing et al., 1993), in mammalian heart membranes (Hanesworth et al., 1993), in cultured vascular smooth muscle cells (Hall et al., 1993) and in adrenal cortex membranes (Bernier et al., 1994).

Several potential functions have been attributed to angiotensin IV, such as modulation of memory acquisition and retrieval, or exploratory behavior in rats and mice (Braszko et al., 1988; Wright et al., 1995). A recent study

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demonstrated the activation by angiotensin IV of specific neuronal pathways (by the *c-Fos* expression technique) in the hippocampus and the piriform cortex (Roberts et al., 1995). In the periphery, angiotensin IV could participate in the control of renal cortical blood flow (Swanson et al., 1992; Harding et al., 1992) although there is still some controversy about the receptor involved (Gardiner et al., 1993). It was also reported that angiotensin IV, in the presence of L-arginine, caused endothelium-dependent vasodilatation of rabbit cerebral arterioles (Haberl et al., 1991). On the other hand, several studies have shown that angiotensin IV may act independently from or even in opposition to angiotensin II. In cultured chick cardiocytes, angiotensin IV can block the ability of angiotensin II to increase protein and RNA synthesis via a mechanism independent of angiotensin AT_1 or AT_2 receptors (Baker and Aceto, 1990). In vascular smooth muscle, the kinetics of Ca^{2+} mobilization are different in response to angiotensin II and angiotensin IV (Dostal et al., 1990).

These findings strongly support the existence of a receptor for angiotensin IV, which has now been designated the AT_4 receptor (Wright et al., 1995). The structure of this receptor is not known at the moment and neither is the intracellular signal that is activated during the signalling process. There is, however, some evidence that the AT_4 receptor is not G-protein-linked and could be a member of a growth factor or cytokine family of receptors (Wright et al., 1995).

As studies on the AT_4 receptor in endothelial cells have been carried out only in bovine tissues, we decided to extend these observations by characterizing a specific binding site for angiotensin IV on cultured porcine aortic endothelial cells and to investigate in more detail its pharmacological properties.

2. Materials and methods

2.1. Cell culture

Porcine aortic endothelial cells were isolated and cultured by a modification of the method of Jaffe et al. (1973). Briefly, freshly collected porcine aortae (Charles River, France) were transported on ice in sterile phosphate-buffered saline (PBS) (Gibco, France) containing 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco) and 2.5 μg/ml fungizone (Gibco). The vessels were rinsed with sterile Hanks' balanced salt solution (HBSS, Gibco). After ligation of one extremity and branch vessels, the aorta was filled with 0.2% collagenase (Sigma) in HBSS and incubated for 15–20 min at room temperature. At the end of the incubation period, the lumen of the aorta was rinsed with HBSS. Free endothelial cells were collected and washed by centrifugation (2000 rpm, 7 min) and resuspended in M199 medium (Gibco) containing 15% fetal bovine serum (Gibco), 6 mM glutamine (Gibco) and 100 U/ml penicillin, 100 μg/ml streptomycin. Cells were seeded in 75-cm^2 culture flasks at 750000 cells/flask and were grown at 37°C under 95% air/5% CO_2. Endothelial cell monolayers were subcultured at 1-week intervals by incubation with 0.05% trypsin-0.02% EDTA (Gibco) and used between passages 3 and 8. The porcine aortic endothelial cells were characterized by morphology with phase-contrast microscopy which demonstrated typical cobblestone appearance and contact inhibition. Detection by means of factor VIII was not possible, as it has been demonstrated that porcine aortic endothelial cells do not express this factor (Giddings et al., 1983).

2.2. Cell membrane preparation

Porcine aortic endothelial cells at confluence between days 4 and 8 were rinsed with Ca^{2+},Mg^{2+}-free PBS, physically scraped from culture flasks and resuspended in cold isotonic buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μM bestatin, 50 μM Plummer's inhibitor, pH 7.4). Cells were homogenized using a Polytron (maximum setting, 2×15 s) and membranes were centrifuged at $40000 \times g$ for 20 min at 4°C. The pellet was resuspended in cold isotonic buffer and rehomogenized as above at the final protein concentration of 100 μg/ml, as determined with the Bio-rad technique, using bovine serum albumin as a standard (Bradford, 1976).

