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Amphibian myocardial angiotensin II receptors are distinct from mammalian AT_1 and AT_2 receptor subtypes

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High-affinity receptors for angiotensin II were identified on *Xenopus laevis* cardiac membranes and characterized by binding-inhibition studies with peptide and non-peptide AII antagonists. Scatchard analysis of the binding data identified a high-affinity site with $K_{d_1} = 1.6$ nM and $B_{max_1} = 3.7$ pmol/mg protein and a low-affinity site with $K_{d_2} = 22$ nM and $B_{max_2} = 9.5$ pmol/mg protein. Treatment with dithiothreitol reduced the number of binding sites by >70%. The rank order of potency for AII analogs was (agent, IC_{50}) [Sar^1,Ile^8]AII, 0.91 nM > AII, 2.0 nM > AI, 5.3 nM > [Sar^1, Ala^8]AII, 19 nM >> CGP42112A, 1.2 μM >>> DuP 753 ≈ PD-123177, >100 μM. The relative potencies of these compounds differ markedly from their activities on the two known mammalian AII receptor subtypes, AT_1 and AT_2. These results indicate that amphibian AII receptors are pharmacologically distinct from both the AT_1 and AT_2 receptors characterized in mammalian tissues.

Angiotensin receptor; Angiotensin; Angiotensin receptor antagonist; *Xenopus laevis*; Myocardium; Heart

1. INTRODUCTION

Angiotensin II (AII)[1] receptors exert a wide variety of effects on many tissues including the vasculature, adrenal, kidney, brain, liver, and heart [1]. The existence of AII receptor subtypes could account for the diverse actions of AII in various target tissues, and on cardiovascular function and sodium homeostasis. Receptor heterogeneity has been suggested by radioligand binding studies [2,3] and by the demonstration of multiple mechanisms of AII receptor signal transduction, including phosphoinositide turnover, inhibition of adenylate cyclase, and Ca^{2+} channel regulation [3,4]. Studies employing recently developed peptide and non-peptide AII antagonists have characterized two AII receptor subtypes (AT_1 and AT_2) with identical affinities for AII and most of its peptide antagonists [5,6]. A receptor that is coupled to the known cellular responses to AII is blocked by the non-peptide antagonist, DuP 753, and is defined as the AT_1 subtype. The function of the AT_2 receptor, which is defined by the inhibition of radioligand binding by novel antagonists such as PD-12377 and CGP 42112A, is not yet known. The two subtypes are present in various proportions in different tissues; AT_1 sites are abundant in adrenal cortex and vascular tissues, and AT_2 sites are highly enriched in uterus [6] and adrenal medulla [7], as well as the PC12 line of adrenal phaeochromocytoma cells [8].

We have recently observed that *Xenopus laevis* follicular oocytes contain endogenous AII receptors which are functionally similar to the AT_1 mammalian receptor in that they mobilize intracellular Ca^{2+} in response to AII [9]. However, the sensitivity of AII-induced calcium responses in the amphibian oocyte to the new antagonists differed markedly from those of the recently-defined mammalian AII receptor subtypes [10]. The low levels of ^{125}I-[Sar^1,Ile^8]AII binding in oocytes did not permit a detailed characterization of the binding parameters of AII receptors in oocyte membranes. For this reason, we could not rule out the existence of amphibian oocyte AII receptor subtype that is not linked to Ca^{2+} mobilization. Subsequently, we screened several *Xenopus* tissues and found high levels of ^{125}I-[Sar^1,Ile^8]AII binding in the heart. In the present study, amphibian cardiac AII receptors were characterized and found to be pharmacologically distinct from the AII receptor subtypes identified in mammalian tissues including the rabbit ventricular myocardium.

