Vol. 105, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1982 Pages 575-581

IDENTIFICATION AND CHARACTERIZATION OF ANGIOTENSIN II RECEPTORS IN CARDIAC SARCOLEMMA

Amal Mukherjee, Padmakar V. Kulkarni, Zohre Haghani, John L. Sutko

From the Departments of Internal Medicine (Cardiology Division) and Radiology, The University of Texas Health Science Center at Dallas, 5323 Harry Hines Blvd., Dallas, Texas 75235

Received February 15, 1982

SUMMARY: We have used [^{125}I] angiotensin II to investigate the presence of specific angiotensin II receptors in beef heart sarcolemmal membranes. The observed binding is saturable, reversible and specific. The apparent equilibrium dissociation constant is 2.23 $\pm$ 0.15 ($\bar{x}$ $\pm$ SEM) and the maximal number of binding sites per mg membrane protein is 32.8 $\pm$ 5.4 fmol ($\bar{x}$ $\pm$ SEM). The specific binding is 80-100% of the total [^{125}I] angiotensin II bound and is directly proportional to membrane protein concentration over the range of 33-173 µg protein per ml. Angiotensin II and its antagonists competed for binding in a potency order of (agent, K_i): angiotensin II, 0.9nM > Sar^1 Ala^8, 7 nM > Sar^1-Ile^8, 51 nM > Sar^1-Leu^8, 427nM > angiotensin I, 1709 nM. The ability to characterize and quantify these receptors should now provide a method for investigating the mechanisms underlying the effects of angiotensin II on myocardial tissues.

The vasoactive peptide, angiotensin II (AII) has been demonstrated to produce both positive inotropic and chronotropic effects on mammalian heart muscle. Depending on the experimental conditions, these actions may involve both direct effects on the myocardium (1-7) and an indirect component resulting from an enhancement of the release of catecholamines from either the adrenal medulla or sympathetic nerve terminals (8,9). The cellular mechanism of the direct component of AII's actions is not clearly understood, although an augmentation of the slow inward Ca^{2+} current appears to be involved (10). The attenuation of the actions of AII by specific AII antagonists, but not by the β-adrenergic blocker, propranolol (6) provides circumstantial evidence for the presence of specific myocardial AII receptors, through which the effects of this vasoactive peptide are mediated.

As a first step in the study of the cellular effects of AII, we have used [^{125}I] AII to investigate the presence and nature of AII receptors in plasma

A preliminary report of this investigation has been presented at the 54th Annual Meeting of the American Heart Association, 1981.

0006-291X/82/060575-07$01.00/0

membranes prepared from bovine myocardium. We have observed the binding of this ligand to be specific, saturable and reversible, properties expected for the binding of a hormone to a physiological receptor.

MATERIALS AND METHODS

Membrane Preparation: Bovine hearts were obtained from a nearby slaughterhouse and brought to the laboratory in ice cold 0.25M sucrose and 20 mM MOPS-Tris, pH 7.4 (buffer A). Approximately 150 g of left ventricular muscle was minced and homogenized in a Waring blender for 5 sec in 300 ml of buffer A. The homogenate was centrifuged at 1000 x g (max) for 10 min. The pellet was washed three times in buffer A by resuspension and centrifugation and then further homogenized in 300 ml of buffer A using three, 30 sec bursts with a Polytron (PT20) at a setting of 7 and centrifuged at 10,000 x g (max) for 10 min. The supernatant was collected and centrifuged at 48,000 x g (max) for 30 min. the pellet was then resuspended in 100 ml of buffer A and mixed with an iso-osmotic Percoll-sucrose solution to give a final Percoll concentration of 25%. The tubes were centrifuged at 48,000 x g (max) for 90 min. The uppermost band was aspirated and diluted with 160 mM NaCl and 20 mM MOPS-Tris, pH 7.4 (buffer B) and centrifuged at 48,000 x g (max) for 10 min. The pellet was resuspended in 25 ml of buffer A and Percoll density gradient centrifugation was repeated as above. Usually a single band was found in the upper fourth of the gradient, and this was aspirated, diluted, centrifuged, wahsed and resuspended in buffer B as above. The final suspension was diluted to (~ 1 mg protein/ml) rapidly frozen in liquid nitrogen and stored at -80° C until assayed. Protein was assayed by the method of Lowry et al. (11) using crystalline bovine serum albumin as a standard.

