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Specific membrane-binding of angiotensin II—the role of degradative enzymes

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The possible role of degradative enzymes was examined in the specific binding of angiotensin II (AII) to cell membranes. Red blood cell membranes did not bind AII specifically under any of the ambient conditions studied, indicating a lack of AII receptors and no role for the degradative enzymes in specific binding. Rabbit aorta smooth muscle cell membranes bound AII specifically, and this binding had similar characteristics to those previously described for this preparation. It is concluded that specific binding of AII to cell membranes does not involve degradative enzymes, and probably represents binding to the biologically active receptor.

Until recently, it has been assumed that specific (preventable or displaceable) binding of a ligand to an isolated membrane preparation indicates the presence of receptors for that ligand in the membrane. However, Cuatrecasas et al (1974) suggested that binding of noradrenaline (NA) to degradative enzymes, especially catechol-*O*-methyl-transferase (COMT), may account for part of the specific binding of NA observed in membrane preparations. Le Morvan et al (1977) investigated a similar possibility for angiotensin II (AII) using guinea-pig aorta smooth muscle preparations and commented 'We cannot conclusively differentiate between binding to the angiotensinases and binding to the angiotensin receptor.' To differentiate possible AII binding to degradative enzymes from that to receptors was the aim of the work in this report. Two isolated membrane preparations were chosen: the rabbit aorta smooth muscle membrane, which has well-characterized binding sites and receptor sites for AII (Baudouin et al 1971; Devynk et al 1973; Peach 1977), and the red blood cells ghost (RBCG) which possess at least two types of angiotensinase enzymes, but apparently no angiotensin receptors (Khairallah et al 1963; Itskovitz & Miller 1967; Kokuba et al 1969; Moore et al 1977).

MATERIALS AND METHODS

Preparation of red blood cell membranes

Rabbit aorta microsomes were prepared from the aortae of 10–15 female rabbits each day that the microsomes were used for binding studies. The aortae, dissected free of adventitia and placed in ice-cold 0·33M sucrose—0·01 Tris-maleate, pH 7·4, containing 1 mM K^+, 1 mM Mg^{2+}, and 1 mM Na_2EDTA, were minced and homogenized with a ground-glass tissue homogenizer operated at high speed, a Polytron operated under refrigeration, and a smooth-walled glass hand homogenizer. The preparation was centrifuged in the cold for 5 min at 5000 *g*, the precipitate washed, and the supernatants pooled and centrifuged at 30 000 *g* to precipitate the mitochondria. The membranes were precipitated by a 4°C 60 min centrifugation at 100 000 *g* and resuspended in 0·01M histidine, pH 7·4, containing 100 mM K^+ and 5 mM Mg^{2+}. 10^{-4}M phenyl methyl sulphonyl fluoride was added to the membrane preparation before binding studies were conducted.

The vesicular nature of the microsomes was assessed by electron microscopy.

Binding studies

Protein concentrations of the membrane preparations used for binding studies, as determined by the method of Lowry et al (1951), were 0·5–1·5 mg ml^{-1} for red blood cell membrane preparations and 0·2–0·8 mg ml^{-1} for rabbit aorta microsome preparations.

Binding studies were at 4° and 37°C for RBCG experiments and at 30°C for experiments using rabbit aorta microsomes. The binding preparations were incubated with AII to determine the nature, extent and time course of the binding of AII to the two preparations. Samples (100 μl) of the reaction mixtures were withdrawn at intervals after addition of AII to the binding preparations. The samples were placed on Millipore filters (HAWP 0·45 μm) and

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washed with 8 ml ice-cold buffer to separate membrane-bound radioactivity, which was retained on the surface of the filters, from free radioactivity. Filters were counted in a Beckman gamma counter and the results in counts min^{-1} plotted against time. According to the currently accepted parameters for binding studies, specific binding was measured by determining the difference between the amount of ^{125}I-AII bound in the presence and in the absence of cold angiotensin. Each binding experiment was repeated at least four times.

RESULTS

Binding studies on RBCGs

Incubation of ^{125}I-AII (10 ng ml^{-1}) with a buffered suspension of red blood cell membranes of protein concentration 0·5–1·5 mg ml^{-1} resulted in measurable binding of the radioactive hormone to membrane preparations (5×10^3–2×10^4 counts min^{-1} mg^{-1} protein) under all conditions studied (Fig. 1). However, ^{125}I-AII binding to red blood cell ghosts was

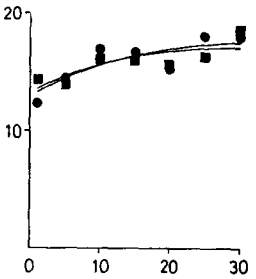

FIG. 1. Binding of ^{125}I-AII to red blood cell membranes in the presence (■) and absence (●) of cold AII in 1000 fold excess concentration. Incubation was at 4°C; binding buffer Tris-HCl 50 M, pH 7·4, containing NaCl 140 mM. Results are of a representative experiment. Ordinate: bound ^{125}I-angiotensin II (counts min^{-1} mg^{-1} protein $\times 10^3$). Abscissa: time (min).

neither displaceable nor preventable by a 1000-fold excess concentration of cold AII. Studies at 4°C were made with Tris-HCl 50 mM, Tris-HCl 50 mM containing NaCl 140 mM and $CaCl_2$ 2·5 mM, Tris-HCl 50 mM containing Na_2EDTA 1 mM, and Tris-HCl 50 mM with sucrose 0·32M and Na_2EDTA 1 mM; all buffers were at pH 7·4. Binding of ^{125}I-AII was examined at 37°C with Tris-HCl 50 mM, pH 7·4, containing NaCl 140 mM and $CaCl_2$ 2·5 mM. ^{125}I-AII binding to the membranes was of the same magnitude at 4° and 37°C and was neither displaceable nor preventable by cold AII in 1000-fold excess concentration. Addition of bovine serum (BSA) to the membrane suspension did not significantly affect the results at 4° or at 37°C in Na^+- and Ca^{2+}-containing buffer.

