SPECIFIC BINDING OF ^{3}H-ANGIOTENSIN II IN RABBIT AORTA

Maryvonne Baudouin, Philippe Meyer, and Manuel Worcel

Unité de Recherches sur l' Hypertension Artérielle (INSERM)
and Département de Médecine Expérimentale
Faculté de Médecine Broussais, Paris 14 - France[+]

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SUMMARY : With the use of highly labelled ^{3}H-angiotensin II, a specific binder of angiotensin was demonstrated in the rabbit aorta. The concentration of sites per mg of aorta is $8.4 \pm 4.8 \cdot 10^{-15}$M. The kinetic constants of the specific binding were identical to those of the biological contractile response. The inhibitory ability of angiotensin derivatives on radioactivity incorporation was proportional to their respective biological activities. These observations suggest that the specific binding sites correspond to those involved in the biological response.

The specific binding of a hormone to its target cells should have the fundamental characteristics of reversibility, great affinity, limited capacity and high specificity (1). The present experiments demonstrate in rabbit aorta the existence of a specific binder of angiotensin II fulfilling these criteria. In addition, the kinetic constants of binding of angiotensin II were found to be similar to those of the muscular contraction of the aorta measured under the same conditions. Such a comparison was not able to be made in previous studies (2, 3).

METHODS. Adult rabbit aortae, dissected free of adventitia, were cut spirally and preincubated for 90 min in oxygenated McIlwain solution (4), pH 7.4, 37°C, containing ^{14}C-Inulin (specific activity 8.3 mCi/mMole ; 20 nCi/ml). The ^{3}H-angiotensin II added to the medium after preincubation had the specific activity of 56 Ci/mMole and the full biological activity of the unlabelled hormone ; this ^{3}H-angiotensin II was obtained by dehalogenation of diodo 5-Valine angiotensin II, as previously reported (5). 12 ml of Instagel™ Packard scintillating fluid were added to hydrolyzed tissue samples for

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[+]Postal address : Hypertension Laboratory, Hopital Broussais, Paris 14 - France -

determination of radioactivity. The determination of bound or "cellular" radioactivity (^{3}H) in tissue was performed by subtracting from the total radioactivity that contained in extracellular fluid volume, assuming that concentrations of ^{3}H in the bath and in the extracellular fluid were identical (6) ; the extracellular fluid volume was determined by ^{14}C-Inulin space.

RESULTS AND DISCUSSION. The time course of incorporation of radioactivity into cellular aorta favoured a reversible phenomenon (Figure 1) : at ^{3}H-angiotensin II concentration of $5 \cdot 10^{-8}M$, the uptake reached equilibrium after 2 min ; at concentration of $7.5 \cdot 10^{-8}M$ equilibrium was reached after 1.5 min. The time courses of cellular uptake and contractile response were identical. Cooling the incubation medium at 4°C reduced the rate (equilibrium after 4 min at $5 \cdot 10^{-8}M$) without affecting the magnitude of the incorporation.

Using a technique of bioassay (7), we found that angiotensin was not degraded during a 2 min incubation in the 600 g supernatant of rabbit aorta homogenate. Therefore radioactivity incorporated in aorta after 2 min was considered as representing essentially intact angiotensin.

The reversibility of cellular uptake was further demonstrated by washing the aorta with hormone-free medium after incubation : at

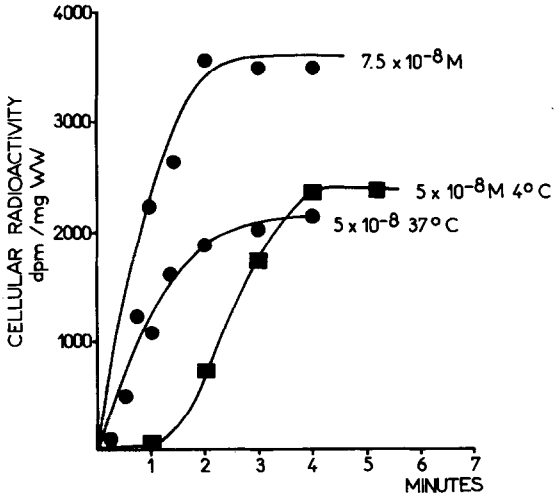

Figure 1 : Time course of incorporation of ^{3}H-angiotensin II into cellular aorta for 2 different initial concentrations. Each point is the mean of 4 experiments. The circles and the squares refer to experiments performed at 37°C and 4°C respectively.

