Biochem J. (1982) **208**, 809–817
Printed in Great Britain

The liver angiotensin receptor involved in the activation of glycogen phosphorylase

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(*Received 22 July 1982/Accepted 17 September 1982*)

Specific angiotensin binding to rat hepatocytes and purified liver plasma membranes was measured by using biologically active [^{3}H]angiotensin (sp. radioactivity 14 Ci/mmol). The kinetic parameters for angiotensin binding to hepatocytes are: K_{+1} (association rate constant), $100\,\mu M^{-1}\cdot min^{-1}$; K_{-1} (dissociation rate constant), $2\,min^{-1}$; K_d (dissociation constant), 30 nM; maximal binding capacity, 0.42 pmol/10^6 cells or 260000 sites/cell. Angiotensin binding to membranes is profoundly affected by GTP (0.1 mM) and NaCl (100 mM); these regulatory compounds greatly enhance both the rate of association and of dissociation and also the extent of dissociation. K_d amounts to 10 nM in the presence of GTP + NaCl and to 1.5 nM in their absence; maximal binding capacity is 0.70 pmol/mg of protein, both with or without GTP + NaCl. The relative affinities of 11 angiotensin structural analogues were deduced from competition experiments for [^{3}H]angiotensin binding to hepatocytes and to membranes (in the latter case, GTP + NaCl were not included, in order to study the higher affinity state of the receptor). These are highly correlated with their biological activity (activation of glycogen phosphorylase in hepatocytes). Binding to membranes occurs in the same concentration range as the biological effect. On the other hand, the existence of numerous spare receptors is suggested by the observation that binding of the agonists to hepatocytes requires 25-fold higher concentrations than those needed for their biological activity. These data clearly suggest that the detected binding sites correspond to the physiological receptors involved in the glycogenolytic action of angiotensin on rat liver.

Although more recently identified than the cyclic AMP-dependent glycogenolysis in liver, it is by now well established that there also is a cyclic AMP-independent Ca^{2+}-mediated glycogenolysis in liver (see De Wulf *et al.*, 1980; Kirk *et al.*, 1980; Exton, 1981; Rasmussen & Waisman, 1981). The hormonal factors that govern this process include the α-adrenergic agonists and two hypertensive peptides, vasopressin and angiotensin. According to the current evidence (see the references cited above) the mechanism involves an increase in cytosolic [Ca^{2+}], which will stimulate phosphorylase kinase and lead to the phosphorylation, i.e. activation, of glycogen phosphorylase, the rate-limiting enzyme in glycogenolysis.

Several reports have been published concerning the hepatic α-adrenergic receptors, using different radioligands, such as the natural hormone noradrenaline, and several α-adrenergic antagonists, such as dihydroergocryptine and phenoxybenzamine. The general consensus is that the α-receptors governing glycogenolysis in liver belong to the α_1-subclass, whereas those implicated in the inhibitory control of adenylate cyclase belong to the α_2-subclass (see Fain & Garcia-Sainz, 1980; Schmelck & Hanoune, 1980; Exton, 1981; Jard *et al.*, 1981).

The vasopressin receptor has also been characterized (Cantau *et al.*, 1980). According to Michell

Abbreviations used: Sar, sarcosine; Ail, alloisoleucine; Hil, β-homoisoleucine.

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et al. (1979) liver vasopressin receptors belong to the V_1 class of receptors as opposed to adenylate cyclase-coupled V_2 receptors involved in the tubular effects of vasopressin in the kidney. The comparison of the kinetics of vasopressin binding to intact hepatocytes and purified liver membranes revealed that vasopressin receptors in the two preparations had similar affinities for antagonists, but differed with respect to their affinity for agonists. This difference could be attributed to the effects of endogenous modulators of receptor function operating in intact cells. Indirect experimental evidence has been provided, indicating that triphosphonucleotides and Mg^{2+} might be the main modulators of receptor function (Cantau *et al.*, 1980).

Apart from a report by Lafontaine *et al.* (1979) using ^{125}I-angiotensin and purified liver plasma membranes, no information was available for the binding specificities of the angiotensin receptor possibly involved in the control of liver glycogenolysis. It seemed therefore appropriate to undertake a study of the angiotensin receptor with [^{3}H]angiotensin. A further incentive was a recent report by Jard *et al.* (1981) indicating that, contrary to vasopressin but similarly to the α_2-adrenergic agonists, angiotensin is able to inhibit hepatic adenylate cyclase in a GTP- and Na^+-dependent manner. The question was thus raised whether liver would possess two classes of angiotensin receptors: one implicated in the control of Ca^{2+}-dependent glycogenolysis, the other involved in the inhibition of adenylate cyclase. In line with the results obtained with vasopressin, special attention was paid to the comparison of the kinetics of angiotensin binding to intact cells and purified liver membranes. The results show that liver (both isolated cells and purified plasma membranes) possesses one class of specific angiotensin receptors that is involved in the control of glycogenolysis. They also show that the binding to membranes is controlled by GTP and Na^+. During completion of the present work, it was reported by Garrison and co-workers (Campanile *et al.*, 1982; Crane *et al.*, 1982) using ^{125}I-angiotensin that purified rat liver membranes contain, however, both high- and low-affinity sites for angiotensin. In addition these authors showed that guanine nucleotides in the presence of a cation (Na^+ or Mg^{2+}) markedly reduce the number of high-affinity sites.

