Vol. 165, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1989 Pages 196-203

IDENTIFICATION OF ANGIOTENSIN II RECEPTOR SUBTYPES

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Received October 9, 1989

SUMMARY. We have demonstrated the existence of two distinct subtypes of the angiotensin II receptor in the rat adrenal gland using radioligand binding and tissue section autoradiography. The identification of the subtypes was made possible by the discovery of two structurally dissimilar, nonpeptide compounds, DuP 753 and EXP655, that show reciprocal selectivity for the two subtypes. In the rat adrenal cortex, DuP 753 inhibited 80% of the total AII binding with an IC_{50} value on the sensitive sites of 2×10^{-8} M, while EXP655 displaced only 20%. In the rat adrenal medulla, EXP655 gave 90% inhibition of AII binding with an IC_{50} value of 3.0×10^{-8} M, while DuP 753 was essentially inactive. The combination of the two compounds completely inhibited AII binding in both tissues.

Angiotensin II (AII), the primary biologically active hormone of the renin-angiotensin system, elicits a variety of physiological effects, including intense arteriolar vasoconstriction, aldosterone biosynthesis and secretion, catecholamine release, stimulation of drinking behavior, glycogenolysis and alteration of renal function (1,2). It exerts these effects through the activation of specific AII receptors on the vasculature, adrenal cortex, adrenal medulla, brain, liver and kidney, respectively. AII receptors from these target organs display slightly different preferences for AII and AIII in terms of binding affinity, agonist potency, and susceptibility to blockade by the octa- and heptapeptide receptor antagonists (3,4), consistent with the existence of subtypes. Multiple mechanisms of signal transduction have also been noted for AII. AII stimulates phosphoinositide turnover and internal release of Ca^{++} (5), activates the opening of dihydropyridine-sensitive Ca^{++} channels (6) and inhibits adenylate cyclase activity (7). Nevertheless, it is still unclear whether a single type of AII receptor is coupled to multiple pathways or multiple receptor subtypes are interacting independently with different signal transducers (8).

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0006-291X/89 $1.50

During the development and evaluation of nonpeptide AII receptor antagonists, we have noted that the AII receptors found in the rat adrenal cortex are quite similar to those found in the vascular system. In both tissues, the binding affinities of two series of AII receptor antagonists, i.e. biphenyl- and carboxybenzamido-imidazoles, correlate significantly with their potencies for the inhibition of AII-induced responses such as Ca^{++} mobilization in rat smooth muscle cells, vasoconstriction in isolated rabbit aorta, and an antihypertensive effect in renal artery-ligated hypertensive rats (9,10). However, it was consistently observed that 20-25% of the specific AII binding found in rat adrenal cortical microsomes was resistant to displacement by our nonpeptide antagonist analogs(9,11). In contrast, the specific binding sites in the rat aortic smooth muscle cells were completely displaceable. In this communication, we will describe the unambiguous demonstration of separate subtypes of AII binding sites in the rat adrenal gland and their selective inhibition by two nonpeptide analogs.

METHODS AND MATERIALS

Binding Assay - Microsomes were prepared according to procedures of Chiu et al. (9). Binding assays were performed by incubating aliquots of a freshly prepared particulate fraction with 0.05 nM [^{125}I]AII and varying concentrations of inhibitor in a final volume of 0.5 ml of assay buffer. After 60 min of incubation at 25°C, the reaction was terminated by addition of cold assay buffer. The bound and free radioactivity were rapidly separated through glass-fibre filters, and the trapped radioactivity was determined by use of a gamma counter.

Autoradiography - Fresh tissue was frozen on powdered dry ice and stored at -70°C until used. Sections were cut at 15 microns and thaw mounted onto gelatin subbed slides. The AII binding was performed according to the procedures of Gehlert et al.(12), except that the dithiothreitol was omitted from the buffer and the incubation with iodinated AII was 60 min. Slides were placed under X-ray film (Kodak XAR-5) and exposed for 1 1/2 days before processing the film to produce the film autoradiograms (Panels A - E). The slides were then fixed with paraformaldehyde vapor at 80°C for two hours, dried under air, delipidated and dipped in emulsion (Kodak NTB-2). The coated slides were exposed for 4-5 weeks, developed with Kodak D-19 and counter-stained with hemotoxylin-eosin to view the histology.