TABLE 1

	TOTAL RADIOACTIVITY		"CELLULAR" RADIOACTIVITY	
	10^{-8}	5×10^{-8}	10^{-8}	5×10^{-8}
PSOAS	425.2 ± 52.2	3446.6 ± 383.6	0	0
OESOPHAGUS	458.2 ± 21.8	3401.0 ± 916.6	0	0
ADIPOSE TISSUE	240.4 ± 23.2	1059.6 ± 87.0	0	0
AORTA	843.8 ± 57.2	4485.8 ± 700.7	516.6 ± 63.0	2520.4 ± 282.5

Incorporation of ^{3}H-angiotensin II (10^{-8}M, and $5 \cdot 10^{-8}$M) in aorta and in different tissues in which angiotensin has no known pharmacological action. Values for non-target tissues are means ± standard deviation of 5 experiments. Values are expressed in dpm per mg of tissue.

37°C, half of the incorporated radioactivity was released into the medium within 2 min, and 90 % of the incorporated radioactivity within 20 min ; at 4°C, half of the incorporated radioactivity was released within 7 min.

The cellular uptake in non-target tissues such as psoas muscle, oesophagus, and adipose tissue was nil after 2 min for a ligand concentration of 10^{-8}M (Table 1). Conversely, the affinity of aorta for ^{3}H-angiotensin II was demonstrated by the measurable uptake occuring after exposition to 10^{-9}M for 2 min.

The cellular uptake of radioactivity was measured at concentrations of ^{3}H-angiotensin II in the medium ranging from 10^{-9}M (biological response threshold) to 5 . 10^{-7}M (above the concentration producing maximal biological response). The tissue was in contact with ^{3}H-angiotensin II for 2 min. An S-shaped curve was obtained for concentrations of ligand ranging from 10^{-9}M to 10^{-7}M (Figure 2). Cellular incorporation reached a plateau between 6.5 . 10^{-8}M and 10^{-7}M.

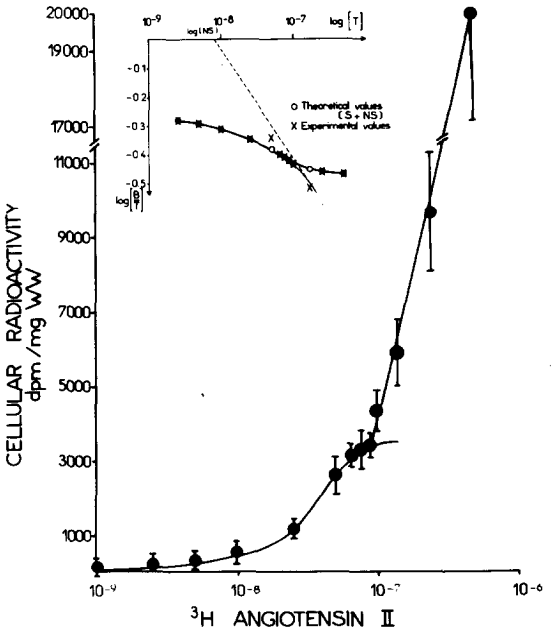

Figure 2 : Concentration dependence of cellular incorporation of ^{3}H-angiotensin II.
Each point is the mean of 9 experiments. Vertical bars indicate the standard deviation.
The corresponding "proportion graph" method is represented in the upper part of the figure : (T) is the total concentration of angiotensin in the bath (Mole/liter), and (B) is the concentration of bound hormone. (Ns) is the concentration of specific binding sites (Mole/1000 g of aorta).

