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IDENTIFICATION AND CHARACTERIZATION OF ANGIOTENSIN II RECEPTOR SUBTYPES IN RABBIT VENTRICULAR MYOCARDIUM

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CGP 42 112 A and DuP 753 block [^{125}I]-angiotensin II binding in rabbit ventricular myocardial membranes in a clearly biphasic manner, indicating the existence of high- and low-affinity sites for these highly selective agents. Assays using concentrations of either agent large enough to prevent high-affinity binding show that their respective high-affinity sites are distinct, and each corresponds to the low-affinity site of the other. The two receptor subsets, present in nearly equal proportions, are also distinguishable by their different sensitivities to dithiothreitol. These findings afford strong evidence for the existence of two distinct angiotensin II receptors in rabbit myocardium, corresponding to the A and B subtypes recently described in adrenals. © 1990 Academic Press, Inc.

Angiotensin II (AII) is a vasoconstrictor peptide that exerts a variety of effects in many nonvascular tissues, including the heart (1,2). In several species it elicits positively inotropic and chronotropic effects on heart muscle; these may involve both direct actions on myocardial cells and indirect components resulting from augmented release of catecholamines (2,3). Moreover, AII appears to play an important role in cardiac hypertrophy (4,5) and recent evidence suggests that it impairs myocardial relaxation by directly influencing the mechanical properties of cardiomyocytes (6).

Several findings suggest that AII receptors are heterogeneous. For instance, in physiological studies with various agonists and antagonists, differences in potency from tissue to tissue have been observed (1,7,8). There is also evidence indicating the existence of multiple second-messenger systems for AII (9-11). Binding sites have been detected in various tissues, including vascular and nonvascular smooth muscle, adrenal cortex, kidney, liver and brain (12), and recently two AII receptor subtypes have been convincingly characterized using highly selective agents (13). Receptors for AII have been identified in cardiac tissue of several species, including man (14-18), but despite the diversity of the cardiac effects of AII, and the potential thera-

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Abbreviations used: AII, angiotensin II; BSA, bovine serum albumin; DTT, dithiothreitol; B_{max}, maximum binding capacity; K_d, dissociation constant.

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peutic value of agents that might interfere specifically with subsets of cardiac receptors, no attempt has been made to differentiate receptor subtypes in this organ. This study, carried out to investigate the possible existence of AII receptor subtypes in the myocardium, clearly demonstrates the presence of two distinct subtypes of high-affinity AII receptors in rabbit cardiac ventricles.

MATERIALS AND METHODS

Materials. [^{125}I]AII (2200 Ci/mmol) was purchased from Anawa (Wangen, Switzerland). The nonpeptidic antagonist DuP 753 (19), formerly known as EX 89 (13), and the peptidic ligand CGP 42 112 A with the formula nicotinic acid-Tyr-(N^{α}-benzyloxycarbonyl-Arg)-Lys-His-Pro-Ile-OH were synthesized by CIBA-GEIGY. Unlabeled AII (human sequence) was obtained from Bachem (Bubendorf, Switzerland). Bovine serum albumin (BSA) was from Sigma.

Membrane preparation. Ventricular membranes from male Chinchilla rabbits weighing 2-3 kg were prepared using a slight modification of a published method (15). The animals were killed by a blow on the head, the hearts removed immediately, and the ventricles dissected free from the atria and fat, cut in quarters, rinsed in ice cold saline, frozen in liquid nitrogen and stored at -80°C. The ventricles were thawed on ice and minced with scissors into pieces about 2-4 mm in size. A homogenate (20 % w/v) was prepared in 0.25 M sucrose, 25 mM Tris pH 7.5 with a Polytron (2 x 30 sec) at setting 6. The homogenate was sedimented at 10 000 g for 20 min, the pellet discarded and the supernatant centrifuged at 47 800 g for 30 min. The pellet was resuspended in 5 ml of 0.6 M KCl and 30 mM histidine at pH 7.0 (per g of tissue) and resedimented at 47 800 g for 30 min. The pellets obtained from the final centrifugations were washed three times and resuspended in 25 mM Tris pH 7.5 and 10 mM $MgCl_2$ using a tight-fitting teflon-pestle homogenizer. All steps above were carried out at 0-4°C. The yield of the crude membrane vesicles was about 1.5 mg of protein/g of ventricles. The membrane preparations were frozen in liquid nitrogen and held in aliquots at -80°C until used. There was no apparent loss of AII binding activity with time. Protein was assayed by the method of Bradford (20) using BSA as standard.

