Identification of atypical (non-AT_1, non-AT_2) angiotensin binding sites with high affinity for angiotensin I on IEC-18 rat intestinal epithelial cells

Roger D. Smith*

Department of Clinical Biochemistry, Addenbrooke's Hospital, Cambridge, CB2 2QQ, UK

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Abstract Specific high-affinity (K_d = 3.4 nM) binding sites for ^{125}I-labelled angiotensin I ([^{125}I]Ang I) were identified on an epithelial cell line (IEC-18) derived from the rat small intestine. The sites, which also have high affinity for Ang II, are insensitive to both AT_1- and AT_2-specific angiotensin receptor antagonists. The rank order of potency with which various angiotensin peptides inhibited [^{125}I]Ang I binding to the cells (Ang I ≥ Ang II > Ang(1–7) > [Sar^1,Ile^8]-Ang II > Ang(3–8) > Ang III) also distinguishes these sites from AT_1 and AT_2 angiotensin receptors.

Key words: Angiotensin I; Atypical angiotensin receptor; Angiotensin receptor antagonist; IEC-18 cell; Intestinal epithelium

1. Introduction

The octapeptide, angiotensin II (Ang II), the principal active component of the renin-angiotensin system, plays a major role in the physiology of the cardiovascular system by affecting diverse target tissues such as vascular smooth muscle, adrenal cortex, pituitary and kidney [1]. The peptide also promotes cardiac hypertrophy and neointimal proliferation following arterial injury [2,3], and is implicated in the pathogenesis of hypertension and atherosclerosis [4]. In addition to these cardiovascular effects, Ang II is also active in non-cardiovascular tissues such as the ovary [5].

Multiple mechanisms of signal transduction have been demonstrated for Ang II. For example, depending on the target cell or tissue, the peptide stimulates phosphoinositide turnover, inhibits adenylate cyclase, activates guanylate cyclase, releases prostaglandins and regulates Ca^{2+} channels (reviewed in [6–8]). In view of these pleiotrophic actions of Ang II, multiple angiotensin receptor subtypes have been postulated for some years [9–12]. However, their existence has only been confirmed in recent years with the introduction of highly-selective non-peptide antagonists which have allowed a classification of angiotensin receptors into AT_1 and AT_2 subtypes [13–16].

AT_1 receptors are defined by their sensitivity to biphenylimidazole antagonists such as DuP 753, whereas AT_2 receptors are defined by their sensitivity to tetrahydroimidazopyridine antagonists such as PD 123319 and PD 123177 [13–16]. Despite differential sensitivities to these antagonists, both AT_1 and AT_2 receptors exhibit high affinity for Ang II, [Sar^1,Ile^8]-Ang II and the heptapeptide, angiotensin III (Ang III), whereas they each have low affinity for the decapeptide, angiotensin I (Ang I) [16].

However, the identification of AT_1 and AT_2 receptors does not exclude the possibility that additional angiotensin receptors may also exist. Here, I describe the presence of angiotensin binding sites which are pharmacologically distinct from either AT_1 or AT_2 angiotensin receptors on IEC-18 rat intestinal epithelial cells.

2. Materials and methods

DMEM, foetal calf serum (FCS), trypsin and antibiotics were from Sigma. IEC-18 cells (a gift of Dr. A. Quaroni) at passages 30–60 were maintained at 37°C in DMEM containing 5% (v/v) FCS, insulin (5 μg/ml: Novo Nordisk), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 in air. Cells were subcultured every 3–4 days using 0.43 mM EDTA/0.05% (w/v) trypsin and seeded into plastic dishes or 24-well cluster plates (Nunc) at a dilution of 1:10. Cells were used after 3–4 days when they formed confluent monolayers. Cultures were free from contamination with *Mycoplasma* as determined by staining with 4′-6-diamidino-2′-phenylindole [17].

Angiotensin peptides were from Bachem. Captopril was from Sigma and aprotinin was from Bayer. DuP 753 and PD 123319 were supplied by DuPont and Parke Davis, respectively, and CGP 42112A was provided by Dr. M. deGasparo. Ang I and Ang II were each iodinated using the soluble lactoperoxidase method [18] as previously described [19], and separated from free iodide using Sep-Pak C_{18} cartridges (Waters).

