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ACCELERATED COMMUNICATION

Novel Angiotensin II Antagonists Distinguish Amphibian from Mammalian Angiotensin II Receptors Expressed in *Xenopus laevis* Oocytes

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SUMMARY

Angiotensin II (AII) stimulates rapid increases in cytosolic Ca^{2+} concentrations in *Xenopus laevis* oocytes after binding to specific receptors located in the surrounding follicular cells. In follicular oocytes, the peptide AII receptor antagonists saralasin (IC_{50} = 25 nM) and CGP 42112A (IC_{50} = 400 nM) were orders of magnitude more potent than the non-peptide antagonists DuP 753 and PD-123177 (IC_{50} > 10 μM) as inhibitors of AII-induced Ca^{2+} mobilization. The relative potencies of the AII antagonists at the *Xenopus* AII receptor were completely different from their activities at the two known mammalian AII receptor subtypes. These results indicate that the ligand-binding domain of the amphibian AII receptor has a unique conformation that distinguishes with high specificity between peptide and non-peptide AII antagonists. The amphibian AII receptor is pharmacologically distinct from the AT_1 receptor subtype, which mediates phosphoinositide hydrolysis and Ca^{2+} mobilization in mammalian adrenal cells.

The pressor octapeptide AII plays an important role in cardiovascular function and sodium homeostasis and has regulatory actions in several other target tissues. AII receptors are present in mammalian tissues including the vasculature, adrenal, kidney, brain, liver, and gonads (1, 2). Several lines of evidence have suggested the existence of AII receptor subtypes that could account for the diverse actions of AII on its numerous target cells. Thus, two types of receptors have been suggested by radioligand binding studies in AII target tissues (3, 4); also, dithiothreitol enhances binding affinity in some tissues and decreases it in others (5, 6). In addition to these indications of receptor heterogeneity, AII receptors are coupled to multiple mechanisms of signal transduction (4, 7, 8). In many tissues, AII stimulates phosphoinositide turnover and inhibition of adenylate cyclase and promotes the opening of Ca^{2+} channels. Until recently, AII receptors have been characterized by binding studies performed with agonist or antagonist peptides that are based on the amino acid sequence of AII. The recent development of non-peptide AII antagonists has led to the identification of two AII receptor subtypes, which have identical affinity for AII and are present in various proportions in adrenal, uterus, and vascular tissue of both rats and humans (6, 9–11). These have been variously described as type 1 and type 2 (10), or type B and type A (9), respectively. The major AII receptor that is coupled to known cellular responses is defined as type 1 or type B by ligand binding studies. We have used the terminology AT_1 and AT_2 to describe the major and minor subtypes of the mammalian AII receptor.[1]

We are currently employing the *Xenopus laevis* oocyte expression system to analyze the signal transduction processes associated with the AII receptor (12). During studies on the expressed mammalian AII receptor, we observed that follicular *Xenopus* oocytes possess endogenous AII receptors that activate the phospholipase C pathway and promote inositol 1,4,5-trisphosphate-induced Ca^{2+} mobilization (12, 13). In the present study, the ligand recognition properties of the amphibian AII receptor were found to differ markedly from those of the mammalian AII receptor subtypes.

Materials and Methods

Ovaries were removed from albino *X. laevis* frogs (Xenopus I, Ann Arbor, MI), and individual stage V/VI oocytes were isolated in modified

[1] The abbreviations AT_1 and AT_2 have been used for AII 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.