Experimental

Biological material

We have used male Wistar-strain albino rats (200–250g body wt.), which were allowed free access to food. Liver cells were isolated as previously described (Vandenheede *et al.*, 1976). Purified plasma membranes were prepared from whole liver by the method of Neville (1968) up to step 11. EDTA (5mM) was present at all steps of membrane preparation. Membranes were stored in liquid N_2 in small portions. These are thawed as needed just before each experiment, washed twice by centrifugation at 27000*g* for 10min in 5mM-Tris/EDTA (pH 7.4)/1mM-$NaHCO_3$ and taken up in 1mM-$NaHCO_3$.

Chemicals

Angiotensin ([Asp^1,Ile^5]angiotensin) was purchased from Schwartz-Mann, Orangeburg, NY, U.S.A., and [Asn^1,Val^5]angiotensin from UCB-Bioproducts, Brussels, Belgium. The following series of angiotensin analogues was obtained from Dr. S. Fermandjian (Service de Biochimie, CEN Saclay, Gif-sur-Yvette, France): [Leu^5]angiotensin, [cyclohexyl-Ala^5]angiotensin, [Sar^1,Hil^5,Ile^8]angiotensin, [dimethyl-Gly^1]angiotensin, [Ala^3]angiotensin, [Sar^1,Ail^5]angiotensin, [Phe^4,Val^5]angiotensin, [Asn^1,D-Arg^2,Val^5]angiotensin, [Sar^1,*N*-methyl-Ile^5,Ile^8]angiotensin and des-Asp^1-angiotensin, the C-terminal heptapeptide from angiotensin. Adenosine 5′-[β,γ-imido]triphosphate, creatine kinase and sodium phosphocreatine were from Boehringer Mannheim G.m.b.H., Mannheim, Germany. GTP was from Sigma Chemical Co., St. Louis, MO, U.S.A. Other sources have been listed previously (Vandenheede *et al.*, 1976; Cantau *et al.*, 1980).

Biological responses to angiotensin and its analogues

Dose-dependent activation of phosphorylase in isolated hepatocytes by angiotensin and its structural analogues was determined as described previously for vasopressin (Keppens & De Wulf, 1979). Pressor effect of angiotensin was studied in the anaesthetized rat by the method of Stürmer (1968).

Preparation, purification and properties of [^{3}H]-angiotensin

(^{3}H-Tyr^4)-labelled [Asn^1,Val^5]angiotensin ([^{3}H]-angiotensin) was prepared by the method of Morgat *et al.* (1970). It was purified by high-pressure liquid chromatography to a specific radioactivity of 14Ci/mmol; its radiochemical purity can be judged from Fig. 1(*c*). We have checked that the labelled angiotensin preparation displayed the same biological activity as the parent peptide as judged by the potency to activate glycogen phosphorylase in hepatocytes and by the vasopressor effect in intact rats.

Measurement of [^{3}H]angiotensin binding to isolated hepatocytes

Cells (0.5×10^6) in 0.5ml of Krebs–Henseleit bicarbonate medium (Krebs & Henseleit, 1932) were

pre-incubated for 20min at 37°C in closed 20ml plastic vials with continuous shaking (120 oscillations/min) in a gas phase of O_2/CO_2 (19:1, v/v) with glucose (10mM), bovine serum albumin (2mg/ml), bacitracin (2mg/ml) and 8-hydroxyquinolinium sulphate (1mM). After this pre-incubation, different amounts of [^{3}H]angiotensin were added. After 3min (except when otherwise stated) 400μl portions were sampled and the reaction was stopped by mixing them with 2vol. of ice-cold Tris/HCl buffer (50mM, pH 7.4) containing 0.15M-NaCl. These samples were immediately filtered at 0°C on Millipore filters (EAWP 02500), prewashed with 5ml of the same buffer. The filters were then washed three times with 7ml of the same ice-cold buffer. The whole procedure took less than 40 s; no wash-off of bound [^{3}H]angiotensin occurred at 0°C, since no release of radioactivity was detected at this temperature even in these diluted conditions. The radioactivity on the filters was counted by liquid-scintillation spectrometry, as previously described (Cantau *et al.*, 1980). Non-specific binding was determined by adding a 200-fold excess of unlabelled angiotensin and was less than 20% of total binding for a concentration of free [^{3}H]angiotensin of 20nM. In preliminary experiments, we have verified that the binding of [^{3}H]-angiotensin increased linearly with the number of cells up to 10^7 cells/ml. The dissociation constants of the angiotensin analogues were deduced from competition experiments designed to determine the dose-dependency for inhibition of [^{3}H]angiotensin (8nM) binding by the unlabelled analogues, as described previously for the vasopressin receptor (Cantau *et al.*, 1980).