2.3. Receptor binding assays

Binding assays were performed in duplicate in assay buffer (isotonic buffer, 0.1% bovine serum albumin, pH 7.4) at 37°C (total volume 250 μl) in the presence of cell membrane preparation (100 μg protein/ml). Non-specific binding was defined in the presence of 1 μM unlabeled angiotensin IV. At the end of the incubation period, the bound radioactivity was rapidly separated from free by addition of ice-cold PBS and vacuum filtration through glass-fiber filters (Whatman GF/C, pretreated for 20 min with 2% bovine serum albumin) on a Brandel apparatus. The receptor-bound radioactivity was determined by liquid scintillation spectrophotometry (Packard 2500 TR, 80–85% efficiency).

Association experiments were performed at 37°C in the presence of 1 nM [^{125}I]angiotensin IV over a time course of 90 min. For dissociation experiments, cell membrane preparations were incubated in the presence of 1 nM [^{125}I]angiotensin IV for 90 min at 37°C. After equilibrium was reached, dissociation was induced by adding 1 μM unlabeled angiotensin IV (final concentration) to each sample. The apparent pseudo-first-order association rate constant, k_{obs}, and the dissociation rate constant, k_{-1}, were determined by using a computer-based non-linear regression curve-fitting program (RS/1, BBN Software).

The association rate constant was then calculated from the equation $k_{+1} = (k_{obs} - k_{-1})/[L]$ where [L] is the radioligand concentration. The apparent kinetic equilibrium dissociation constant, K_d, was derived from the equation $K_d = k_{-1}/k_{+1}$.

Saturation equilibrium binding assays were performed on endothelial cell membranes incubated in the presence of increasing concentrations of [^{125}I]angiotensin IV (0.1 nM–7 nM, with isotopic dilutions at highest concentrations) for 90 min at 37°C. Competition equilibrium binding assays were done with 0.2 nM [^{125}I]angiotensin IV displaced by varying concentrations of competitive ligands (0.01 nM to 100 μM, in half-log dilutions).

The data were analysed with the non-linear curve-fitting program, LIGAND (Munson and Rodbard, 1980) for the determination of the maximum number of binding sites, B_{max}, and of the equilibrium dissociation constant, K_d, and for analysis of competition data and determination of associated K_i values.

In order to assess a potential G-protein linkage of the angiotensin IV site, binding assays were carried out as for competition binding assays, but after a 60-min preincubation at room temperature of the membrane preparation in the presence of increasing concentrations of GTPγS (0–100 μM).

2.4. Drugs

The following drugs were used: [^{125}I][Tyr^2]angiotensin IV (human; spec. act., 81.4 TBq/mmol, NEN, France), angiotensin II-(3–8) (angiotensin IV) (human, Bachem), angiotensin III (Sigma), angiotensin II (human, Sigma), angiotensin I (Sigma), Nle-Tyr-Ile-NH_2 (Neosystem Laboratoire, France), [Sar^1,Ile^8]angiotensin II (Sigma), angiotensin II-(1–7) (Bachem); losartan (DuP 753: (2-n-butyl-4-chloro-5-hydroxymethyl-1 [(2′-(1H-tetrazol-5-yl) biphenyl-4-yl) methyl] imidazol, potassium salt, DuPont de Nemours, USA); PD 123177 (1-(4-amino-3-methyl-phenyl) methyl-5-diphenylisoethyl-4,5,6,7-tetrahydro-1H-imidazo [4,5-C] pyridine-6-carboxylic acid) (synthesized by the Chemistry Department, Synthélabo Recherche, Bagneux, France); CGP 42112A (nicotinic acid-Tyr-(N^{α}-benzyl-oxycarbonyl-Arg) Lys-His-Pro-Ile-OH, Bachem).

The peptides were prepared and diluted in the incubation buffer (in the presence of bovine serum albumin 0.1%). Other drug solutions were prepared in dimethylsulphoxide (30%) at 10 mM and subsequent dilutions were made in incubation buffer.

3. Results

Specific binding of [^{125}I]angiotensin IV on porcine aortic endothelial cells increased as a function of time and an apparent steady state was reached between 30 and 90 min (Fig. 1A). Following addition of 1 μM unlabelled angiotensin IV, specific binding was rapidly and fully reversible (Fig. 1B). The pseudo-first-order association rate constant, k_{obs}, was found to be 0.57 min^{-1} ($n = 4$) and the dissociation rate constant, k_{-1}, 0.175 min^{-1} ($n = 6$). The association rate constant, k_{+1}, calculated from k_{obs} and k_{-1}, was determined to be $3.98 \cdot 10^8$ M^{-1} min^{-1}. Based on these rate constants, the apparent kinetic K_d value was 0.44 nM.