ABBREVIATIONS: AII, angiotensin II; ACh, acetylcholine; saralasin, [Sar^1, Ala^8]angiotensin II; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Barth's solution (82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, 1 mM NaH_2PO_4, pH 7.8). The oocytes were microinjected with the Ca^{2+}-specific photoprotein aequorin (50 ng/oocyte), and AII-induced light emission was determined as previously described (12). Aequorin was obtained from Dr. John R. Blinks (Department of Pharmacology, Mayo Foundation, Rochester, MN). mRNA from rat adrenal cortex was prepared as previously described (14). There is a large degree of variability in endogenous AT receptor activity in oocytes from different frogs (13). In order to examine endogenous AT receptors, we investigated oocytes that possessed at least 20-fold responses to AII (100 nM) on their first day after the ovary was surgically removed. Oocytes possessing less than 2-fold responses to AII (5 μM) were coinjected with mRNA and aequorin, and exogenous (expressed) AT receptor activity was examined on day 3. IC_{50} values, defined as the concentration of antagonist that inhibited control (no antagonist) responses by 50%, were determined from dose-response curves consisting of at least six inhibitor concentrations. AII responses were normalized to control responses in each experiment. Saralasin was purchased from Peninsula Laboratories (Belmont, CA), and AII was obtained from Bachem Inc. (Torrance, CA). The peptide AII antagonist CGP 42112A [nicotinic acid-Tyr-(N^{α}-benzyloxycarbonyl-Arg)Lys-His-Pro-Ile] was provided by Dr. M. de Gasparo (Ciba-Geigy Basel, Switzerland). The non-peptide AII antagonists DuP 753 [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*-imidazol[4,5-c]pyridine-6-carboxylic acid·HCl] were provided by Dr. P. C. Wong (DuPont, Wilmington, DE). mRNA coding for the muscarinic M1 receptor (15) was synthesized by *in vitro* transcription (14) from cDNA, which was provided by Dr. T. I. Bonner (National Institute of Mental Health, National Institutes of Health, Bethesda, MD).

Results and Discussion

Xenopus oocytes possess an endogenous AII receptor that is coupled to the generation of inositol 1,4,5-trisphosphate-induced Ca^{2+} mobilization in AII-stimulated oocytes (13). The resulting increases in cytoplasmic Ca^{2+} can be followed by measuring AII-induced light emission from oocytes injected with the Ca^{2+}-specific photoprotein aequorin (12). Two peptide AII antagonists, saralasin and CGP 42112A, and two non-peptide antagonists, DuP 753 and PD-123177, were examined for their abilities to inhibit AII-induced light emission in aequorin-injected oocytes. The dose-response curves in Fig. 1 show that the rank order of inhibitory potency was saralasin > CGP 42112A ≫ DuP 753 = PD-123177. The non-peptide antagonist DuP 753 was much less potent than predicted by its actions on type 1 (AT_1) mammalian AII receptors (6, 9–11) and was of similarly low activity as PD-123177. Neither of these antagonists inhibited the amphibian receptor at less than micromolar concentrations (Table 1). In contrast, receptor binding studies have shown that mammalian AT_1 receptors in rat uterus, adrenal, liver, and smooth muscle are 3 orders of magnitude more sensitive to DuP 753 (IC_{50} = 20–60 nM) than to PD-123177 (IC_{50} ≥ 100 μM). Also, AT_2 receptors, which are abundant in the rat and human uterus, rat adrenal medulla, and the pheochromocytoma PC12W cell line, are 3 orders of magnitude more sensitive to PD-123177 (IC_{50} = 3 nM) than to DuP 753 (IC_{50} ≥ 100 μM). Neither of the non-peptide antagonists exhibited agonist activity at the amphibian receptor at concentrations up to 10 μM.

