ANGIOTENSIN RECEPTORS IN RAT UTERINE MEMBRANES

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1. Introduction

Angiotensin II is a polypeptide hormone possessing a potent contractile effect on various smooth muscle cells [1]. Hormone-receptor interaction is the first event in the series of molecular processes leading to a hormonal effect on a target cell. Extensive investigations performed during the last decade have demonstrated that receptors for polypeptide hormones are located on the plasma membrane (for review, see [2]). Previous studies from our laboratory have characterized angiotensin II receptor sites of rabbit aortae plasma membranes [3]. In the present investigation, we report the principal properties of angiotensin II receptors of the plasma membrane of rat uterine myometrial cells which appear to be essentially similar to those of rabbit aorta smooth muscle cells.

2. Methods

Wistar female rats (weighing between 180 and 200 g) were injected intramuscularly with 60 $\mu g \cdot 100\ \mu g^{-1}$ diethylstilbestrol daily for 2 days before an experiment. They were killed by a blow on the head. Plasma membranes from myometrial smooth muscle were prepared according to the method of Kidwai et al. [4] with some modifications. The myometria from the uterine horns of 6–8 rats were carefully separated from endometria, cut up finely with razor blades and homogenized with the use of an all-glass Potter-Elvehjem homogenizer for 1 min repeated two times at a speed of approximately 1400 rev/min. The homogenate (H_1) (in 0.25 M mannitol and 1 mM Tris–EDTA, pH 7.1) was centrifuged at 115 000 *g* for 40 min. The sediment [5] was resuspended in a minimum volume of buffer, layered on the top of a discontinuous gradient (2 ml 33% sucrose (d 1.14) below and 1 ml 8% sucrose (d 1.08) above) and centrifuged at 115 000 *g* for 90 min. The plasma membrane fraction (P), being the least dense component, was concentrated at the interphase d 1.08/1.14 and the rest of the homogenate (H_2) ($d > 1.14$) representing mitochondria, endoplasmic reticulum and nuclei, sedimented. The plasma membrane fraction thus obtained was removed by aspiration and centrifuged at 115 000 *g* for 40 min in 10 mM Tris–HCl buffer, pH 7.4. 5′Nucleotidase measured according to Song and Bodansky [5] and adenylcyclase activity determined using the method of Krishna et al. [6], were considered as plasma membrane marker enzymes. As detailed in a previous publication [7], the specific activity of 5′Nucleotidase was 6 times higher in the plasma membrane fraction (P) than in the whole homogenate H_1. The specific activity of adenylcyclase was augmented in a similar fashion: the basal and isoproterenol (10^{-5} M) and NaF-stimulated activities all being 2–3 times higher in the membrane preparation (P) than in the whole homogenate H_1.

The binding of angiotensin II to membranes was studied within 4 hr of preparation of the membranes, the membranes being kept at 0°C, with the use of tritiated (Asn^1, Val^5)-angiotensin II retaining the full biological activity of the unlabelled hormone [8]. Two batches of [^{3}H]angiotensin II with specific activities of 39 and 70 $Ci \cdot mmole^{-1}$ respectively, were used. The incubation medium contained 100 mM KCl, 5 mM $MgCl_2$ and 10 mM histidine buffer, pH 6.8, in a volume of 0.5 ml. The concentration of membrane protein in the incubation medium, determined by the method of Lowry et al. [9] was

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50–100 $\mu g \cdot ml^{-1}$. Incubation was performed at 29°C, after which membrane-bound and unbound radioactivity were separated by filtration on Millipore filters HAWP 0.45 μm as described in previous publications [3]. Binding studies were performed in the presence and absence of 1000-fold excess of unlabelled angiotensin II; specific binding was considered, according to the currently accepted procedure, as the difference between the radioactivity bound to membranes in the absence and in the presence of non-radioactive angiotensin.