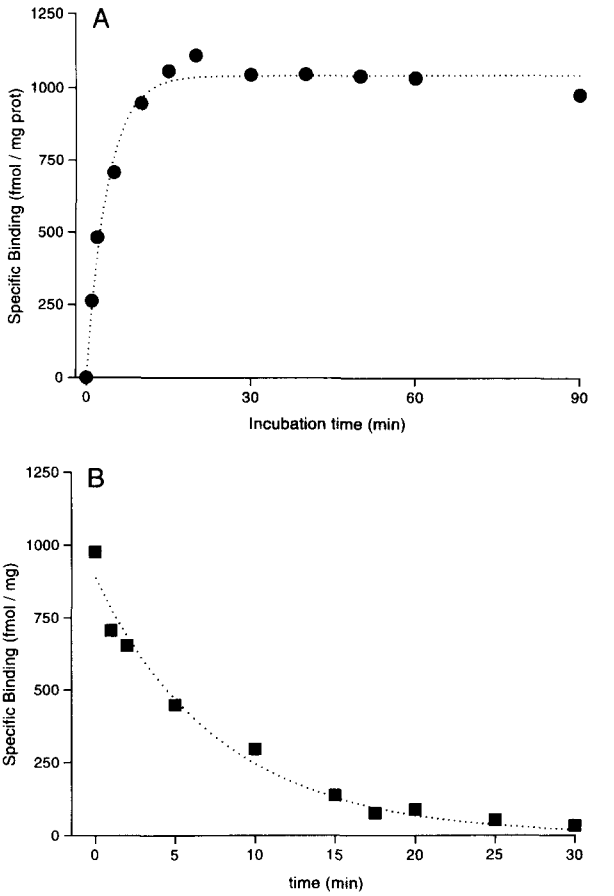

Fig. 1. Kinetic analysis of 1 nM [^{125}I]angiotensin IV binding in porcine aortic endothelial cells at 37°C. Data presented represent results from typical association (A) and dissociation (B) experiment.

Data from a typical equilibrium saturation binding experiment (90 min at 37°C) and its Scatchard transformation are shown in Fig. 2. Data analysis was consistent with a single high-affinity site for [^{125}I]angiotensin IV binding, with $K_d = 3.87 \pm 0.60$ nM and $B_{max} = 9.64 \pm 1.44$ pmol/mg protein ($n = 5$). At the level of the K_d, the specific binding represented more than 90% of the total binding of [^{125}I]angiotensin IV.

The specificity of angiotensin IV binding sites was evaluated in competition experiments. As shown in Fig. 3, the peptides angiotensin IV, angiotensin III and Nle-Tyr-Ile-NH_2 (a tripeptide with high affinity for AT_4 receptors, Sardinia et al., 1994a) inhibited specific [^{125}I]angiotensin IV binding with nanomolar affinity, while angiotensin II and derived peptides exhibited only micromolar affinity. Saralasin and selective AT_1 (losartan) or AT_2 (PD 123177,

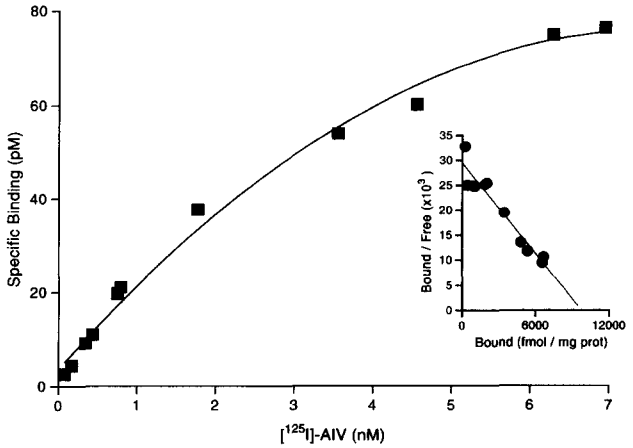

Fig. 2. Saturation isotherm and Scatchard transformation (inset) of [^{125}I]angiotensin IV to porcine aortic endothelial cell membranes at 37°C for 90 min. Data presented represent results from a typical experiment.