Measurement of [^{3}H]angiotensin binding to purified liver membranes

Except when otherwise stated, binding assays were done at 37°C in 0.5ml containing triethanolamine/HCl, pH 7.4 (50mM), $MgCl_2$ (3mM), NaCl (100mM), GTP (0.1mM), bovine serum albumin (1mg/ml), bacitracin (0.5mg/ml), 8-hydroxyquinolinium sulphate (1mM) and different concentrations of [^{3}H]angiotensin; adenosine 5'-[β,γ-imido]triphosphate (30μM) and a GTP-regenerating system composed of creatine kinase (0.1mg/ml) and sodium phosphocreatine (10mM) were also added. The reaction was initiated by the addition of the washed membranes (corresponding to 20μg of protein) and stopped, except when otherwise indicated, 15min later by the addition of 2ml of ice-cold Tris/HCl buffer (10mM, pH 7.4) containing 3mM-$MgCl_2$. The diluted samples were filtered on Millipore filters (EAWP 02500) as described for the experiments with hepatocytes. Non-specific binding was determined in the presence of 1μM-unlabelled angiotensin and was less than 15% for a concentration of [^{3}H]angiotensin of 5nM. It has been verified that binding of [^{3}H]angiotensin is a linear function of the amount of membranes used up to at least 100μg of protein, as determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The dissociation constants of the angiotensin analogues were deduced by competition experiments with 1nM-[^{3}H]angiotensin (see the experiments with hepatocytes) in the absence of NaCl and GTP (and of the GTP-regenerating system).

Stability of angiotensin

From the work of Lafontaine *et al.* (1979) we knew that angiotensin is extensively metabolized by liver preparations. Fig. 1 illustrates the degree of hormonolysis by hepatocytes and purified plasma membranes. Hepatocytes were mixed with increasing concentrations of angiotensin. Portions were withdrawn either immediately or after 15min of pre-incubation and added for 90s to a series of fresh cell suspensions, for the estimation of their potency to activate glycogen phosphorylase. It can be seen that after the 15min pre-incubation period, the K_a for angiotensin is shifted from 0.3nM (Fig. 1*a*) to 2nM (Fig. 1*b*). However, when 1mM-8-hydroxyquinolinium sulphate is present during the pre-incubation, no hormonolysis is apparent since the K_a remains at 0.2nM (Fig. 1*b*). Figs. 1(*c*) and 1(*d*) illustrate the degree of stability of angiotensin when incubated with purified plasma membranes. Without 8-hydroxyquinolinium sulphate, extensive degradation occurs, as indicated by the almost complete disappearance (Fig. 1*d*) of the peak of radioactivity typical of non-incubated [^{3}H]angiotensin (Fig. 1*c*) and the appearance of an important peak representing degradation products (Fig. 1*d*). The inclusion of the proteinase inhibitor in a more diluted system offers a satisfactory protection, as shown also by the absence of degradation products (Fig. 1*d*); these conditions were retained for the binding assays. The same Figure (*e* and *f*) also illustrates that the radioactivity associated with the plasma membranes and afterwards released by an acid treatment is barely distinguishable from the original label similarly treated, showing that no significant hydrolysis of angiotensin occurred once bound to its receptor.

Results

Specific binding of [^{3}H]angiotensin to intact hepatocytes was time-dependent and essentially reversible on the addition of an excess of unlabelled peptide (Fig. 2). The amount of specifically bound hormone increased progressively up to a plateau value; the level of this plateau and the time required to reach it were clearly dependent on the hormone