3. Results

The time-course of the specific [^{3}H]angiotensin II binding to various myometrial subcellular fractions is represented in fig.1. The concentration of angiotensin binding sites varied from one preparation to another, possibly in relation to the homogenization procedure, and was 7–20-fold higher in plasma membrane than in whole uterine homogenate H_1. The enrichment of angiotensin binding sites in a purified plasma membrane fraction appears thus to be even higher than that of the two marker-enzymes adenylcyclase and 5'nucleotidase. This result strongly suggests that

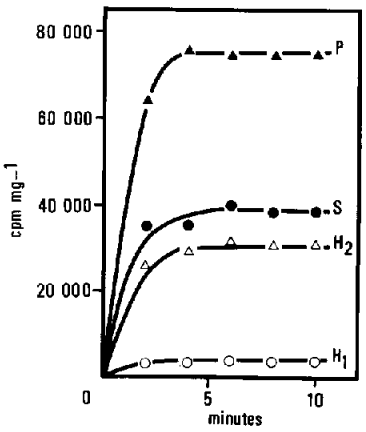

Fig.1. Specific displacable binding of 5 × 10^{-8} M angiotensin II on subcellular extracts from rat myometrium. P, S, H_2 and H_1 represent various subcellular fractions as described in the methods. P corresponds to the plasma membrane enriched fraction. The incubation was performed at 29°C and the binding expressed in cpm per mg of protein.

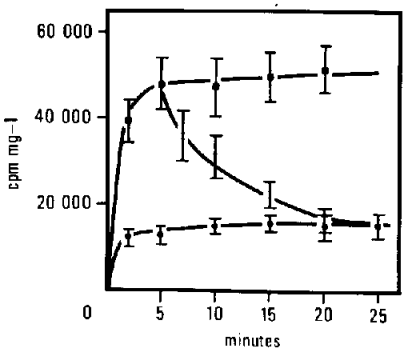

Fig.2. Uptake of 5 × 10^{-8} M [^{3}H]angiotensin II at 29°C by plasma membrane fraction, in the absence (——) and presence (– – –) of 5 × 10^{-5} M unlabelled angiotestin II. Dissociation was followed as the exchange of bound [^{3}H]angiotestin with 5 × 10^{-5} M unlabelled hormone added after five min of incubation. Each point is a mean value from five independent determinations. The vertical bar represents the standard error of the mean.

angiotensin receptor sites are located in the plasma membrane and indicates that angiotensin receptors, as other peptide hormone receptors [10], may be considered as useful markers for this membrane fraction.

The [^{3}H]angiotensin II binding to uterine membranes was not modified by preincubation of the membranes at 29°C in the incubation medium, (at least) for up to 75 min. At 29°C, the specific binding of 5 × 10^{-8} M [^{3}H]angiotensin II to the plasma membrane fraction reached equilibrium within 5–6 min (fig.2). The equilibrium of [^{3}H]angiotensin II binding was maintained at least up to 25 min, suggesting the absence of marked inactivation of the ligand during this period of time.

Reversibility of the angiotensin binding was demonstrated by the addition of 1000-fold unlabelled angiotensin which resulted in a complete dissociation of the specific binding of 5 × 10^{-8} M [^{3}H]angiotensin II within 15 min at 29°C (fig.2). Analysis of kinetic studies represented in fig.3 indicated an association rate constant K_1 of 1.6 × 10^{-5} $M^{-1} \cdot sec^{-1}$ and a dissociation rate constant K_{-1} of 1.3 × $10^{-3} \cdot sec^{-1}$.