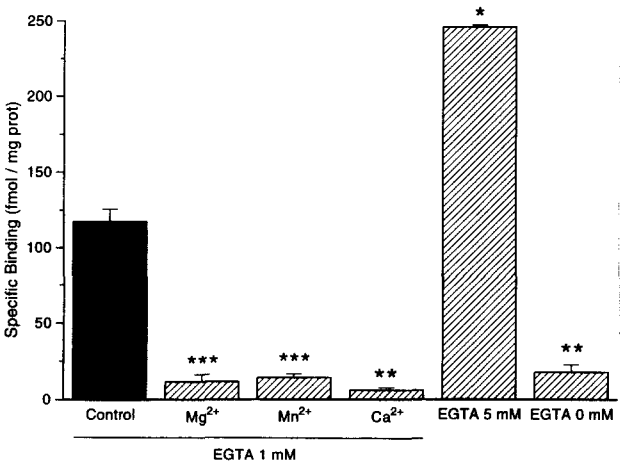

Fig. 4. Effect of divalent cations and EGTA on binding of 0.2 nM [^{125}I]angiotensin IV to porcine aortic endothelial cell membranes at 37°C for 90 min. Mean values ± S.E.M. of 3–4 experiments/group.

CGP 421112A) ligands had no significant effect up to 100 μM. On the other hand, the addition of the sulfhydryl reducing agent, dithiothreitol (5 mM), to the incubation buffer did not modify the [^{125}I]angiotensin IV-specific binding (data not shown).

Binding of [^{125}]angiotensin IV to porcine aortic endothelial cells was sensitive to the presence of divalent cations. As shown in Fig. 4, addition of Mg^{2+}, Mn^{2+} or Ca^{2+} (as $MgCl_2$, $MnCl_2$, 5 mM, $CaCl_2$, 2.6 mM) to the incubation buffer resulted in a 90–95% inhibition of [^{125}I]angiotensin IV-specific binding at 0.2 nM. A similar inhibition was observed when the ion chelator, EGTA, was omitted from the incubation buffer. Furthermore, if the concentration of EGTA was increased from 1 mM (incubation buffer) to 5 mM, there was a significant increase of the angiotensin IV binding at 0.2 nM (Fig. 4). To further characterize this effect, ligand saturation experiments were carried out in order to directly examine the effect of EGTA on the binding parameters of [^{125}I]angiotensin IV. Addition of 5 mM EGTA in the assay buffer did not modify the affinity of [^{125}I]angiotensin IV on porcine aortic endothelial cell membranes ($K_d = 5.7 \pm 1.3$ nM, not different from control value in the presence of 1 mM EGTA). In contrast, the number of [^{125}I]angiotensin IV binding sites was significantly increased, 2-fold ($B_{max} = 17.8 \pm 2.5$ pmol/mg protein, $n = 5$, $P < 0.05$ when compared with control values).

The non-hydrolyzable GTP analog, GTPγS, was used to study a possible G-protein interaction with the angiotensin IV binding site in porcine aortic endothelial cells. Exposure of porcine aortic endothelial cell membranes to GTPγS did not significantly modify specific [^{125}I]angiotensin IV binding, even at concentrations as high as 100 μM (data not shown). These results suggest that the angiotensin IV binding site in porcine aortic endothelial cells may not be G-protein-linked.