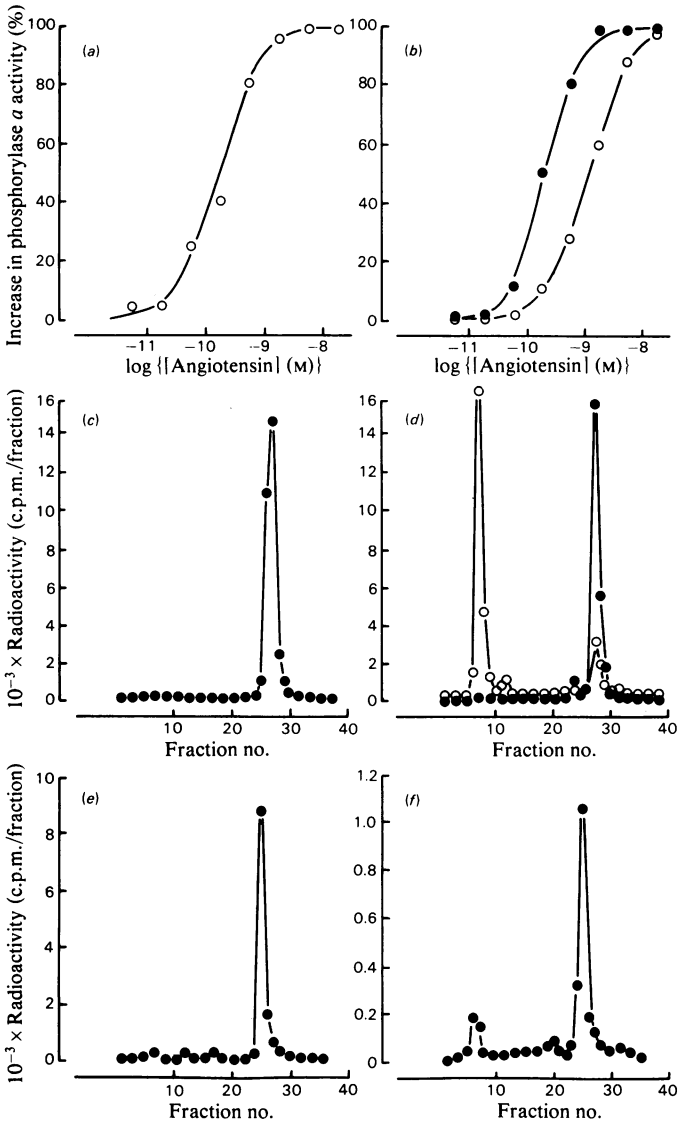

Fig. 1. *Stability of the angiotensin preparations used*

(*a* and *b*) Hepatocytes (10^6 cells/ml) were mixed with increasing concentrations (from 0.16nM to 0.4μM) of angiotensin. Portions (50μl) were withdrawn either at once (*a*) or after 15min of pre-incubation with (●) or without (○) 1mM-8-hydroxyquinolinium sulphate (*b*); these were then added to a series of fresh cell suspensions (5 × 10^6 cells/ml); 90s later, samples were taken for the assay of glycogen phosphorylase. (*c* and *d*) Plasma membranes (50μg of protein) were incubated for zero (*c*) or for 30min (*d*, ○) at 30°C with 5nM-[^{3}H]angiotensin in a final volume of 0.1ml in the absence of hydroxyquinolinium sulphate, or of 0.5ml for the incubation in the presence of 1mM-8-hydroxyquinolinium sulphate (*d*, ●). Proteins were precipitated with an equal volume of trichloroacetic acid (5%, w/v); the acid was extracted by repeated washes with diethyl ether. Portions were analysed by high-pressure liquid chromatography equipped with a Waters column (μ-bondpack C18). Elution at 1.2ml/min was by a linear gradient made from the following media: A, methanol/water/1M-ammonium acetate, pH4.0 (10:40:1, by vol.); B, methanol/water/1M-ammonium acetate, pH4.0 (40:10:1, by vol.); 0.6ml fractions were collected (*e* and *f*) Plasma membranes (150μg of protein) [in the presence of 1mM-8-hydroxyquinolinium sulphate] were incubated for 30min at 30°C with 10nM-[^{3}H]angiotensin in a final volume of 5ml. The radioactive material retained by the membranes was precipitated by three centrifugations at 20000 *g* for 10min at 0°C. The final pellet was treated for 10min at room temperature with 0.5ml of 0.1M-HCl. The supernatant (20000 *g* for 10min) was analysed by high-pressure liquid chromatography (*f*). (*e*) Shows the elution profile of similarly treated [^{3}H]angiotensin in the absence of membranes. This Figure shows results that are representative of several (two to three) similar experiments.