Confluent cell monolayers were washed with ice-cold binding medium (BM; 130 mM NaCl, 5.1 mM KCl, 1.3 mM $CaCl_2$, 1.3 mM $MgCl_2$, 0.1 μM KI, 0.1% (w/v) protease-free BSA, 100 U/ml aprotinin, 1 mM captopril, 50 mM HEPES, pH 7.4) prior to incubation at 4°C in BM containing [^{125}I]Ang I or [^{125}I]Ang II, with or without additions, as indicated in the individual figure legends. After two washes with ice-cold BM, IEC-18 cells were harvested into solubilisation buffer (1% (v/v) Triton X-100, 10% (v/v) glycerol, 0.1% (w/v) BSA, 25 mM HEPES, pH 7.5), and cell-associated radioactivity was measured in a gamma-counter. Non-specific binding was determined in the presence of an excess (10 μM) of unlabelled Ang I or Ang II.

3. Results

[^{125}I]Ang I bound specifically and saturably to IEC-18 cells at 4°C. Specific binding increased with time to reach a plateau value at ~4 h (Fig. 1). This incubation time was therefore employed in subsequent experiments.

In principle, the apparent binding of [^{125}I]Ang I to IEC-18 cells could have resulted from proteolytic conversion of [^{125}I]Ang I to [^{125}I]Ang II during the incubation, with the latter (rather than the former) peptide actually binding to the cells. In order to exclude this possibility, [^{125}I]Ang I (0.5 nM)-containing BM was preincubated with IEC-18 cells and then allowed

*Corresponding author. Fax: (44) (1223) 21-6862. *Address from 1/1/1995:* Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA.

Abbreviations: Ang, angiotensin; AT, angiotensin receptor; BM, binding medium; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; HEPES, *N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulphonic acid.

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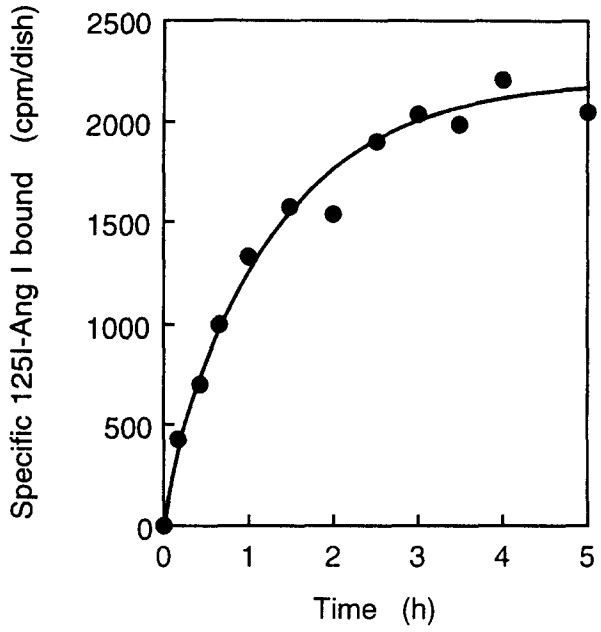

Fig. 1. Time course of [^{125}I]Ang I binding to IEC-18 cells. Cells were incubated for the indicated times at 4°C with [^{125}I]Ang I (0.25 nM) in the presence or absence of an excess (10 μM) of unlabelled Ang I. After washing, the specific binding of [^{125}I]Ang I was determined. Each point represents the average of duplicate determinations.

to rebind to a cell line (RIE-1 [19]) known to express AT_1 receptors (Fig. 2). These cells bind specifically nanomolar concentrations of [^{125}I]Ang II, but not [^{125}I]Ang I. However, when [^{125}I]Ang I was preincubated with IEC-18 cells, no specific binding of the radiolabel to RIE-1 cells was subsequently observed (Fig. 2). Hence, there was no significant conversion of [^{125}I]Ang I to [^{125}I]Ang II during incubation with IEC-18 cells.