We have previously reported that the peptide antagonist saralasin inhibits amphibian AII receptor-mediated Ca^{2+} responses in oocytes (13). The amino acid sequence of CGP 42112A is related to that of angiotensin, and in mammals this peptide is about 3 orders of magnitude more selective for AT_2 than AT_1 receptors (9). In *Xenopus* oocytes, CGP 42112A was a less potent inhibitor of the amphibian response (IC_{50} = 400 nM) than was saralasin (IC_{50} = 25 nM), consistent with their rank order of potency at AT_1 receptors (9). Both of the peptide antagonists exhibited weak partial agonist activity at high concentrations; thus, 10 μM saralasin and CGP 42112A elicited 3 ± 0.5- and 2 ± 0.2-fold light responses, respectively, compared with 20–40-fold light responses evoked by 500 nM AII. (Light emission is expressed as peak/basal ± standard error of four to six observations, throughout this report.) The peptide antagonist CGP 42112A (2 μM) inhibited the AII-induced response in a time-dependent manner ($t_{1/2}$ = 11 min), with complete blockade by 30 min, which was in contrast to the rapid blockade ($t_{1/2}$ = 0.7 min) induced by saralasin (Fig. 2A). The lower affinity of CGP 42112A might reflect a slower association or faster dissociation rate constant for binding to the amphibian AII receptor. The inhibition by CGP 42112A was partially reversible; after washing for 1 hr, CGP 42112A-treated oocytes recovered about 70% of their initial responsiveness to AII (Fig.

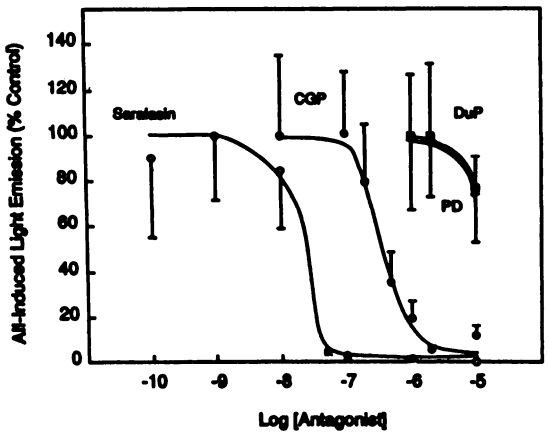

Fig. 1. Relative potencies of AII antagonists for amphibian AII receptor-mediated light responses. Oocytes were incubated with increasing concentrations of AII antagonists [saralasin, (○), CGP 42112A, (●), PD-123177, (△), and DuP 753, (■)] for at least 30 min before AII-induced light emission responses were measured. Each *point* represents the mean ± standard error of data from 5–10 oocytes. Data are expressed as percentages of the control levels, which were 5–10 × 10^4 photons/sec.

TABLE 1

Selective actions of AII antagonists on amphibian and expressed mammalian AII receptor activity in the oocyte

Oocytes (5–10/dose) were incubated with increasing concentrations of AII antagonists saralasin, (CGP 42112A, DuP 753, and PD-123177) for at least 30 min before AII-induced light responses were measured, as described for Fig. 1. Amphibian AII receptor responses were measured in freshly isolated oocytes 1–24 hr after injection of aequorin; mammalian AII receptor responses were measured 2–3 days after coinjection of aequorin and rat adrenal mRNA (13). IC_{50} is defined as the concentration at which 50% of the maximal responses is inhibited (see Materials and Methods).

Antagonist	Inhibitory potencies of AII antagonists (IC_{50})	
	Amphibian	Mammalian
Peptide		
Saralasin	25 nM	<50 nM
CGP 42112A	400 nM	>10 μM
Non-peptide		
DuP 753	>10 μM	400 pM
PD-123177	>10 μM	>10 μM