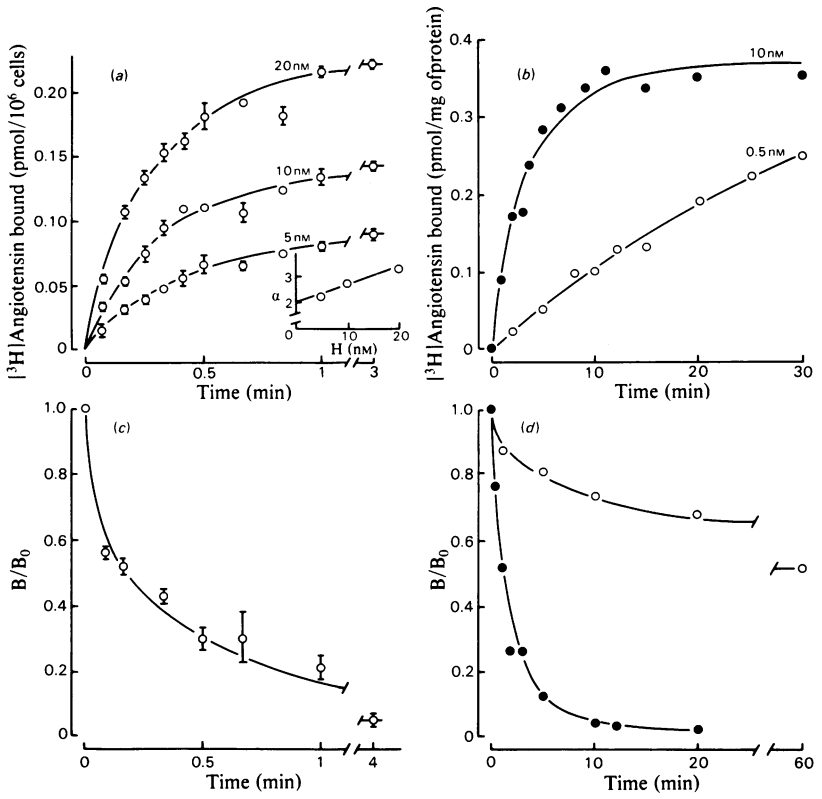

Fig. 2. *Time courses of* [^{3}*H*]*angiotensin binding to hepatocytes and purified liver membranes*

(*a* and *b*) These Figures show results for association. Purified liver membranes (15 μg of protein), with (●) or without (○) 0.1 mM-GTP and 100 mM-NaCl (*b*) or isolated hepatocytes (0.5×10^6 cells) (*a*) were incubated in the presence of the indicated concentrations of [^{3}H]angiotensin. Specific binding is given as means ± S.E.M. for three experiments (hepatocytes) or as means of two experiments done in triplicate (membranes). In the case of the experiments with hepatocytes, where three different hormone concentrations were used, binding can be expressed as $B = B_{eq.}(1 - e^{-\alpha t})$, where $\alpha = K_{-1} + K_{+1}H$, B = specific binding at time *t*, $B_{eq.}$ = specific binding at equilibrium, K_{+1} and K_{-1} are the rate constants of the formation and dissociation of the hormone–receptor complexes and H = free hormone concentration. The inset gives α as a linear function of H, from which K_{+1} and K_{-1} values of 100 $\mu M^{-1} \cdot min^{-1}$ and 2 min^{-1} can be estimated, yielding a $K_d = K_{-1}/K_{+1} = 20$ nM. (*c*) and (*d*) show results for dissociation. (*c*) After 5 min of pre-incubation of hepatocytes with 10 nM-[^{3}H]angiotensin, an excess of unlabelled hormone (2 μM) was added. Values represent fractions of B_0 (specific binding at zero min) and are given as means ± S.E.M. for three experiments yielding a K_{-1} of 2 min^{-1}. (*d*) After 15 min of pre-incubation of plasma membranes (20 μg of protein) with 5 nM-[^{3}H]angiotensin with (●) or without (○) 0.1 mM-GTP and 100 mM-NaCl, 5 μM-unlabelled angiotensin was added. Values of B/B_0 are means of two experiments done in triplicate. From the approximately exponential curve, a $t_{\frac{1}{2}}$ of 1.5 min can be deduced, yielding an estimated value of about 0.5 min^{-1} for K_{-1}.

concentration used. The observed time course of [^{3}H]angiotensin binding could be accounted for by a pseudo-first-order type of reaction. Indeed, only a small fraction of the hormone gets bound at equilibrium, so that the free hormone concentration corresponds to the total concentration. From these data, we have calculated the values for K_{+1} and K_{-1} to be 100 $\mu M^{-1} \cdot min^{-1}$ and 2 min^{-1} respectively (see the legend to Fig. 2). Specific [^{3}H]angiotensin binding to hepatocytes was freely and almost completely reversible within 1 min, yielding a dissociation rate constant equal to 2 min^{-1}. Binding of [^{3}H]angiotensin to purified liver plasma membranes was markedly dependent on the presence of GTP and NaCl. In the presence of these effectors, angiotensin binding is a completely reversible and fairly rapid process (although slower than the one observed on intact hepatocytes). In the absence of GTP and NaCl, angiotensin binding was slow and partially irreversible (Fig. 2).