[^{125}I] AII Binding Assay: [^{125}I]AII with a specific activity of (~ 1800 Ci/mmol) was either purchased from New England Nuclear, Boston, Massachusetts or AII (Calbiochem, San Diego, California) was iodinated in our laboratories using the method of Hunter and Greenwood (12). The labeled material was purified on a carboxymethyl cellulose column as described by White and Ojeda (13) for the purification of other peptides. Beef heart membranes (50-100 µg protein/ml) were incubated with [^{125}I] AII (0.05-5.0 nM) at 37°C for 10 min in a total volume of 0.15 ml containing 53 mM NaCl and 7 mM MOPS-Tris, pH 7.4 (assay buffer). Binding reactions were terminated by the addition of 2 ml of ice cold buffer B and filtration through GF/C filter disks (Whatman). The filters were washed three times with 5 ml of ice cold buffer B, dried, and counted in a triton-toluene liquid scintillation mixture with an efficiency of 90% in a Beckman LS7500 liquid scintillation spectrometer. Nonspecific binding was defined as [^{125}I] AII which could not be displaced by 10 µM unlabeled AII. Specific binding defined as the total [^{125}I]AII bound minus the nonspecific binding, is shown in all the figures. Specific binding was generally 80-100% of the total [^{125}I]AII bound to the membranes and filter paper. Using these direct binding methods, the number and affinity of AII binding sites in membranes were analyzed according to Scatchard (14) with regression lines by the method of least squares. Six to seven data points were obtained for each Scatchard analysis. Replacing NaCl by 50 mM Tris HCl pH 7.4 did not after either the K_D or maximal binding of AII to the receptors.

RESULTS AND DISCUSSION

Preliminary characterization of the membrane fraction has revealed: Na/Ca exchange transport activities of 60-80 nmoles Ca^{2+} uptake/mg protein/min (15); Na,K-ATPase activities of 40-80 µmoles Pi/mg protein/ hr;

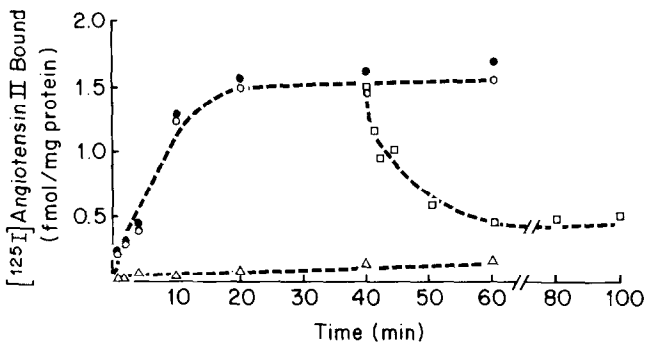

Figure 1. The time course of the association (O–O) and dissociation (□ - □) of [^{125}I] angiotensin II with beef heart sarcolemmal membranes. [^{125}I] angiotensin II (0.22 - 0.25nM) with (Δ-Δ) and without (O–O) unlabeled angiotensin (10^{-5}M) was incubated with membranes (0.12–0.33 mg/ml) at 23°C. At the specified time intervals, 0.1 ml samples were removed, diluted in 2 ml ice-cold buffer B and filtered as described in the Methods. Specific binding (O–O) was defined as the difference between incubation mixtures with unlabeled angiotensin II (Δ-Δ) and otherwise identical mixtures without unlabeled angiotensin II (O–O). Dissociation (□ - □) was induced by addition of unlabeled angiotensin (10^{-5}M) after 40 min incubation of membrane and [^{125}I] angiotensin II at 23°C. The results expressed here represent the means from two experiments performed in duplicate.

(-) [^{3}H] dihydroalprenolol binding of 1.2-1.5 pmoles/mg protein and [^{3}H] quinuclidinyl benzilate binding of 3.5 pmoles /mg protein (unpublished results).