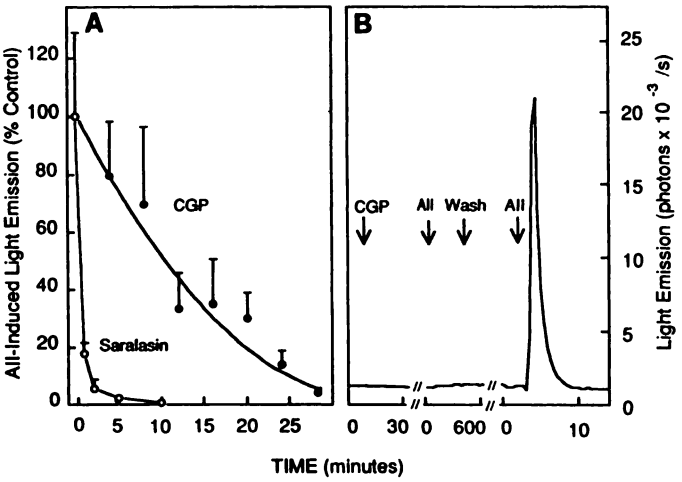

Fig. 2. A, Time course of CGP 42112A and saralasin blockade of AII receptor activity. CGP 42112A (5 μM) (●) and saralasin (500 nM) (○) were preincubated with aequorin-injected oocytes for increasing times before AII-induced light emission responses were measured. Each *point* represents the mean ± standard error of data from three or four oocytes. B, Reversibility of CGP 42112A blockade of AII receptor activity. The time course of light emission is representative of five responses in aequorin-injected oocytes that were treated individually with CGP 42112A (2 μM) followed by 500 nM AII, washed for 10 hr, and exposed to 500 nM AII.

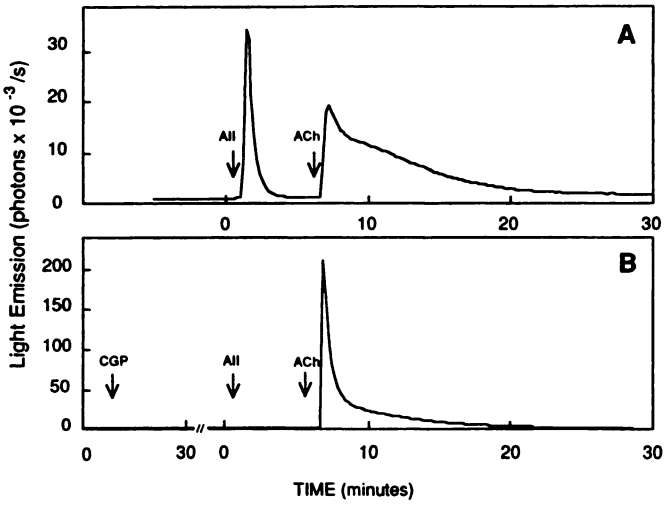

Fig. 3. Specificity of CGP 42112A inhibition of AII receptor activity. The time course of light emission is representative of responses in five oocytes that were coinjected 1 day previously with M1 mRNA (50 pg/oocyte) and aequorin. These oocytes were treated individually without (A) or with (B) CGP 42112A (2 μM for 30 min) followed by 500 nM AII and 500 nM ACh.

2B). This is consistent with the partial recovery observed with saralasin at the amphibian receptor (13). The lack of complete recovery and the partial agonist activity of CGP 42112A suggest that a degree of receptor internalization could be occurring, as observed with saralasin in cultured bovine adrenocortical cells (16). Depletion of surface AII receptors by rapid receptor internalization would be one explanation for the lack of AII responses immediately after antagonist wash-out.

The inhibitory action of CGP 42112A on agonist-induced light responses was specific for the AII receptor. In oocytes expressing M1 muscarinic receptors after coinjection of aequorin and M1 receptor mRNA (synthesized from muscarinic M1 cDNA), treatment with CGP 42112A (2 μM, 30 min) had no effect on light responses to 500 nM ACh (control, 105 ± 15; CGP 42112A, 129 ± 18) but completely blocked light responses induced by 500 nM AII (Fig. 3). During these studies, we observed that ACh-stimulated responses of M1-injected oocytes (88 ± 21) were reduced by 60% after AII pretreatment (36 ± 5). This was not attributable to depletion of aequorin, because the Ca^{2+} ionophore ionomycin elicited similar levels of light emission from control oocytes (503 ± 72) and oocytes exposed to AII and ACh (615 ± 89). The reverse was also true; in M1-injected oocytes exposed first to 500 nM ACh, the responses to 500 nM AII were reduced by 87%. These observations are consistent with the occurrence of receptor-mediated heterologous desensitization in oocytes expressing ACh, neurotensin, and serotonin receptors after injection of rat brain mRNA (17, 18). Such an effect could be due to desensitization at the receptor-effector level but is more likely to reflect depletion of a common agonist-sensitive calcium pool within the endoplasmic reticulum of the oocyte, because the AII responses were reduced by only 15% after ACh treatment if atropine (5 μM) was added before AII stimulation (data not shown). It is interesting that, whereas the amphibian receptor was inhibited by both peptide antagonists, it was relatively insensitive to the non-peptide antagonists. When the cDNAs are available, it will be important from both developmental and structure-function aspects to compare sequences between the amphibian and mammalian AT receptors, as well as with receptors from other species along the phylogenetic tree.