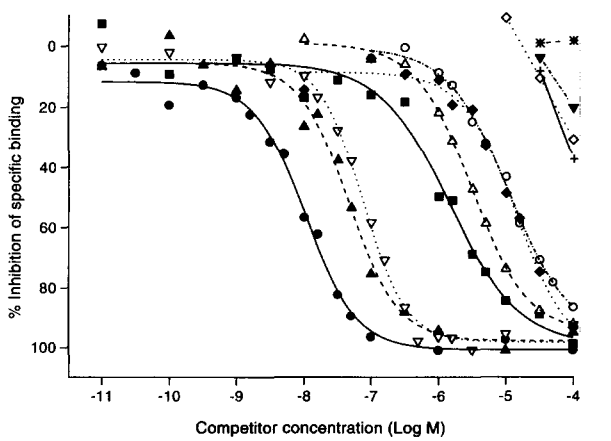

Fig. 3. Representative concentration–effect curves for the inhibition of 0.2 nM [^{125}I]angiotensin IV binding to porcine aortic endothelial cell membranes at 37°C for 90 min. ●, angiotensin IV; ▲, Nle-Tyr-Ile-NH_2; ▽, angiotensin III; ■, angiotensin II; △, angiotensin I; ○, angiotensin II-(1–7); ◆, [Sar^1,Ile^8]angiotensin II; ◇, saralasin; ▼, losartan; +, CGP 42112A; *, PD 123177. Mean values ± S.E.M. of 3–5 experiments/curve.

4. Discussion

In this study, we have characterized a new binding site for [^{125}I]angiotensin IV in cultured porcine aortic endothelial cells. The results obtained indicate that [^{125}I]angiotensin IV binding is saturable, reversible, time-dependent and reaches equilibrium in approximately 30 min at 37°C. Binding remains stable for at least 1 h (Fig. 1A). Scatchard analysis indicated the presence of a single binding site which is abundant ($B_{max} = 9.64 \pm 1.44$ pmol/mg protein) and of high affinity ($K_d = 3.87 \pm 0.60$ nM) in porcine aortic endothelial cells. Similar results have been obtained in other tissues, such as bovine aortic endothelial cells (Hall et al., 1993), bovine adrenal cortex (Swanson et al., 1992), guinea pig hippocampus (Harding et al., 1992;

Bernier et al., 1994) and mammalian heart membranes (Hanesworth et al., 1993).

The higher K_d value of 3.87 nM obtained in porcine aortic endothelial cells under equilibrium conditions, compared with the kinetically determined value of 0.44 nM, is consistent with previously reported results for angiotensin IV binding in various tissues. This high value was not due to a slow dissociation rate, which would imply that binding equilibrium was not reached for low concentrations of ligand (see, for comparison, Bernier et al., 1994, in bovine adrenal cortex membranes). In fact, total dissociation was obtained after 30 min (with 1 nM [^{125}I]angiotensin IV), while equilibrium experiments were performed with a 90-min incubation period. The other possibility could consist of partial degradation of the ligand during the incubation in the presence of porcine aortic endothelial cell membranes for 90 min, due to the presence of proteinases not affected by inhibitors present in the assay. If this is the case, the higher K_d values derived from pseudo-equilibrium studies would be an underestimation of the actual affinity of angiotensin IV for its binding site, around 0.4 nM.

These considerations prompted us to perform concentration-displacement experiments in the presence of a low concentration of [^{125}I]angiotensin IV (0.2 nM), therefore, minimizing the problem of ligand affinity in the determination of potencies of various compounds to displace specific angiotensin IV binding (according to Cheng and Prusoff, 1973). Under these experimental conditions, the non-peptide angiotensin receptor antagonists, losartan and PD 123177, selective, respectively, for the angiotensin AT_1 and AT_2 receptors as well as the AT_2 peptidic ligand, CGP 42112A, or the non-selective AT_1/AT_2 antagonists, saralasin and [Sar^1,Ile^8]angiotensin II, were ineffective up to 100 μM to displace [^{125}I]angiotensin IV binding. Therefore, the binding site for [^{125}I]angiotensin IV in porcine aortic endothelial cells appears to be clearly distinct from AT_1 and AT_2 receptors (Timmermans et al., 1991). Concentration-displacement experiments with angiotensin IV and other ligands presented in Fig. 4 confirmed the high affinity of the binding for [^{125}I]angiotensin IV. According to the rank order of affinity of competiting displacers (angiotensin IV > angiotensin III > angiotensin II > angiotensin I > angiotensin II-(1–7); Table 1), the N-terminal position of angiotensin IV appears to play a critical role for binding of the peptide on its site. Recently, Sardinia et al. (1994b) have described the structure-binding relationship of N-terminal modified angiotensin IV analogs, supporting the importance of N-terminal stucture for binding of angiotensin IV. The same rank order of affinity has been reported for these peptides whatever the tissue in which the AT_4 receptor has been described (Hall et al., 1993). These results reveal a pharmacological profile which appears to be specific for [^{125}I]angiotensin IV binding site and quite similar to the profile of AT_4 receptors described so far (Wright et al., 1995).