2. MATERIALS AND METHODS

^{125}I-[Sar^1,Ile^8]AII (2200 Ci/mmol) was obtained from New England Nuclear (Boston, MA). [Sar^1,Ala^8]AII and [Sar^1,Ile^8]AII were purchased from Peninsula Labs (Belmont, CA). AII and AI

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[1] *Abbreviations:* AII, angiotensin II; Ca^{2+}, calcium; DTT, dithiothreitol. The abbreviations AT_1 and AT_2 have been used for angiotensin II receptor subtypes as recommended by a Committee on Nomenclature for Angiotensin Receptors, convened by the Council for High Blood Pressure of the American Heart Association.

were obtained from Bachem Inc. (Torrance, CA). The peptide AII antagonist, nicotinic acid-Tyr-(N^{α}-benzyloxycarbonyl-Arg)Lys-His-Pro-Ile (CGP 42112A) was provided by Dr M. de Gasparo (Ciba-Geigy Basel, Switzerland). The non-peptide AII antagonists, DuP753 (2-*n*-butyl-4-chloro-5-hydroxy-methyl-1-(2'-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl imidazole, potassium salt) and PD-123177 (1-(4-amino-3-methylphenyl)methyl-5-diphenyl-acetyl-4,5,6,7-tetrahydro-1*H*-imidazole[-4,5-*c*]pyridine-6-carboxylic acid · HCl) were provided by Dr P.C. Wong (DuPont, Wilmington, DE)[1].

Heart tissue was removed from *Xenopus laevis* (Xenopus 1, Ann Arbor, MI) frogs and immediately homogenized with a polytron in ice-cold, freshly prepared 20 mM $NaHCO_3$. The homogenate was stirred on ice for 20 min and then filtered through nylon mesh. After centrifugation at 100 × *g* for 10 min at 4°C, the supernatant was removed and centrifuged at 30 000 × *g* for 20 min at 4°C. The particulate fraction was washed by resuspending the pellet in homogenization buffer and centrifuging again at 30 000 × *g* for 20 min. The final pellet was resuspended in ice-cold binding buffer at a concentration of 1 μg/μl for binding studies.

Cardiac membranes (25–80 μg protein/tube) were incubated with ^{125}I-[Sar^1,Ile^8]AII (50 pM) in 400 μl binding buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 2 mM EGTA, 100 mM NaCl, 1.0% bovine serum albumin, pH 7.4) with increasing concentrations of AII and antagonist ligands. The incubations were terminated after 2 h at room temperature by rapid filtration and the membranes were collected on glass fiber filters (Whatman GFC). The specific binding was defined as the difference between the total radioactivity bound to membranes and that bound in the presence of 1 μM unlabeled AII. In typical experiments, the total binding was between 10 000 and 35 000 cpm and the non-specific binding was between 400 to 500 cpm. All experiments were performed in duplicate with a variation of less than 10% and were repeated 2–3 times. The binding data were analyzed using an iterative non-linear regression analysis computer program ('Ligand', Dr P.J. Munson, NICHD, NIH, Bethesda, MD).

3. RESULTS

In our previous report [10], the amphibian oocyte AII receptor was shown to be pharmacologically distinct from the mammalian AT_1 and AT_2 receptors, a finding that extends previous observations on differences in AII-related peptide agonist activities between amphibian and expressed mammalian AII receptors [9]. Because the levels of ^{125}I-[Sar^1,Ile^8]AII binding to oocyte membranes were too low to perform quantitative receptor analysis, we screened several tissues from *Xenopus* frogs in order to find a suitable source for radioligand binding studies. In autoradiographic studies with ^{125}I-[Sar^1,Ile^8]AII, AII receptors were observed in skin, ovary, kidney and heart; no specific binding was seen in the liver, spleen or air sac (data not shown). We chose the heart to perform binding studies since ^{125}I-[Sar^1,Ile^8]AII binding was abundant, and could be directly compared with the recent characterization of AII receptor subtypes in mammalian cardiac tissue [11].