The concentration-dependent binding of [^{125}I]Ang I to IEC-18 cells is shown in Fig. 3. Scatchard analysis of the data (insert to Fig. 3) generated a linear slope, indicating the presence of a single class of high-affinity [^{125}I]Ang I binding sites on the cells

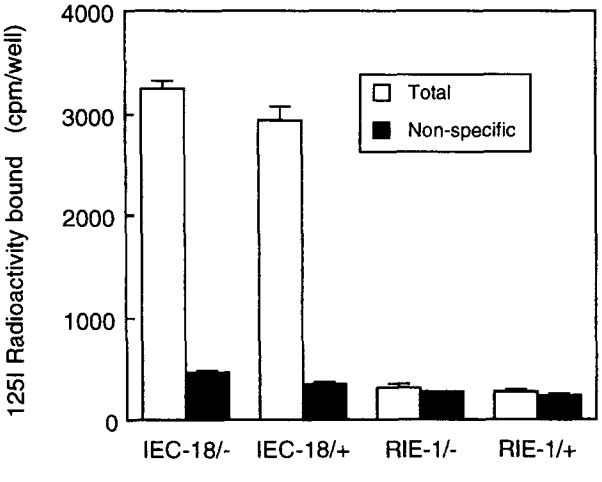

Fig. 2. Rebinding of [^{125}I]Ang I to IEC-18 and RIE-1 cells. Binding medium containing [^{125}I]Ang I (0.5 nM) was incubated for 4 h at 4°C in the presence (+) or absence (−) of IEC-18 cells. Medium was then incubated with fresh cultures of IEC-18 or RIE-1 cells as indicated. The total and non-specific binding of the radiolabel to each cell type was then determined. Each column represents the mean of triplicate determinations (± S.E.M.).

with a K_d value of 3.4 nM and a B_{max} of 280 fmol/10^6 cells (which corresponds to 170,000 binding sites per cell, assuming one molecule of [^{125}I]Ang I binds per site).

Unlabelled Ang I inhibited [^{125}I]Ang I (0.25 nM) binding to IEC-18 cells in a dose-dependent fashion, with an ID_{50} of ~3 nM (Fig. 4). However, neither the AT_1-specific antagonist, DuP 753 [13–15], nor the AT_2-specific antagonist, PD 123319 [13–15], had any effect on [^{125}I]Ang I binding to IEC-18 cells, even when these agents were present at micromolar concentrations (Fig. 4). In control experiments, DuP 753 inhibited [^{125}I]Ang II binding to RIE-1 cells with an ID_{50} of ~30 nM and PD 123319 inhibited [^{125}I]Ang II binding to Swiss 3T3 cells (which express AT_2 receptors [20]) with an ID_{50} of ~3 nM (data not shown). The AT_2-specific ligand, CGP 42112 [12], similarly had no effect on [^{125}I]Ang I binding to IEC-18 cells at concentrations up to 10 μM (Fig. 4). The angiotensin binding sites on IEC-18 cells are therefore atypical, being insensitive to both AT_1-, and AT_2-specific ligands.

The potencies with which various angiotensin peptides inhibited [^{125}I]Ang I (0.25 nM) binding to IEC-18 cells is shown in Fig. 4. It is apparent that, in addition to Ang I, the sites also have high affinity (ID_{50}~5 nM) for Ang II, although this was slightly less than that of Ang I (ID_{50}~3 nM). They also have moderate affinity (ID_{50}~30 nM) for Ang(1–7), but only low affinity (ID_{50}~3 μM) for Ang(3–8) and very low affinity (ID_{50}~30 μM) for Ang III (Fig. 4). The sites also have low affinity (ID_{50}~1 μM) for the non-specific angiotensin receptor antagonist, [Sar^1,Ile^8]-Ang II (Fig. 4). Thus, in addition to their insensitivity to AT_1- and AT_2-specific ligands, the rank order of potency with which angiotensin peptides inhibit [^{125}I]Ang I binding (Ang I ≥ Ang II > Ang(1–7) > [Sar^1,Ile^8]-Ang II > Ang(3–8) > Ang III) also differentiates the atypical sites on IEC-18 cells from both AT_1 and AT_2 angiotensin receptors (each of which have high affinity