The concentration dependence of angiotensin binding studied with [^{3}H]angiotensin concentration ranging from 5 × 10^{-9} M to 5 × 10^{-7} M indicated

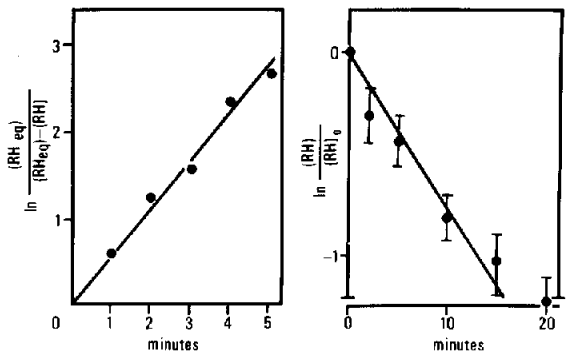

Fig.3. Determination of rate constants by analysis of the association and dissociation kinetic studies. [^{3}H]angiotestin II concentration was 5×10^{-8} M and the temperature of incubation was 29°C. [RH] = concentration of the complex hormone–receptor at a given time. [RHeq] = concentration of the hormone complex at equilibrium. [RHo] = concentration of the complex at the time 0 of the dissociation studies.

the presence of two classes of binding sites (fig.4). The high affinity binding sites were saturated at a [^{3}H]angiotensin II concentration of 10^{-7} M. According to the Scatchard plot [11], the high affinity binding sites have an apparent dissociation constant K_d 29°C value of 2×10^{-8} M and a binding capacity of 0.7 pmole · mg^{-1} protein. The low affinity binding sites appeared to be not saturated at a [^{3}H]angiotensin II concentration of 5×10^{-7} M. The K_d value estimated by the Scatchard plot (2×10^{-8} M) is

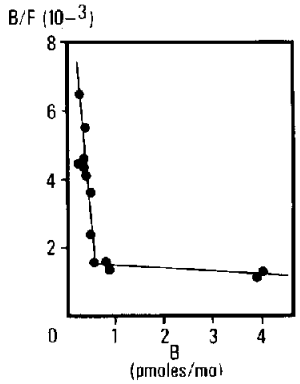

Fig.4. Scatchard analysis of steady state specific binding data at 29°C for plasma membrane fraction with concentrations of [^{3}H]angiotensin II from 5×10^{-9} M to 5×10^{-7} M.

close to the K_d value of 8.3×10^{-9} M, calculated from the ratio $\frac{K_{-1}}{K_1}$.

The specificity of [^{3}H]angiotensin II binding was demonstrated by the inhibitory effect of various compounds added at a concentration of 10^{-6} M on the binding of 5×10^{-8} M [^{3}H]angiotensin II. Results are shown in table 1. Substitution of valine by isoleucine in position 5 did not affect the displacement. The 2–8 angiotensin and 3–8 angiotensin fragments displaced the octapeptide strongly. The inhibitory effect of the competitive antagonist Sar^1, Ile^8-angiotensin II was greater than that of the two other competitive inhibitors Ala^8 and Ile^8-angiotensin II. The decapeptide angiotensin I, which is the angiotensin II precursor [1], had no inhibitory effect, demonstrating that angiotensin I does not interact with angiotensin II receptors and that angiotensin I is not converted into angiotensin II by uterine membrane converting-enzyme during the period of incubation. L-Noradrenaline and prostaglandin $F_{2\alpha}$, two compounds possessing a myotropic activity but being structurally different from angiotensin II, did not affect its binding, demonstrating that they interact with receptors different from those of angiotensin II.

4. Discussion

The demonstration of angiotensin specific binding sites in plasma membrane was not unexpectedly given the rapidity of the onset of uterine contraction in response to angiotensin II and the various membrane phenomena elicited by the hormone, such as membrane depolarization [12], intracellular penetration of extracellular calcium [13] and inhibition of adenylcyclase when this enzyme was previously stimulated by isoproterenol [14].