Time Course and Reversibility of Binding

The time course of specific [^{125}I]AII binding to beef heart membranes was investigated at 23°C using 220-250 pM [^{125}I]AII and 120-133 µg/ml membrane protein. [^{125}I]AII was bound to the membranes and a steady state was reached after approximately 20 min which was maintained for at least 60 min suggesting that there was no inactivation of the ligand or loss of the binding sites during the incubation period (Figure 1). The nonspecific binding was less than 10% of the total binding and this did not increase appreciably with time. At 37°C [^{125}I]AII binding was more rapid, reaching a steady state values in 2-3 minutes (data not shown). The rate of dissociation of specifically bound [^{125}I]AII from beef heart membranes was determined by adding excess of unlabeled AII (10 µM) to equilibrated mixtures of [^{125}I] AII and beef heart membranes. The half time ($t_{1/2}$) of the dissociation was 8 minutes. The dissociation of the bound hormone was not complete. Similar

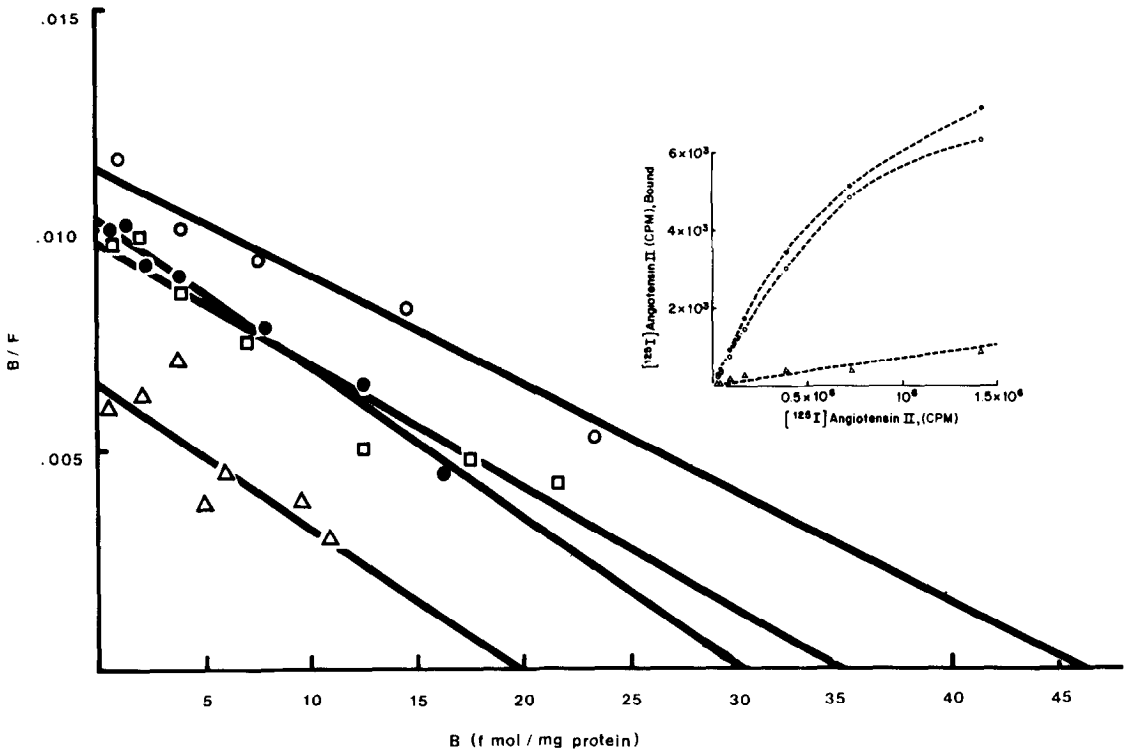

Figure 2. Scatchard analysis of [^{125}I] angiotensin II binding to beef heart sarcolemmal membrane in four separate experiments. B/F is the ratio of bound to free [^{125}I] angiotensin II. The slope, determined by linear regression analysis, is equal to $-1/K_D$. The maximal number of binding sites was determined from the intercept of the lines with the abscissa. Inset. Binding of [^{125}I] angiotensin II to beef heart sarcolemmal membrane as a function of increasing concentrations of [^{125}I] angiotensin II. O–O: Total binding; Δ–Δ , nonspecific binding.

findings also have been observed for AII binding to rat mesenteric arteries (16), calf cerebral cortex membranes (17) and anterior pituitary membranes (18).

Saturability of [^{125}I]AII Binding to Beef Heart Membranes

The concentration dependence of AII binding was studied with [^{125}I] AII concentrations ranging from 0.05–5.0 nM at 37°C. The binding of AII to beef heart membranes with increasing concentrations of the [^{125}I]AII is shown in Figure 2. Scatchard analysis of the data revealed a single class of binding sites with an equilibrium dissociation constant, K_D, of 2.23 ± 0.15 nM ($\bar{x}$ ± SEM). The maximal number of binding sites was 32.8 ± 5.4 ($\bar{x}$ ± SEM) fmol/mg protein.