RESULTS AND DISCUSSION

Adrenal cortical microsomes are a rich source of AII receptors which have been extensively characterized, especially in terms of the binding affinities of a variety of angiotensin peptides (13,14) and nonpeptide AII receptor antagonists (9,10). To differentiate the subtypes of AII receptors, four compounds were used (see Figure 1). Saralasin, a peptide analog of AII, is a potent and specific AII antagonist which blocks all known AII receptors. As shown in figure 2A, saralasin inhibited the specific binding of [^{125}I]AII to rat adrenal cortical microsomes in a concentration-dependent, monophasic fashion yielding an IC_{50} value of 1.0×10^{-9} M. Complete displacement was achieved at a concentration of 1×10^{-7} M. S-8308, the first nonpeptide AII antagonist reported by Furukawa and coworkers (15), was previously shown to be

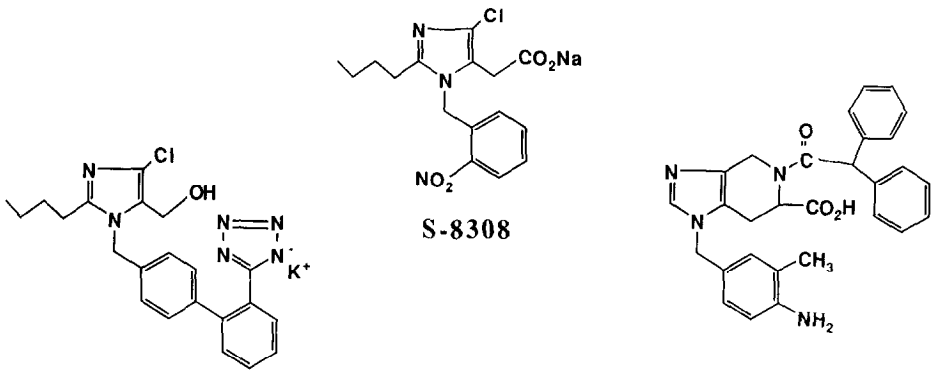

Fig. 1. Structures of selected nonpeptide AII receptor antagonists. Extensive pharmacology has been previously described for S-8308 (15,16) DuP 753 (10) and EXP655 (17).

a weak but specific AII antagonist exerting its action at the receptor level (16). It was found to inhibit the specific binding in a monophasic manner, yielding an IC_{50} value of 1.5 X 10^{-5} M, with complete displacement at 1 X 10^{-4} M (data not shown, see 16). In contrast, DuP 753, a potent orally active analog derived from modification of S-8308 (10) exhibited a biphasic displacement as shown in figure 2A. It inhibited the specific binding of [^{125}I]AII in a concentration-dependent manner from 10^{-9} M to 10^{-7} M . A plateau of constant binding (about 28% of the total) was followed over a two-log concentration increase of DuP 753 beyond which another concentration-dependent displacement was observed. Inhibitory constants (IC_{50}'s) approximated for these two sites were 1.7 X 10^{-8} M and 1 X 10^{-4} M. Based on this observation, a distinction can be made, between the two binding sites described here as either DuP 753-sensitive or DuP 753-insensitive sites. An intriguing observation was the relative inactivity of EXP655, a structual analog of S-8308 (compound #13 of ref. 17), which inhibited only 20% of the total specific binding at 3 x 10^{-5} M. This is particularly puzzling because EXP655 was claimed to be an antihypertensive agent possessing high affinity for AII receptors (17). On the contrary, we found that this compound was inactive in antagonizing AII-induced rabbit aortic contractions at concentrations up to 10^{-5} M and in lowering blood pressure in renal artery-ligated hypertensive rats at doses up to 30 mg/kg, i.v. (data not shown).

The coincidence between the amount of AII binding resistant to displacement by DuP 753 and that displaceable by EXP655 led us to further investigate whether the DuP 753-insensitive sites were sensitive to EXP655. To test this hypothesis, the ligand-binding profile of the DuP 753-insensitive site was examined in the presence of a saturating concentration (10^{-5} M) of DuP 753. Figure 2B illustrates that the residual [^{125}I]AII binding (expressed as 100%) was inhibited by saralasin, and by EXP655, in a concentration-dependent, monophasic manner with IC_{50} values of 1.8 x 10^{-9} M and 1 X 10 $^{-7}$ M, respectively. Not shown in the figure was S-8308 which gave the same result as