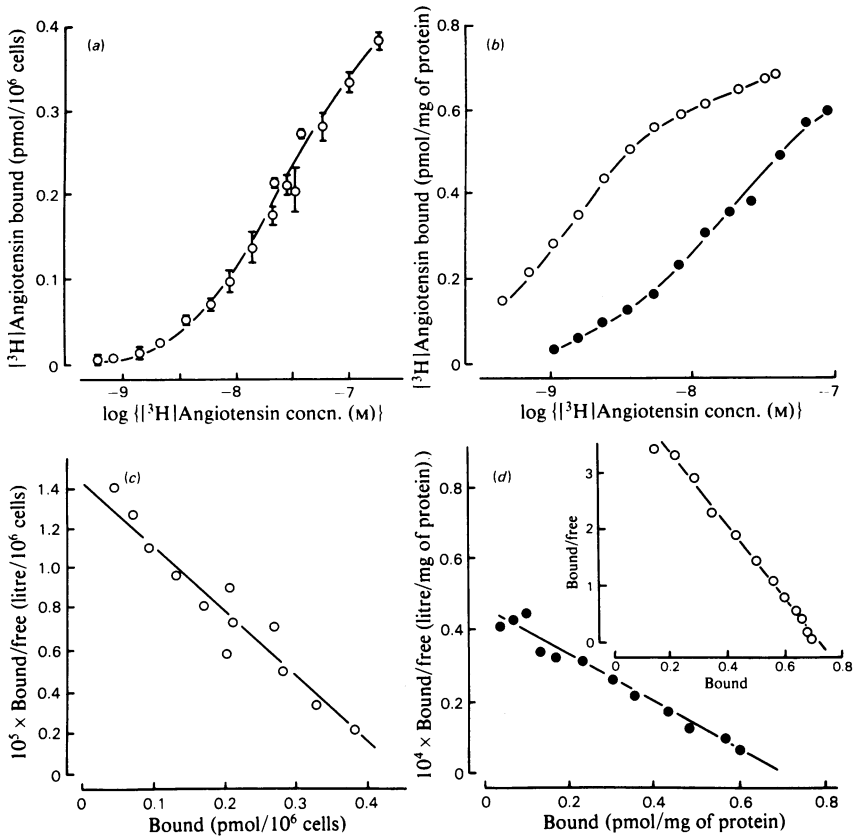

Fig. 3. *Dose-dependency of specific [3H]angiotensin binding to isolated hepatocytes and purified liver membranes*
(*a* and *b*) Values for specific [3H]angiotensin binding are given as means of three experiments ±S.E.M. (hepatocytes; *a*) or as means of two experiments done in triplicate in the absence (○) or presence (●) of 0.1 mM-GTP and 100 mM-NaCl (membranes; *b*). (*c*) and (*d*) show Scatchard (1949) plots of the data of (*a*) and (*b*) respectively. K_d values and maximal binding capacities estimated from the regression lines are respectively: hepatocytes, 30 nM and 0.42 pmol/10^6 cells; membranes without GTP and NaCl, 10 nM and 0.70 pmol/mg of protein; membranes in the presence of GTP and NaCl, 1.5 nM and 0.70 pmol/mg of protein.

Angiotensin binding to hepatocytes was saturable (Fig. 3). Scatchard (1949) analysis revealed the presence of one class of sites with an apparent dissociation constant of 30 nM. When determined in the presence of GTP and NaCl, dose-dependent angiotensin binding to purified liver membranes also indicated the presence of only one class of sites with a dissociation constant of 10 nM (Fig. 3). Although angiotensin binding to membranes incubated in the absence of GTP and NaCl was only partially reversible (Fig. 2), determination of dose-dependent binding after 30 min incubation in the presence of the labelled hormone led to a linear Scatchard plot with an apparent dissociation constant of 1.5 nM (Fig. 3). Altogether these results indicate that GTP and NaCl convert angiotensin binding sites on liver membranes into a low-affinity state and that these sites in intact cells are possibly in the low-affinity state. The following maximal binding capacities were calculated from the regression lines: 0.75 pmol/mg of membrane protein (with or without GTP + NaCl) and 0.42 pmol/10^6 cells (or 260000 angiotensin receptor sites/hepatocyte).

As also obtained for vasopressin (Cantau *et al.*, 1980) dose-dependent activation of glycogen phosphorylase in intact hepatocytes with [3H]angiotensin (Fig. 4) occurred in a much lower concentration range ($K_a = 0.6$ nM; see also Table 1) than that for binding ($K_d = 25$ nM; see also Fig. 3 and Table 1). This observation suggests the existence of numerous spare receptors with some amplification step(s) between hormone binding and the final physiological response.

Next, experiments were designed to check the

inherent assumption made that the sites revealed by [^{3}H]angiotensin binding represent the biological receptors mediating the angiotensin-induced phosphorylase activation. We have compared the relative biological potencies of a series of angiotensin analogues and their abilities to interact with the detected binding sites on hepatocytes and purified membranes. Except for one peptide (identified as partial agonist in Table 1), the agonistic peptides activated glycogen phosphorylase in liver cells as much as the parent peptide, yielding parallel dose–response curves when plotted as in Fig. 4. They also inhibited [^{3}H]angiotensin binding to the same extent as unlabelled angiotensin, both with hepatocytes and with liver membranes; the studies with the membranes were performed in the absence of GTP and NaCl in order to detect the high-affinity state of the receptor (see Fig. 3). The experimental results obtained for the series of analogues are given in Table 1 and expressed as pK_a ($-\log K_a$) and pK_d ($-\log K_d$) values. The highly significant correlations between pK_a and pK_d values of fully agonistic analogues clearly are in favour of the existence of specific angiotensin receptors involved in transmitting the hormonal signal to the final activation of glycogen phosphorylase in intact cells (Fig. 5). Fig. 5(*a*) shows that the pK_a values and the corresponding pK_d values for membranes are of the same order of magnitude. On average, the agonistic angiotensin analogues were bound to the cells with a K_d about 25 times higher than their respective K_a, over the range of two orders of magnitude (Table 1 and Fig. 5*b*).