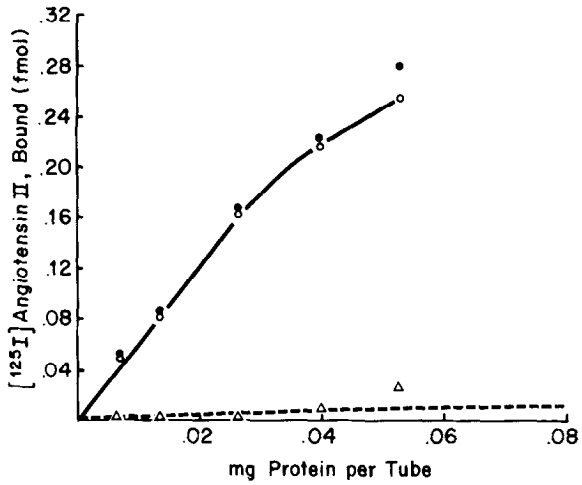

Figure 3. Specific binding of [^{125}I] angiotensin II to beef cardiac sarcolemmal membrane as a function of increasing concentration of membrane protein. The data points represent the means from two separate experiments. Total binding O-O; nonspecific binding Δ-Δ; specific binding O-O are shown.

Specific binding of [^{125}I]AII to beef heart membranes was proportional to membrane protein concentrations over the range of 33-176 μg protein per ml (Figure 3).

Displacement of [^{125}I] AII Binding to Beef Heart Membranes

The ability of selected angiotensin ligands to inhibit binding of [^{125}I]AII was tested. The order of potency of ligands in inhibiting [^{125}I]AII binding was AII > Sar^1-Ala^8 AII > Sar^1-Ile^8 AII > Sar^1-Leu^8 AII > AI. The IC_{50} values and the apparent dissociation constants of the drugs are shown in Table 1. Our data show that AII is 20-400 times more potent than its antagonists and about 2000 times more potent than AI in displacing [^{125}I]AII from specific binding sites. Bennett and Syder (17) and Gunther et al. (16) have found, however, in brain and mesenteric artery that certain AII antagonists displayed equal or greater potency than did native AII. Similar findings have also been obtained by us with rat anterior pituitary membranes (18). The reasons for this discrepancy may be attributable to either species or tissue differences, or it may also be due to partial degradation of AII antagonists by beef heart sarcolemmal membrane fraction.

The present data indicate that AII binds specifically to cardiac membranes in a rapid, saturable and reversible manner as expected for the

Table 1

INHIBITION CONSTANTS FOR BINDING OF ANGIOTENSIN DRUGS TO BOVINE MYOCARDIAL MEMBRANES

Agents	IC_{50} nM	K_i nM
Angiotensin II	1	0.9
Sar^1-Ala^8 AII	20	17
Sar^1-Ile^8 AII	60	51
Sar^1-Leu^8 AII	500	427
Angiotensin I	2000	1709

Values are mean of two separate experiments done in duplicate. Sarcolemmal membranes (50-100 µg protein/ml) were incubated at 37°C for 10 min with 0.38 nM [^{125}I]AII and various concentrations of unlabeled drugs. Apparent K_i values were calculated from the equation:

$$K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_D}}$$

where IC_{50} represents the concentration of each agent which inhibits 50% of the specific binding, [L] the concentration of [^{125}I]AII and K_D, the dissociation constant of [^{125}I]AII obtained from equilibrium studies.

binding of a hormone to its physiological receptors. The direct identification of myocardial AII receptors and the ability to characterize and quantify them should now facilitate further study of the cellular mechanism of action of this hormone and of the possible regulation of its receptor in normal and pathophysiologic states.

ACKNOWLEDGEMENTS: The authors wish to thank Dr. James T. Willerson for his support and continued interest in these studies. The authors also acknowledge the technical help of Mr. John White and the secretarial assistance of Ms. Gayle Blust.

This work was supported by the NIH Ischemic Heart Specialized Center of Research Grant #HL-17669, the Harry S. Moss Heart Fund, and a grant from the American Heart Association.

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