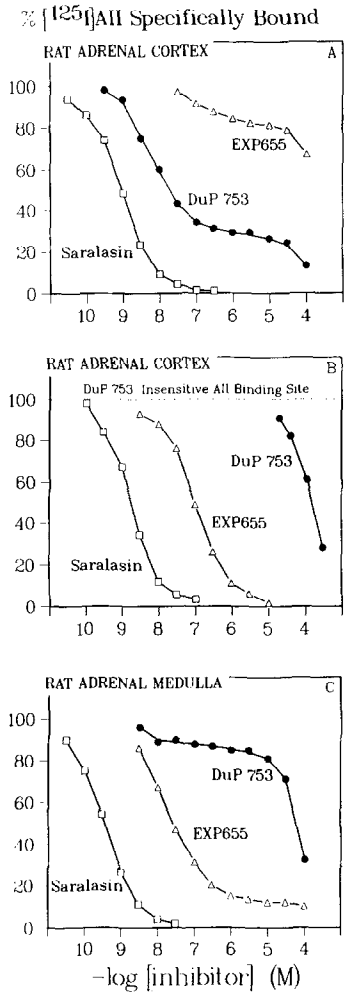

Fig. 2. Inhibition of the specific binding of [^{125}I]AII to rat adrenal cortical and medullary microsomes by saralasin, DuP 753 and EXP655. A) adrenal cortical microsomes containing both the DuP 753 sensitive and insensitive sites, B) adrenal cortical microsomes in the presence of 10^{-5} M DuP 753; in this condition only the DuP 753-insensitive site was measured, and C) adrenal medullary microsomes containing predominately the DuP 753-insensitive site.

that previously noted (IC_{50} = 1 x 10^{-5} M). As expected, DuP 753 inhibited the residual binding only at high concentrations, with an IC_{50} value of 1 X 10^{-4} M.

Autoradiographic examination of [^{125}I]AII binding to the rat adrenal gland was undertaken to explore the localization of AII receptors and possible anatomical differentiation of subtypes. As shown in Figure 3A, [^{125}I]AII densely labeled the outer layers of the adrenal cortex as well as the entire adrenal medulla, as reported by Catt et al. (18). Most of the cortical labeling appeared to be over the zona glomerulosa with moderate labeling of the zona fasciculata. Unlabelled AII potently inhibited the labeling in both cortex and medulla (Figure 3B). In the presence of 10^{-5} M DuP 753, the labeling over the cortex was significantly reduced and the resistant sites were found to be distributed uniformly around the outer layer of the cortex (Figure 3C). The

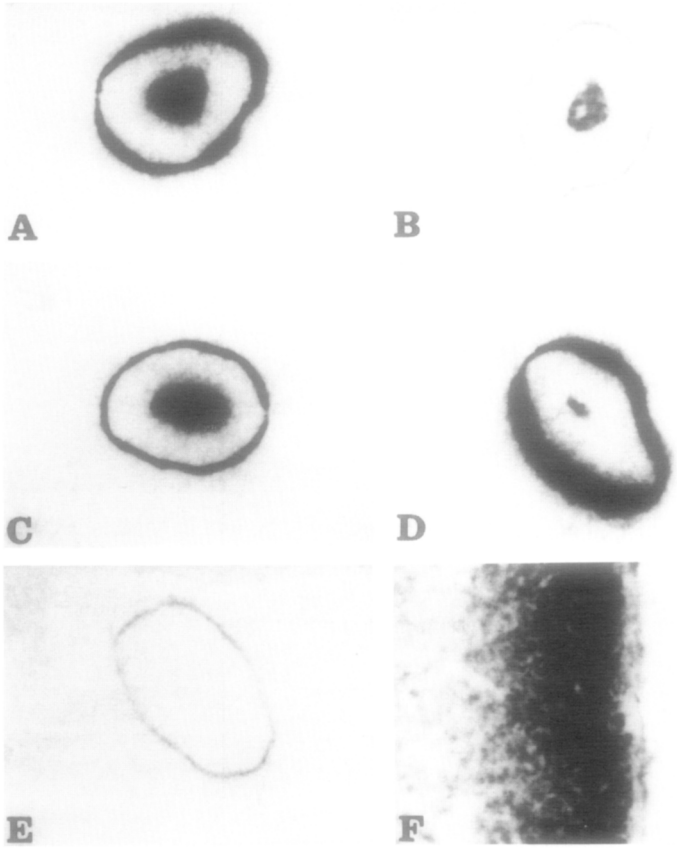

Fig. 3. Autoradiograms of ^{125}I-AII binding to sections of rat adrenal gland. Additions to film autoradiograms: A. None (Total binding) B. 10^{-7} M unlabeled AII to indicate equipotent reduction of cortical and medullary labeling (3 X 10^{-7} M blocks all binding, similar to 3E). C. 10^{-5} M DuP 753 (10^{-4} M gives identical results). D. 10^{-6}M EXP655. E. 10^{-5} M DuP 753 + 10^{-6} M EXP655 F. Emulsion autoradiogram of a section incubated with 10^{-5} M DuP 753 to illustrate localization of resistant binding sites over the zona glomerulosa.

labeling of the adrenal medulla, however, was not appreciably affected by DuP 753. By contrast, 10^{-6} M EXP655 had no apparent effect on cortical labeling, but almost totally eliminated the labeling of the medulla (Figure 3D). When both compounds were applied in combination, the AII labeling of both regions was completely abolished (Figure 3E).