We have also checked that [^{3}H]angiotensin binding to hepatocytes and membranes was not inhibited by other cyclic AMP-independent glycogenolytic agonists, such as vasopressin and α-adrenergic agonists, nor by glucagon (results not shown).

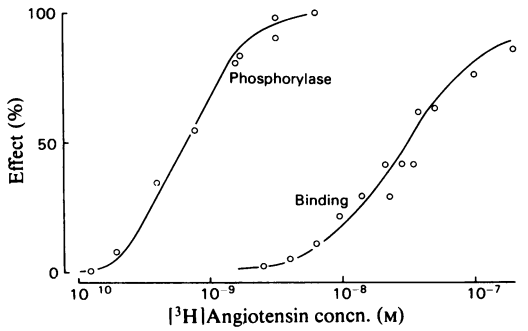

Fig. 4. *Relationship between phosphorylase activation and [^{3}H]angiotensin binding*
Data of a typical experiment are expressed as percentage values of the corresponding maxima.

Discussion

The data confirm and extend the observations of Lafontaine *et al.* (1979) and the very recent reports by Campanile *et al.* (1982) and Crane *et al.* (1982) on the presence of specific angiotensin-binding sites on purified liver membranes. However, these authors have used ^{125}I-angiotensin (of an unspecified biological activity) and a direct comparison with our data is therefore not easy. In particular, they have reported on the presence of binding sites with higher

Table 1. *Comparison of the pK_a and pK_d values of angiotensin structural analogues*
Apparent affinity constants for phosphorylase activation (K_a) were computed as described by Bréant *et al.* (1981) and expressed in terms of pK_a($-\log K_a$). Dissociation constants for the binding were calculated from data obtained in competition experiments with [^{3}H]angiotensin (see the Experimental section) and also expressed in terms of pK_d. Peptide 12 is a partial agonist. Values listed are means ± S.D. (*n*) except when only one was available.

	Hepatocytes		Membranes
	pK_a	pK_d	pK_d
1. Angiotensin	9.5 ± 0.1 (5)	8.0 ± 0.2 (6)	8.7 ± 0.1 (5)
2. [Asn^1,Val^5]Angiotensin	8.9 ± 0.2 (2)	7.6 ± 0.2 (5)	8.5 ± 0.1 (3)
3. [Leu^5]Angiotensin	9.2 ± 0.1 (3)	7.5 ± 0.2 (4)	8.6 ± 0.2 (3)
4. [cyclohexyl-Ala^5]Angiotensin	9.4 ± 0.1 (3)	7.9 ± 0.2 (3)	8.8 ± 0.1 (3)
5. [Sar^1,Hil^5,Ile^8]Angiotensin	7.4 ± 0.1 (4)	6.0 ± 0.3 (2)	7.5 ± 0.3 (3)
6. [dimethyl-Gly^1]Angiotensin	8.3 ± 0.1 (4)	7.1 ± 0.3 (3)	8.0 ± 0.2 (3)
7. [Ala^3]Angiotensin	8.8 ± 0.2 (3)	7.5 ± 0.2 (2)	8.0 ± 0.1 (3)
8. [Sar^1,Ail^5]Angiotensin	10.4 ± 0.1 (2)	8.4 ± 0.1 (2)	9.0 ± 0.1 (3)
9. [Phe^4, Val^5]Angiotensin	7.8 ± 0.2 (3)	6.5	7.5 ± 0.1 (3)
10. Des-Asp^1-angiotensin	8.1	6.7	7.6 ± 0.2 (3)
11. [Asn^1,D-Arg^2,Val^5]Angiotensin	7.0 ± 0.1 (2)	5.9	7.1 ± 0.2 (3)
12. [Sar^1,*N*-methyl-Ile^5,Ile^8]Angiotensin	7.3 ± 0.1 (3)	6.8 ± 0.1 (2)	7.8 ± 0.1 (3)