Binding assay. Equilibrium binding studies were performed at 25°C for 50 min in Tris pH 7.5 containing $MgCl_2$ 10 mM and BSA 0.2% using [^{125}I]-AII as radioligand and the peptidase inhibitors antipain, phosphoramidon, leupeptin, pepstatin, bestatin, amastatin, each at 1 µg/ml (all obtained from Novabiochem, Läufelfingen, Switzerland) and bacitracin 100 µg/ml. Control experiments showed that under these conditions equilibrium was reached and the binding was linear to the amount of protein used. Bound and free ligand were separated by addition of 4 ml of ice-cold Tris pH 7.5 and subsequent rapid filtration through Whatman GF/F filters presoaked in 0.1% BSA. This was followed by three additional washes with 4 ml of the same buffer and radioactivity trapped on the filter was then measured in a γ-counter (Pharmacia LKB, Uppsala, Sweden) at 80% efficiency. Nonspecific binding was determined in the presence of 10 µM unlabeled AII. The integrity of the radioligand before and after incubation was determined by chromatography on reversed-phase plates (RP-18W/UV_{254}, Machery-Nagel, Germany) using the solvent system NaCl 3%/acetonitrile (60:40, v/v). The plates were read on a Berthold radioactivity scanner (Regensdorf, Switzerland). Controls showed that under the incubation conditions used 80-85% of the radioligand remained intact.

Data analysis. The binding data were analyzed as reported previously (13), using the LIGAND iterative curve-fitting program (21) and the four-parameter logistic dose-response method of De Lean et al. (22). The data were obtained from experiments each performed in duplicate, and the geometric means are given when dose-response curves were averaged.

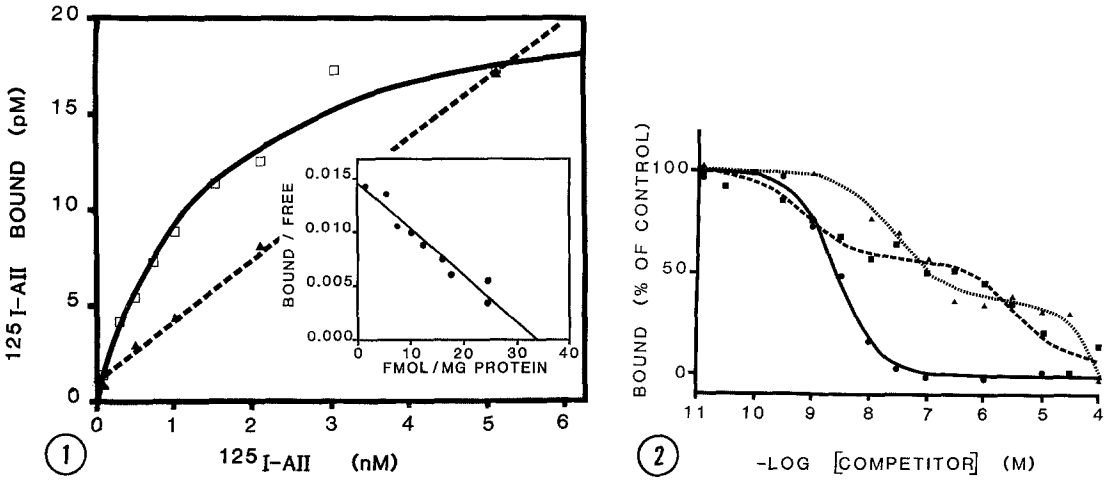

Fig. 1. Saturation binding of [^{125}I]AII to rabbit ventricular membranes. Specific (□) and nonspecific binding (▲) as a function of increasing concentrations of [^{125}I]AII is shown. A Scatchard analysis of the data is given in the inset. The values given are representative of three experiments.

Fig. 2. Competition curves with AII (●), CGP 42 112 A (■) and DuP 753 (▲). The experiments were carried out in the presence of 0.5 nM [^{125}I]AII.

RESULTS AND DISCUSSION

Saturation binding of [^{125}I]AII to rabbit ventricular membranes is depicted in Fig. 1. The data demonstrate the presence of specific sites with high affinity for AII. Analysis of the data as shown in the Scatchard plot suggests the presence of a single class of receptors with a K_d of 1.6 nM and a B_{max} of 33 fmol/mg protein. These results were corroborated in competition binding experiments (n = 5) using AII as ligand (Fig. 2). Monophasic displacement of [^{125}I]AII with a K_d of 2.5 nM (range 1.2-3.5 nM; n = 5) and a B_{max} of 56 fmol/mg protein (range 33-85 fmol/mg) with a Hill slope of 1.0 (range 0.8-1.1) was obtained, also suggesting the existence of a single class of high-affinity sites. These results are in agreement with the findings of Mukherjee et al. (14) and Baker et al. (15), indicating the occurrence of a single class of AII binding sites in bovine and rabbit heart membranes having affinities and binding capacities similar to those found in this study. Baker et al. (15) show a close correlation between binding to high-affinity receptors and the inotropic effects of AII and several hormone analogs on the myocardium indicating the physiological relevance of the occupancy of these sites for some of their cardiac actions.