The *Xenopus* oocyte is a frequently used model system for the expression of exogenous mRNAs for a wide variety of mammalian proteins (19). Several receptors, including those for substance K (20), serotonin (21), and thyrotropin-releasing hormone (22), have been cloned by taking advantage of this expression system. Injection of *Xenopus* oocytes with mRNA from several tissues, including rat adrenal cortex (12), brain (23), and liver (24) and bovine adrenal (25), results in expression of functional AT receptors. However, the presence of endogenous AII receptors complicates the use of the oocyte system for the cloning of AII receptors from other species and could lead to the cloning of a protein that up-regulates amphibian receptor expression or amplifies endogenous receptor activity. Thus, the ability to isolate expressed AII receptor activity versus endogenous AII receptor activity with AII antagonists should facilitate cloning strategies and structure-function studies on the mammalian AII receptor.

The AII receptors present in rat adrenal cortex are predominantly of the AT_1 subtype and mediate the stimulation of Ca^{2+} mobilization and aldosterone biosynthesis induced by AII in that tissue (9, 10). Indeed, all of the AII receptor/signal transduction pathways and physiological functions so far examined have been attributed to AT_1 receptors (26). We prepared rat adrenal cortex mRNA and coinjected it with aequorin into control oocytes that did not possess high endogenous AII receptor activity (light responses less than 2 ± 0.3). As shown previously (12), these oocytes expressed mammalian AT receptors, which were linked to Ca^{2+} mobilization, 2–3 days after mRNA injection (50–100-fold responses). The non-peptide AT_1 receptor antagonist DuP 753 inhibited the expressed AII receptor activity, with complete blockade at 100 nM, a concentration that had no effect on amphibian AII receptors (Table I). PD-123177, the non-peptide AT_2 receptor antagonist, minimally inhibited the expressed AII receptor response at micromolar concentrations (Table I). These results indicate that, as ex-

pected, the expressed mammalian receptors linked to Ca^{2+} mobilization were of the AT_1 variety. Consistent with this, CGP 42112A only marginally inhibited the expressed AT_1 receptor at concentrations (1 μM) shown to block the endogenous amphibian response by 95% (Table I). These findings clearly demonstrate the ability of these novel antagonists to differentiate expressed from endogenous AII receptors in the oocyte.

These results have shown that the amphibian AII receptor is 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 (13). The amphibian AII receptor is functionally similar to the mammalian AT_1 receptor but is almost devoid of recognition for the non-peptide AII antagonists. These distinctions could reflect structural differences arising at the level of the gene or during post-transcriptional modification or post-translational processing of the protein. Many of the known membrane receptors exhibit molecular heterogeneity and evolutionary differences between species. The very low affinities of the non-peptide antagonists for the amphibian AII receptor may reflect their pharmacological development by progressive chemical modifications to enhance the potency of the original imidazole inhibitor, based on their abilities to antagonize AII action at the rat vascular smooth muscle receptor (27). It is clear that the *Xenopus* oocyte offers a valuable system in which to determine the nature of the function and signal transduction pathway(s) of the AII receptor subtypes. In addition, the ability to selectively block amphibian receptors without inhibiting expressed AII receptors could facilitate the use of the oocyte expression system in AII receptor cloning strategies and, ultimately, in structure-function studies on the cloned receptor subtypes.

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