Initial experiments indicated that specific binding of 50 pM ^{125}I-[Sar^1,Ile^8]AII to *Xenopus* cardiac membranes reached equilibrium between 1 and 2 h at room temperature, and was linear between 10 and 160 μg protein. The levels of ^{125}I-[Sar^1,Ile^8]AII binding were higher in fresh tissue (89 ± 7.0 fmol/mg protein) than in frozen tissue (38 ± 2.3 fmol/mg protein) and were reduced by 85% when incubations were performed at 4°C. The binding of ^{125}I-[Sar^1,Ile^8]AII was reversible as evidenced by the ability of unlabeled AII to displace the bound peptide from membrane receptors (data not shown). Scatchard analysis of the equilibrium binding data gave curvilinear plots that were best fit by a two-site model (Fig. 1), giving a high-affinity site with K_{d_1} = 1.6 ± 0.18 nM and B_{max_1} of 3.7 ± 0.34 pmol/mg, and a low-affinity site with K_{d_2} = 22 ± 2.5 nM and B_{max_2} = 9.5 ± 1.0 pmol/mg. These data are consistent with reports of two classes of binding sites in mammalian heart membranes [12,13]. The two sites identified by Scatchard analysis could represent two distinct AII receptors, or could reflect the existence of one AII receptor in different affinity states. In this regard, mammalian high-affinity AII receptors are well known to be regulated by guanine nucleotides [4,12,13].

The recent development of several novel AII receptor antagonists has permitted the characterization in several tissues of two AII receptor subtypes, AT_1 and AT_2, which have identical affinity for AII and the majority of its peptide analogs. AT_1 and AT_2 receptors are differentially affected by dithiothreitol, which inhibits AT_1 receptor binding but enhances AT_2 receptor binding [10]. In amphibian cardiac receptors, DTT markedly reduced the binding of ^{125}I-[Sar^1,Ile^8]AII. Scatchard analysis revealed that DTT reduced the B_{max} of both binding sites but only marginally affected binding affinities (K_{d_1} = 1.2 ± 0.19 nM, B_{max_1} = 2.9 ± 0.35 fmol/mg; K_{d_2} = 18 ± 2.1 nM, B_{max_2} = 2.8 ± 0.38 pmol/mg).

The major mammalian AII receptor subtype, AT_1, which appears to mediate all physiological responses so far studied, binds DuP 753 with high affinity (K_i = 25–60 nM) but does not recognize PD-123177. CGP 42112A is a weak antagonist (K_i = 0.6–3.7 μM) at this receptor, in contrast to its high affinity (K_i = 0.1–1 nM) for the AT_2 receptor [6,11]. The AT_2 receptor also binds PD 123177 (IC_{50} = 3 nM) but does not recognize

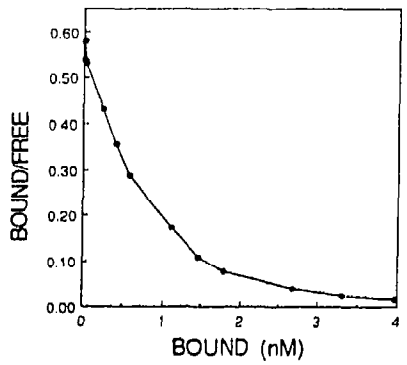

Fig. 1. Rosenthal plot of ^{125}I-[Sar^1,Ile^8]AII binding to *Xenopus* cardiac membranes. The binding data are representative of 3 competition experiments performed in duplicate (see Fig. 2) analyzing using an iterative non-linear regression analysis computer program (see section 2). Membrane protein content was 25 μg/tube.

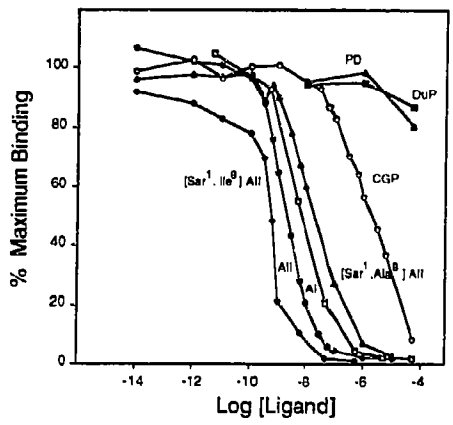

Fig. 2. Competitive inhibition of ^{125}I-[Sar^1,Ile^8]AII binding to *Xenopus* cardiac membranes. The competition curves are representative of 3 experiments performed in duplicate. The unlabeled ligands used were [Sar^1,Ile^8]AII (♦), AII (○), AI (□), [Sar^1,Ala^8]AII (▲), CGP 42112A (○), PD-123177 (△) and DuP 753 (■). Membrane protein content was 25 μg/tube. Total and non-specific binding ranged between 10 to 15 000 cpm and 400–500 cpm, respectively.