However, the results suggesting an interaction with a single population of high-affinity receptors do not exclude the existence of more than one class of sites to which AII binds with very similar affinities.

Figure 2 shows that two selective compounds (13) CGP 42 112 A and DuP 753 displace [^{125}I]AII in a biphasic manner, indicating the presence of two sub-

types of AII receptor in the myocardium. CGP 42 112 A has a high affinity (K_d = 0.5 nM, range 0.4-0.7 nM; n = 3) for one site and occupies the second only at concentrations several orders of magnitude higher (K_d = 3 µM, range 0.8-10 µM). DuP 753 likewise binds with high affinity (K_d = 31 nM, range 22-60 nM; n = 4) to one population of AII receptors and has a very weak affinity (K_d = 33 µM, range 30-40 µM) for a second population.

These binding data are in good accord with the findings of Whitebread et al., who characterized two subtypes of AII receptors in rat and human adrenals (13). They first proposed the following nomenclature for the previously unrecognized AII receptor subtypes: type A for those showing a high affinity for the peptidic agent CGP 42 112 A and type B for those with high affinity for DuP 753.

To characterize the individual sites of the biphasic displacement curves observed in cardiac membranes, competition binding experiments were carried out using AII, CGP 42 112 A and DuP 753 in the presence of the selective agents in concentrations blocking their respective high-affinity sites (Fig. 3). In the presence of DuP 753 (3 µM), i.e. with the putative type A receptors left unblocked, the remaining binding sites have a high affinity for the peptidic agent CGP 42 112 A (K_d = 0.7 nM), but a negligible affinity for DuP 753 (K_i = 40 µM), as shown in Fig. 3A. In the experiments carried out in the presence of 0.3 µM of CGP 42 112 A (Fig. 3B), i.e. with the putative type B receptors unmasked, DuP 753 has an affinity more than 1000 times higher (K_d = 25 nM) than for subtype A (Fig. 3A) and a low affinity for CGP 42 112 A (K_d = 4 µM). These data are in good agreement with the affinities of the high- and low-affinity sites derived from the biphasic displacement curves shown in Fig. 2, and hence fully explain the complex binding characteristics observed in these experiments. The two receptor populations have an almost identical high affinity for AII (1.5 nM and 1.2 nM in the presence of CGP 42 112 A and DuP 753 respectively; see Figs. 3A and 3B), which accounts for the failure to resolve more than one site in binding studies on myocardial membranes using the natural hormone. However, by using the highly selective agents, the presence of at least two types of high affinity AII receptors with inverse affinities for two chemically unrelated compounds can clearly be demonstrated.

From inhibition experiments performed with these highly selective agents the proportions of the receptor subtypes can be estimated (Fig. 4). Using DuP 753, 33% of the AII binding sites were not blocked and can therefore be classified as type A receptors, whereas 67% were sensitive to this compound and can be assigned to the B subtype. In the inverse experiments using CGP 42 112 A as competitor, 41% of the sites were found to be type A and 59% type B. These independent data demonstrate the presence of nearly equal proportions of these AII receptors subtypes in membranes of rabbit heart ventricles.