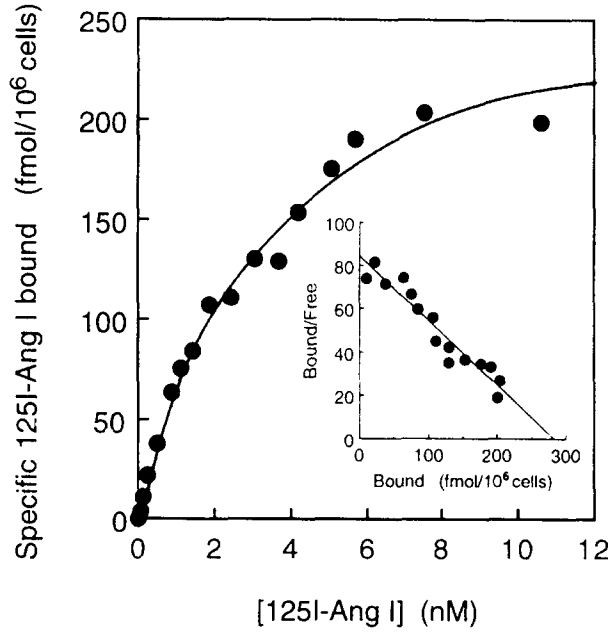

Fig. 3. Concentration-dependent binding of [^{125}I]Ang I to IEC-18 cells. Cells were incubated for 4 h at 4°C in the presence of [^{125}I]Ang I in the concentration range 0.1–11 nM. After removing an aliquot of medium for the determination of free [^{125}I]Ang I concentration, specific [^{125}I]Ang I binding was determined. The insert shows Scatchard analysis of the same data. Each point represents a single determination.

for Ang II, Ang III and [Sar[1],Ile[8]]-Ang II, but only low affinity for Ang I [16]).

When the experiment of Fig. 4 was repeated using [^{125}I]Ang II in place of [^{125}I]Ang I, similar results were obtained (Fig. 5). [^{125}I]Ang II binding was also insensitive to DuP 753 and PD 123319, and the rank order of potency with which angiotensin peptides inhibited [^{125}I]Ang II binding was Ang I > Ang II ≫ Ang III (Fig. 5).

4 Discussion

To date, there are few reports of high affinity angiotensin binding sites that are insensitive to both AT_1- and AT_2-specific angiotensin receptor antagonists. However, such sites have been described on two species of *Mycoplasma* which commonly infect eukaryotic cell cultures, namely *M. hyorhinis* and *Acholeplasma laidlawii* (although this phenomenon does not appear to be a general feature of *Mycoplasmataceae* since atypical sites were not present on *M. hominis*) [21,22]. In view of these findings, it is therefore essential to exclude *Mycoplasma* infection when evaluating putative atypical angiotensin binding sites on cultured eukaryotic cells. Accordingly, the IEC-18 cells used in the present study were free of such contamination as assessed by staining with 4′-6-diamidino-2′-phenylindole [17] (data not shown).

Atypical angiotensin binding sites have also been described on chick embryo chorioallantoic membrane [23] and turkey adrenal gland [24], as well as oocytes [25] and cardiac membranes [26] isolated from *Xenopus laevis*. However, in common with the AT_1 receptor [16], both the turkey adrenal site and the *Xenopus* cardiac site have high affinity for Ang II, Ang III and [Sar[1],Ile[8]]-Ang II, low affinity for Ang I and are able to mediate inositol phosphate production and elevation of intracellular [Ca^{2+}] [24,26–28]. Since the turkey and *Xenopus* receptors also share 60–75% amino acid sequence with the AT_1 receptor [24,27], it therefore appears likely that they represent the avian and amphibian homologues of the mammalian receptor (which have acquired their unusual pharmacological properties as a result of evolutionary divergence).