DuP 753 [5,7,8]. The rank order of potency of the several AII agonist and antagonist compounds in *Xenopus* cardiac muscle was [Sar^1,Ile^8]AII > AII > AI > [Sar^1,Ala^8]AII >> CGP 42112A; the non-peptide antagonists, DuP 753 and PD-123177, did not compete with ^{125}I-[Sar^1,Ile^8]AII binding at micromolar concentrations (Fig. 2, Table I). Computer-assisted analysis of the binding data demonstrated that none of the peptide antagonists could be fit to a single site model which is consistent with the two classes of AII binding sites revealed by Scatchard analysis (Fig. 1).

4. DISCUSSION

The characterization of *Xenopus* myocardial AII receptors has revealed distinct pharmacological differences between amphibian and mammalian cardiac receptors. While the amphibian ovarian AII receptor is functionally similar to the mammalian AT_1 receptor in terms of Ca^{2+} mobilization, it is almost unaffected by the non-peptide AII receptor antagonist, DuP 753, which selectively binds to mammalian AT_1 receptors [5,7]. In the present binding study, we found that *Xenopus* cardiac AII receptors do not recognize either DuP 753 or PD-123177, an antagonist which specifically binds to the mammalian AT_2 receptor [5,7,8]. Furthermore, CGP-42112A is three orders of magnitude less potent than AII in amphibian cardiac tissue, in contrast to its equipotency at the mammalian AT_2 receptor [6,11]. In *Xenopus* myocardium, AII was twofold more potent than AI, in contrast to mammalian tissue where AII is 2–3 orders of magnitude more potent than AI [13–15]. However, the relatively higher potency of AI in the amphibian heart might reflect its conversion to the octapeptide agonist during incubation for the binding assay.

Table I

Inhibition constants for binding of AII agonists and antagonists to *Xenopus* myocardial membranes

Agent	IC_{50}
[Sar^1,Ile^8]AII	0.91 ± 0.084 nM
Angiotensin II	2.0 ± 0.16 nM
Angiotensin I	5.3 ± 0.57 nM
[Sar^1,Ala^8]AII	19 ± 2.2 nM
CGP-42112A	1.2 ± 0.13 μM
DuP 753	> 100 μM
PD-123177	> 100 μM

IC_{50} is defined as the concentration at which 50% of the maximum binding of ^{125}I [Sar^1,Ile^8]AII is inhibited and is calculated by computer analysis from 3 experiments performed in duplicate.

These results extend our previous observations on the pharmacological difference between functional responses mediated by amphibian AII receptors and mammalian AII receptors expressed in *Xenopus* oocytes [9,10]. Since AII receptor subtypes have not been characterized in the mammalian ovary, we could not rule out the possibility of tissue-specific differences. In this study, we could directly compare our binding data in frog heart with the AII receptor subtypes recently characterized in the rabbit heart [11]. In that report, AT_1 receptors, which recognized DuP 753 with much higher affinity than CGP 42112A and with one order of magnitude less affinity than AII itself, composed about 33% of the total AII receptor population. AT_2 receptors, at which CGP 42112A was equipotent with AII and showed fivefold higher affinity than DuP 753, comprised the remaining 67%. DTT was shown to strongly inhibit the AT_1 receptor and to markedly enhance AII binding to the AT_2 receptor. In contrast, the amphibian heart AII receptor does not behave like either of the mammalian cardiac receptor subtypes. It does not recognize DuP 753, unlike the mammalian AT_1 receptor. Also, it does not behave like the AT_2 receptor since AII exhibits three orders of magnitude higher affinity for amphibian receptor than CGP 42112A, and because DTT dramatically reduces AII binding rather than enhancing it. These results demonstrate that AII receptors in *Xenopus laevis* and mammalian cardiac muscle are pharmacologically distinct in their binding affinities for novel peptide and non-peptide antagonists, consistent with molecular heterogeneity of the AII receptors and evolutionary differences between species.

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