The binding of AII to red blood cell membranes denatured by heat treatment (either 1 h at 70°C or 20 min at 84°C) was of the same magnitude and exhibited the same characteristics as binding of the peptide to untreated membranes. Binding of ^{125}I-AII to denatured RBCGs was of the magnitude 5×10^3–2×10^4 counts min^{-1} mg^{-1} protein, and, as with untreated membranes, was neither displaceable nor preventable by cold AII.

Binding studies on rabbit aorta microsomes

Results of binding studies on rabbit aorta microsomes using unlabelled and ^{125}I-AII confirmed the results of previous investigations with respect to the existence of AII specific binding sites (Fig. 2). Binding of ^{125}I-AII to microsomal membranes was

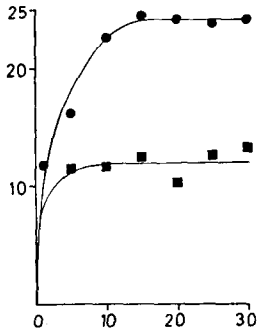

FIG. 2. Binding of ^{125}I-A-II to rabbit aorta microsomes in the presence (■) and absence (●) of cold AII in 1000x fold excess concentration. Incubation was at 30 °C; binding buffer 0·01 M histidine, pH 7·4, containing 100 mM K^+ and 100 mM Mg^{2+}, with PMSF 10^{-4} M. Results are of a representative experiment. Ordinate: bound ^{125}I-angiotensin II (counts min^{-1} mg^{-1} protein $\times 10^3$). Abscissa: time (min).

found to reach equilibrium within 15 min, while that of the radioactive peptide in the presence of cold AII reached equilibrium within 5 min; the amount of AII bound at equilibrium remained constant during the 30 min incubation studied. Specific binding was determined (from four studies) to be 50–53% of total binding. The presence of Na^+ in the binding buffer at a concentration of 120 mm was found to affect neither the course of AII binding nor the magnitude of specific binding (specific binding = 51% of total binding in the presence of Na^+).

DISCUSSION

Specific binding of a hormone to elements of a binding preparation may be interpreted as specific binding 1) to hormone receptors, and/or 2) to hormone-specific degradative enzymes. Differentiation be-

tween the two is therefore a most important prerequisite to the assumption that results obtained from binding studies are in fact due to hormone-receptor interaction. Red blood cell membranes are known to be rich in angiotensinases (Khairallah et al 1963); they presumably do not contain AII receptors, as there have been no reports of a biological effect of AII on RBCGs. A red blood cell membrane preparation was consequently chosen to serve as a control binding preparation for the present series of experiments.

Red blood cell membranes did not specifically bind AII at 37° or at 4 °C; at the latter temperature the action of any enzymes in the preparation would be effectively inhibited. AII did not bind specifically to the membranes in the presence of Na^+, which has been reported to stimulate AII binding to bovine adrenal cortex preparations (Glossmann et al 1974) —but not to vascular smooth muscle (Devynck et al 1973)—and to enhance the biological response to AII in a whole rat blood pressure assay system (Schaechtelin et al 1974). Finally, there was no AII specific binding to red blood cell membranes in the presence of Ca^{2+}, which Schaechtelin et al (1974) reported to be twice as effective as Na^+ in enhancing the response to AII in the rat pressor system.

The absence of specific binding of AII to the membrane preparations as indicated by the above results, and by the fact that ^{125}I–AII binding to heat-denatured membranes was of the same magnitude and exhibited the same characteristics as binding of the peptide to untreated membranes, represents a direct confirmation of the assumption that red blood cell membranes do not contain AII receptors. That AII does not bind specifically to the angiotensinase enzymes found in abundance on the red blood cell membrane also indicates that it must be a hormone-receptor interaction that is responsible for the results obtained from AII binding studies on biological membranes such as the rabbit aorta microsome. The apparent lack of enzyme-ligand binding for AII is particularly significant in the light of the recent proposal that the measurable specific binding of the pressor substance NA to elements of plasma membrane preparations is mainly to its degradative enzyme COMT rather than to its β-andrenoceptors (Cuatrecasas et al 1974).

That AII receptors were present on rabbit aorta microsomes as prepared was demonstrated by means of binding studies reproducing the results of Baudouin et al (1971) with respect to the existence and kinetics of specific binding to aortic microsomes. Results of Devynck et al (1973) with respect to the effect of Na^+ on AII binding were also confirmed.

We have, therefore, shown that when isolated biological membrane preparations exhibit specific binding to AII, it is extremely unlikely that binding to degradative enzymes is part of the specific binding.

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