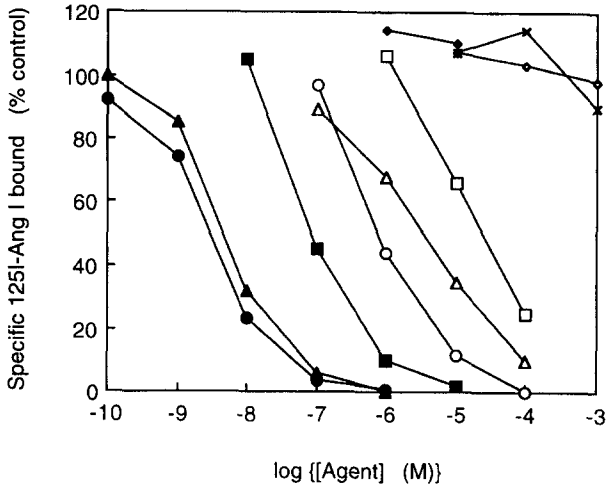

Fig. 4. Inhibition of [^{125}I]Ang I binding to IEC-18 cells by various agents. Cells were incubated for 4 h at 4°C in the presence of [^{125}I]Ang I (0.25 nM) and the indicated concentrations of Ang I (●), Ang II (▲), Ang(1–7) (■), [Sar[1],Ile[8]]-Ang II (○), Ang(3–8) (△), Ang III (□), CGP 42112 (♦), PD 123319 (◇) or DuP 753 (×). After washing, the specific binding of [^{125}I]Ang I was determined. Each point represents the average of duplicate determinations.

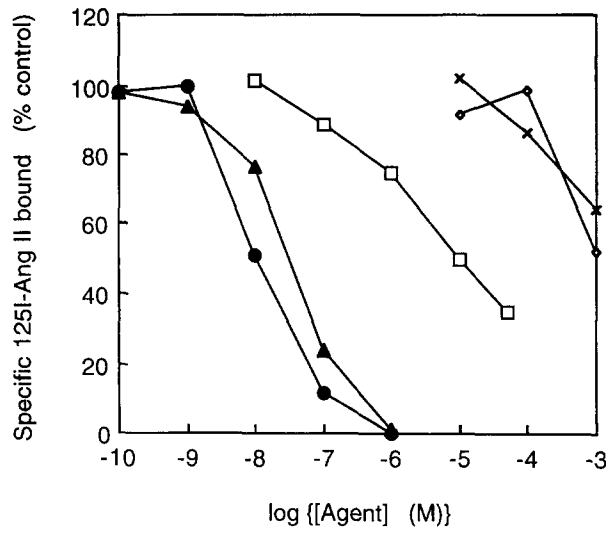

Fig. 5. Inhibition of [^{125}I]Ang II binding to IEC-18 cells by various agents. Cells were incubated for 4 h at 4°C in the presence of [^{125}I]Ang II (0.25 nM) and the indicated concentrations of Ang I (●), Ang II (▲), Ang III (□), PD 123319 (◇) or DuP 753 (×). After washing, the specific binding of [^{125}I]Ang I was determined. Each value represents the average of duplicate determinations.

Thus far, the only previous report of atypical angiotensin binding sites on any mammalian cell is a site present on mouse Neuro-2A neuroblastoma cells [29–31]. Like the IEC-18 site, the Neuro-2A site also has low affinity for Ang III [29], although the affinities of this site for Ang I and [Sar[1],Ile[8]]-Ang II have not been reported. However, it has not been clear whether or not the Neuro-2A cells used in these studies were contaminated with *Mycoplasma*, and, if contaminated, which strain was present. It therefore remains to be determined whether or not the Neuro-2A site is endogenous to those cells, and, if so, what its relationship is to the IEC-18 site.

Recent studies suggest that some smaller fragments of Ang II, particularly Ang(3–8) and Ang(1–7), are also biologically active, and that they also act via receptors that are distinct from AT_1 and AT_2 receptors. For example, Ang(3–8) binds to a site present in several tissues which is insensitive to both DuP 753 and PD 123177 [32], and the cardiovascular actions of Ang(1–7) are unaffected by DuP 753 or PD 123319 [33].

Although the function, if any, of the atypical site on IEC-18 cells remains to be determined, its identification supports the hypothesis that additional (non-AT_1, non-AT_2) angiotensin receptors also exist. Further characterisation of such receptors would be facilitated by the development of antagonists specific for each site. Indeed, such an antagonist has recently been described for the Ang(1–7) receptor [34]. However, the unequivocal existence of additional 'AT_n' receptors will only be demonstrated by isolating and sequencing cDNAs encoding these putative receptors.

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