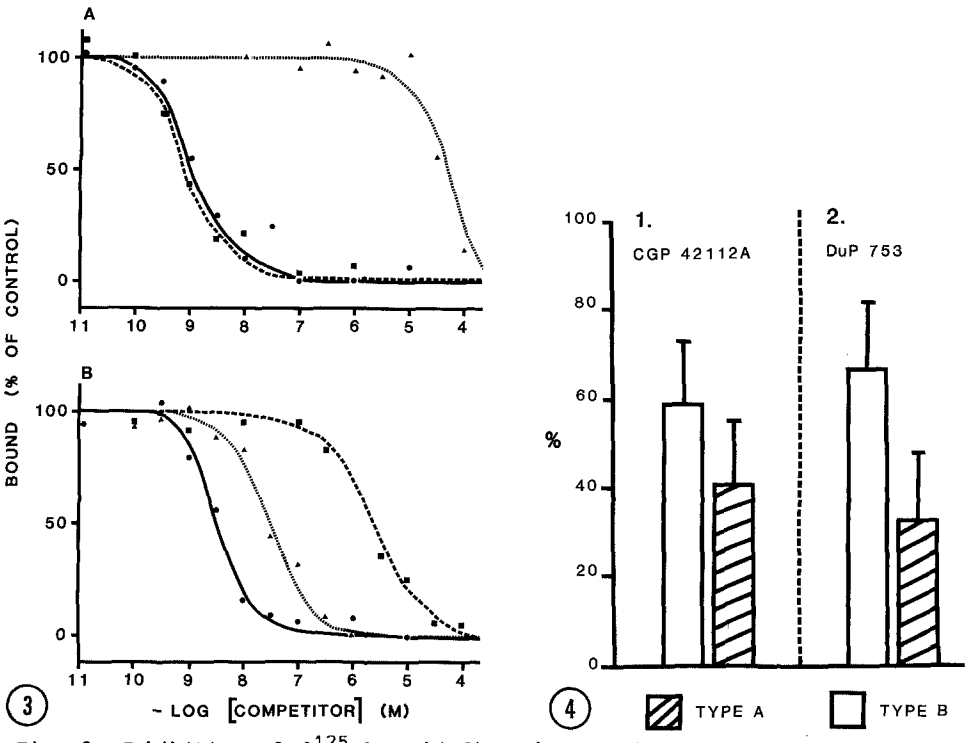

Fig. 3. Inhibition of [^{125}I]AII binding, by AII (•), CGP 42 112 A (▪) and DuP 753 (▲) to subtypes of membranes of rabbit myocardial ventricular membranes. In panel A, subtype B was blocked with 3 µM DuP 753. In panel B, subtype A was blocked with 0.3 µM CGP 42 112 A. The experiments were carried out in the presence of 0.5 nM [^{125}I]AII and the data given are representative of two experiments each.

Fig. 4. Proportions of AII receptor subtypes (%) in rabbit myocardial membranes as determined in competition binding experiments by their sensitivity to subtype-selective concentrations of CGP 42 112 A and DuP 753. Panel 1 shows the results of experiments in which the receptors sensitive (type A) and resistant (type B) to CGP 42 112 A (0.3 µM) were determined. The results using DuP 753 (3 µM) to define the proportions are shown in panel 2 (type B sensitive and type A insensitive to this agent). The experiments were carried out in the presence of 0.5 nM [^{125}I]AII. The values given are means ± SD of 7 experiments each.

In addition to the classification based on receptor selectivity, we have confirmed that the two subtypes are differently affected by the disulfide-reducing agent DTT (13). The binding of angiotensin II to the type B receptor is strongly inhibited by this agent, whereas binding to the type A receptor is markedly enhanced, reaching a maximum at 3 mM DTT (Fig. 5). This fact strengthens the evidence for the distinct nature of the subtypes of AII receptors in the myocardium.

Recently, two distinct AII receptor subtypes (A and B) have been identified on the basis of their characteristic affinities for the peptidic and nonpeptidic ligands as used in this study as well as by their different susceptibilities to DTT (13). In human uterus, only subtype A was found, and in vascular smooth-muscle cells only subtype B, whereas both subtypes were present in adrenals (13). The latter finding was confirmed by others using a similar

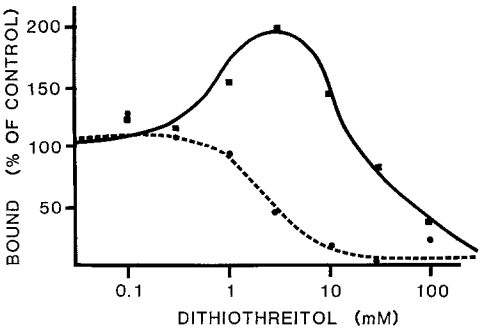

Fig 5. Effect of various concentrations of dithiothreitol on the binding of 0.5 nM [^{125}I]AII to subtypes of AII receptors of rabbit myocardial receptors. The experiments were carried out in the presence of 3 µM DuP 753 (■) and 0.3 µM CGP 42 112 A (●), respectively, to investigate the effects of DTT on type A and type B angiotensin II separately (see Figs. 2 and 3). The data given are representative of two experiments each.

approach (19,23) and extended by observations suggesting that different post-receptor coupling mechanisms are linked to the two AII receptor subtypes (24,25). The nomenclatures so far used to define these entities have been inconsistent, the one being variously referred to as subtype AII-B, AII-1, or AII_{α}, and the other as AII-A, or AII-2, or AII_{β} (13,19,25), and the adoption of a standard classification into AT_1 and AT_2 subsets, respectively, has now been proposed by the Committee of the American Heart Association for the Nomenclature of Angiotensin Receptors (26). However, regarding all criteria there is good agreement between the susceptibilities of the two AII receptor subtypes originally described (13) and those found in this and other studies, suggesting that these entities have similar, if not identical properties.

Morphological studies provide evidence for the existence of AII receptors on cardiac myocytes as well as in distinct loci such as components of cardiac innervation (16-18). However, the lack of tools to investigate AII receptor subtypes in the way described above has hitherto precluded effectual investigation of the location and functions of these distinct entities and possible pathophysiological implications of their coexistence in this vital organ. Studies on the existence and location of AII receptor subtypes in the heart should provide an important insight into the molecular mechanisms involved in the action of this hormone on cardiac functions and the alterations that may occur in pathologic conditions.

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