The identity of the plasma membrane binding sites to the receptors initiating the angiotensin contractile response is supported by various arguments. The first one is the rapidity of [^{3}H]angiotensin II binding to plasma membrane, which occurs in a period of time very close to that necessary to obtain a maximal contraction. The second argument is the similarity of the concentration of angiotensin inducing half-maximal contraction (ED_{50}) and that responsible for half-occupation of high affinity bin-

Table 1
Inhibitory effect on [^{3}H]angiotensin II binding (%)

^{a}NH$_2$ –	Asp	– Arg	– Val	– Tyr	– Val	– His	– Pro	– Phe		100
	a —	—	—	—	Ile	—	—	—		104
		—	—	—	—	—	—	—		106
			—	—	—	—	—	—		81
	Sar	—	—	—	—	—	—	Ile		68
	—	—	—	—	—	—	—	Ile		40
	—	—	—	—	—	—	—	Ala		41
	b —	—	—	—	—	—	—	—	– His – Leu	3
	L-Noradrenaline									2
	PGF$_{2\alpha}$									0

[a] Angiotensin II
[b] Angiotensin I.

Inhibitory effect of angiotensins, angiotensin analogues and fragments, L-Noradrenaline and PGF$_{2\alpha}$ on 5×10^{-8} M[^{3}H] angiotensin II binding to plasma membrane fraction d < 1.14. Labelled hormone and the other drugs (10^{-6} M) were added simultaneously, and the incubation was performed at 29°C for 5 min. The displacement of [^{3}H]angiotensin II by Asn^1, Val^5-angiotensin II was considered as 100%. Each value is the mean of 4–8 determinations.

ding sites (apparent dissociation constant or K_d value). The ED_{50} of the angiotensin dose-response curve on oestrogen pretreated uteri was reported to be $1.0 \pm 0.1 \times 10^{-8}$ M [15] which is very close to the K_d value of 2×10^{-8} M reported here. Similarly, the angiotensin concentration responsible for the maximal contraction (2×10^{-7} M) is almost identical to the [^{3}H]angiotensin II concentration of 10^{-7} M which saturates the high affinity binding sites.

The angiotensin-elicited contraction of uterine muscle is probably not a linear function of receptor-occupancy. The main reason is that angiotensin contraction is partially mediated by endogenous prostaglandins whose suppression changes both the ED_{50} and the angiotensin concentration inducing a maximal contraction [16]. The non linearity between receptor occupancy and contraction may be the explanation for the slight difference observed between the ED_{50} and the K_d values. It may also provide an explanation for another difference observed between the binding and the biological effect. The inhibitory effect of angiotensin fragments and analogues, as determined in our experimental conditions, reflects the affinity of these compounds to receptors. The affinity of the 3–8 hexapeptide for receptor site is higher than that suggested by the analysis of its contractile effect: the inhibitory effect of this compound on [^{3}H]angiotensin II binding is 81% of that of unlabelled angiotensin, whereas its ED_{50} value of 2.2×10^{-6} M is more than 100 times higher than that of angiotensin II [17]. It would be extremely important to determine what factor contributing to the contraction is less sensitive to the hexapeptide than to the octapeptide angiotensin II, and particularly to know whether this difference stems from a difference in the stimulatory effect on endogenous prostaglandins.

The present investigation shows that uterine membrane receptor sites are similar to those of rabbit aorta membrane [3,18] in the sense that these receptors interact with the C-terminal end of the angiotensin molecule. This is clearly demonstrated by the fact that angiotensin fragments devoid of the first or the first two amino-acid residues are able to interact with angiotensin receptors in a way very similar to the intact octapeptide. If the N-terminal end of the angiotensin molecule, although necessary for a complete biological effect, is not involved in the binding process to the receptor site, one may suggest that the N-terminal end of angiotensin interacts with a membrane component distinct from the binding site demonstrated in the present experiment. Angiotensin binding sites in bovine adrenal cortex seem to be

somewhat different, as their affinity for the 3–8 angiotensin II fragment is less important than that of smooth muscle cell receptors [20]. Prostaglandins not only mediate partially the contractile effect of angiotensin II on rat uterus as previously mentioned, but also have a long-lasting potentiating effect on angiotensin action [17]. The lack of effect of prostaglandins on [^{3}H]angiotensin binding demonstrates that their potentiating effect on angiotensin does not involve a modification of the angiotensin-receptor mechanism, at least on oestrogen-pretreated rat uteri.

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