It was apparent from the autoradiogram of the rat adrenal gland that DuP 753-resistant sites are present in the cortical zona glomerulosa and predominate in the medulla. To characterize these AII receptors further, [^{125}I]AII specific binding to rat adrenal medullary microsomes was studied (Figure 2C). Saralasin inhibited the binding in a concentration-dependent fashion with an IC_{50} value of 4 X 10^{-10} M. About 90% of the total binding was resistant to displacement by DuP 753 which is consistent with the results obtained by autoradiographic techniques. In contrast, the inhibition by EXP655 was concentration-dependent and nearly monophasic, yielding an IC_{50} value of 3

X 10^{-8} M. Interestingly, 10% of the binding was resistant to EXP655, which complements the 10% inhibition seen with DuP 753 below 1 x 10^{-5} M.

The present studies using radioligand binding and autoradiographic techniques clearly demonstrate the existence of two subtypes of AII receptors in the rat adrenal gland. This unequivocal distinction was made possible by the strong, reciprocal specificity displayed by the two seemingly related nonpeptide antagonists. The adrenal cortex, particularly the zona glomerulosa, contains predominately the DuP 753-sensitive receptor whereas the medulla harbors almost exclusively the DuP 753-insensitive, EXP655-sensitive type of AII receptor. It is important to note that while both compounds were derived from a common nucleus, as shown by S-8308, DuP 753 is an orally active antihypertensive agent and EXP655 shows no effects on blood pressure.

The function of the two AII subtypes is not completely clear, but the predominant, DuP 753-sensitive site in the adrenal cortex may mediate the stimulation of aldosterone biosynthesis and Ca^{++} mobilization induced by AII in that tissue. AII stimulates the release of catecholamines from adrenal medulla by acting on AII receptors which are perceived as a single class of high affinity binding sites (19), but appear to be distinct from AII receptors found elsewhere (20,21,22) and may represent the EXP655-sensitive sites. It is apparent that these selective antagonists will permit more precise studies of the functional consequence of AII receptor activation in the adrenal gland and other tissues, without the confusion of total AII blockade.

We have previously decribed the detection of a single population of high affinity binding sites for [^{3}H]AII in the adrenal cortex, with an apparent dissociation constant (Kd) of 1.2 X 10^{-9} M and a maximal binding capacity of 2.6 pmol/mg of protein (9). While these results are in good agreement with those reported by other investigators (23,24,25), our present results indicate that there are two populations of binding sites, both of which show similar affinity for AII. Previously, Devynck and coworkers (26) detected the presence of two populations of specific receptors using [^{3}H]AIII as the radioligand, and suggested that the one with a higher affinity but with much smaller capacity was specific for AIII while the other with a lower affinity but with greater capacity was the AII receptor, distinct from that of AIII. Unfortunately, these interesting observations were not confirmed by other investigators (27,28). Additional evidence indicates that AIII is equipotent with AII in stimulating the synthesis and secretion of aldosterone both *in vitro* (29) and *in vivo* (30). Saralasin appears to be more selective in blocking the effect of AII (29) whereas Ile^7-AIII is more effective against AIII (31). It is conceivable that many of these uncertainties might be resolved by the use of these receptor subtype-specific blockers.

On the basis of our present findings, we can conclude that AII binds to two distinct populations of AII receptors with similar avidity and potency. These two receptor subtypes are not readily distinguishable by profiling with AII peptide homologs and analogs, but are unquestionably identifiable by the use of either of the nonpeptide antagonists, DuP 753 or EXP655. Analogies can

be drawn from other receptor systems such as the alpha adrenergic receptors, which can be divided into $alpha_1$ and $alpha_2$ by their susceptibility to blockade by either prazosin or yohimbine, respectively (32,33), and the histaminergic receptors which can be distinguished as H_1 or H_2 by the use of diphenhydramine or cimetadine, respectively (34). In keeping with the international system of receptor classification, we propose that the AII receptors sensitive to DuP 753 be designated as AII-1 sites and those sensitive to EXP655 be known as AII-2 sites.[1] Having established this subclassification, DuP 753 and EXP655 will become the prototypic and subtype-specific tools for unraveling the function(s) of different AII receptors in health and diseases.

ACKNOWLEDGMENTS. We thank Tam Nguyen, William Roscoe, and Sheila Diamond for their excellent technical assistance and Anna Wayland for the preparation of this manuscript.

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