Half of this uptake was obtained at $3 \cdot 10^{-8}$M. The kinetic characteristics of the biological response were also very similar since half of the maximal, and the maximal contractile responses were obtained at 10^{-8}M and 10^{-7}M respectively (Figure 3). At concentrations of ^{3}H-angiotensin II above 10^{-7}M, the incorporation of radioactivity rose rapidly and the proportion of bound radioactivity varied directly with the concentration of ligand, suggesting a non-specific binding. This assumption has been checked by proportion graph methodology (8) which enables a wider range of ligand concentration to be used and is therefore preferable to the classical graphical methods (9). The observed parameters have been compared to a theorical association of one specific (S) and one non-specific (NS) system (Figure 3) A highly significant adequacy of fit has been found by means of a 360 IBM computer. The binding parameters of the specific system obtained by computerized analysis were Ks (association constant) 13.1 $\pm$ 7.7 nM^{-1}, and Ns (concentration of moles of sites per mg of aorta) $8.4 \pm 4.8 \cdot 10^{-15}$.

The binding index (concentration of sites x association constant) of the non-specific system was $N_{NS}\ K_{NS} = 0.48 \pm 0.06$. Incubating the aorta at 60°C during 60 min (8 experiments performed with ^{3}H-angiotensin II concentrations ranging from 10^{-8} to $5 \cdot 10^{-7}$M), suppressed the specific binding with limited capacity, and left a non-specific binding operating like a partition system $N_{NS_1}\ K_{NS_1} = 0.32 \pm 0.10$.

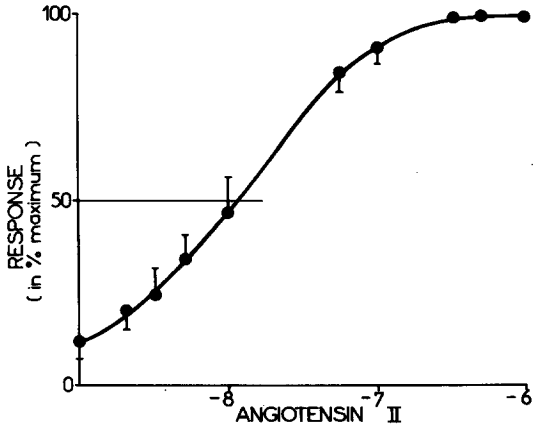

Figure 3 : Dose-response curve to angiotensin II.
Abcissa : log. of molar concentration in the medium.
Ordinate : vasoconstrictive response in % of the maximal response.

TABLE 2

	2×10^{-8}	2.5×10^{-8}	3×10^{-8}	10^{-7}	5×10^{-7}	10^{-6}	BIOLOGICAL ACTIVITIES
ANGIO II	25 %	29 %	33 %				100
2-ORN-ANGIO II	5 %			32 %			20
4-PHE-ANGIO II	0			25 %	24 %		10
6-ALA-ANGIO II	0			0	0	13 %	0.8
3-8 HEXAPEPTIDE				0	13 %	29 %	0.1
8-ALA-ANGIO II				0	0	9 %	0
L-NORADRENALINE				0	0	0	
BRADYKININ				0	0	0	

Comparison between the inhibitory effect of various compounds on radioactivity incorporation and their respective biological activities. The concentration of ^{3}H-angiotensin II was constantly $6.5 \cdot 10^{-8}M$.

Angiotensin and its octapeptide analogues were all in the NH^{2}-1 Asp-5 Val form. Each percentage is the mean of 4 determinations. Biological activities from ref. 10.

The specificity of the high affinity binding has been tested by studying the inhibitory effect of various compounds on the cellular incorporation of ^{3}H-angiotensin II. Unlabelled angiotensin II, angiotensin fragments or analogues, l-noradrenaline or bradykinin were introduced at different concentrations in the bath together with a constant concentration ($6.5 \cdot 10^{-8}$M) of ^{3}H-angiotensin II, and incubated for 2 min. The concentrations of unlabelled angiotensin II varied from $2 \cdot 10^{-8}$M to $3 \cdot 10^{-8}$M, the total concentration of angiotensin in the bath (tritiated and unlabelled) remained therefore within the limits of saturation of the specific system. At concentration of $3 \cdot 10^{-8}$M, unlabelled angiotensin II reduced the incorporation by 33 % (Table 2). Much higher concentrations of angiotensin derivatives were necessary to obtain an inhibitory effect of approximately equal magnitude. A fairly good correlation was found between the inhibitory ability of the different compounds and their respective biological activities. No inhibitory effect was found with two other vasoactive compounds, l-noradrenaline and bradykinin even at concentrations of 10^{-4}M. These observations strongly indicate that the saturable binding sites demonstrated here may be those involved in the biological response.

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