Table 1
Competition data for [^{125}I]angiotensin IV to porcine aortic endothelial cells membranes

Inhibitors	K_i (μM)
Angiotensin IV	0.011 ± 0.001
Nle-Tyr-Ile-NH_2	0.030 ± 0.007
Angiotensin III	0.079 ± 0.019
Angiotensin II	1.46 ± 0.30
Angiotensin I	3.14 ± 0.41
Angiotensin II-(1–7)	10.8 ± 1.6
[Sar^1,Ile^8]angiotensin II	14.4 ± 6.8
Saralasin	> 100 [a]
Losartan	> 100 [a]
CGP 42112A	> 100 [a]
PD 123177	> 100 [a]

Mean values ± S.E.M. of 3–7 experiments/competitor. [a] Less than 40% displacement was observed with the highest concentration of competitor.

[^{125}I]Angiotensin IV binding in porcine aortic endothelial cells is not modified by the sulfhydryl reducing agent, dithiothreitol (5 mM), suggesting that disulfide bridges are not important in the binding of angiotensin IV to these sites. It was recently reported that, in preliminary experiments on solubilized AT_4 receptors, reduction of receptor preparations, causing the loss of one or more disulfide links covalently joining receptor subunits, does not alter the binding characteristics of the receptor, in agreement with the present results (Wright et al., 1995). The lack of effect of dithiothreitol on angiotensin IV binding is in marked contrast with the well-characterized effect of the reducing agent on angiotensin AT_1 or AT_2 receptors. Radioligand binding to AT_1 receptors is markedly reduced in the presence of dithiothreitol, suggesting that disulfide bridges are essential for activation of the AT_1 receptor, while binding to AT_2 receptors has been reported to be enhanced (Whitebread et al., 1989; Gehlert et al., 1991). In the presence of higher concentrations of dithiothreitol (up to 100 mM), an increase of specific binding of [^{125}I]angiotensin IV to bovine adrenal cortex membranes has been reported recently (Jarvis and Gessner, 1994). No significant effects on the apparent density of AT_4 receptors were observed, but the affinity was increased. We cannot rule out at the moment the possibility that similar effects could be observed in porcine aortic endothelial cells exposed to high concentrations of dithiothreitol.

Interestingly, in bovine adrenal cortex membranes, the effect of dithiothreitol on angiotensin IV affinity was potentiated by EDTA and binding was dramatically decreased in the presence of divalent cations (Jarvis and Gessner, 1994). In porcine aortic endothelial cells, the divalent cations Mg^{2+} or Mn^{2+} at 5 mM and Ca^{2+} at 2.6 mM also inhibited the binding of [^{125}I]angiotensin IV. Moreover, in saturation equilibrium binding assays performed in the presence of 5 mM EGTA, there was a 2-fold increase of the number of binding sites compared with control conditions (1 mM EGTA), without any change of

angiotensin IV affinity. Taken together, these results suggest that, in the presence of divalent cations, there are fewer binding sites accessible to the ligand. Whether these observations are only artefactual (binding studies performed on membrane preparations) or reflect a true characteristic of the AT_4 receptor deserves further evaluation.

A major question concerns the intracellular signal activated by the AT_4 receptor. The lack of effect of GTPγS on [^{125}I]angiotensin IV binding on porcine aortic endothelial cells supports the view that this binding site is not coupled to a classical G-protein. Similar results have been reported previously (Hall et al., 1993; Hanesworth et al., 1993; Miller-Wing et al., 1992). More recently, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of solubilized as well as membrane-bound receptors indicates that the AT_4 receptor exists in both monomeric and heterodimeric forms (165 and 60 kDa subunits), more consistent with a member of a growth factor or cytokine family of receptors than with a classical G-protein-coupled receptor (Wright et al., 1995).

In conclusion, this report describes the characteristics of a binding site for [^{125}I]angiotensin IV in porcine aortic endothelial cells, clearly distinct from the AT_1 and AT_2 angiotensin II receptors. According to its pharmacological characteristics, this binding site closely resembles the AT_4 receptor recently characterized (Sardinia et al., 1994b; Wright et al., 1995). The presence of specific binding for [^{125}I]angiotensin IV on porcine aortic endothelial cells supports the concept that angiotensin IV may play a important role in cardiovascular regulation.

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