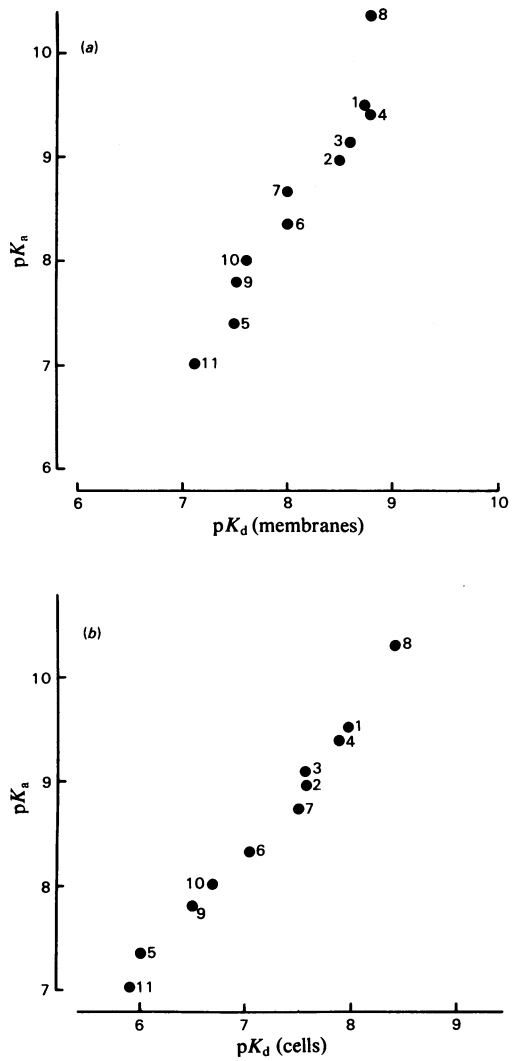

Fig. 5. *Correlation between pK_a and pK_d values for full agonists*

(*a*) Correlation between pK_a values and pK_d values for membranes ($r = 0.968$). (*b*) Correlation between pK_a values and pK_d values for cells ($r = 0.988$). The numbers represent the analogues listed in Table 1.

affinity [Lafontaine *et al.* (1979): $K_d = 0.1$ nM; Campanile *et al.* (1982) and Crane *et al.* (1982): $K_{d1} = 0.21$–0.46 nM] than those found in our study ($K_d = 1.5$ nM in the absence of GTP + NaCl). In addition, the latter authors also describe a class of low-affinity binding sites ($K_{d2} = 3$–4 nM) overall resulting in a curvilinear Scatchard plot, in contrast with our results (Fig. 3). This discrepancy can also be caused by minor differences in the procedures for the preparation of liver membranes, and/or by the use of different temperatures in the incubations. Our main contribution has been to provide a characterization of angiotensin receptors on isolated hepatocytes incubated in experimental conditions where the glycogenolytic response to angiotensin could be expressed and was measured.

The strong correlation (Fig. 5) between the glycogenolytic potencies of a series of angiotensin analogues and their affinities for binding to isolated hepatocytes clearly suggest that the detected binding sites are the hormonal receptors involved in the control of glycogenolysis in liver by angiotensin. However, it must be pointed out that for all angiotensin analogues studied dose-dependent phosphorylase activation is displayed in a 25-fold lower concentration range than dose-dependent binding. The existence of numerous spare receptors implying that a maximal response is elicited by the occupation of only a small fraction of the receptors available could account for the observed differences between pK_a and pK_d values. This situation is not at all exceptional and has, e.g., been shown to occur with the vasopressin-mediated control of liver glycogen breakdown (Cantau *et al.*, 1980). It seems clear that some intermediary event serves as an amplifying step between hormone binding and the final biological response. As discussed previously for vasopressin (Kirk *et al.*, 1979, 1981; Cantau *et al.*, 1980), inositol lipid degradation could function in this manner. Whether such a degradation is involved for angiotensin cannot yet be assessed on the basis of the data available (Billah & Michell, 1979) since these studies have been carried out in the absence of specific inhibitors of angiotensin hydrolysis (see the Experimental section).

The results obtained with purified membranes suggest another possible interpretation for the observed difference between pK_a and pK_d values. As reported by Crane *et al.* (1982) and independently shown in the present study, angiotensin-binding sites on purified liver membranes can exist under two affinity states. A high-affinity state could be detected in the absence of GTP and Na^+. Although it was difficult to determine the dissociation constant for angiotensin binding to the high-affinity state owing to the partial reversibility of the binding process, we show that the apparent K_d values for angiotensin and angiotensin analogues are close to the corresponding K_a values. It is therefore possible that the native state of the angiotensin receptor in intact cells is a high-affinity state rapidly converted into a low-affinity state under the concerted influence of binding to the receptor of an agonist and of endogenous modulators of receptor function including GTP and univalent ions.

Studies of angiotensin binding to intact hepatocytes and purified membranes failed to reveal any marked heterogeneity of angiotensin receptors

with respect to their recognition patterns towards a series of angiotensin structural analogues. Although there is evidence from the results obtained with purified membranes that angiotensin receptors can exist under different affinity states, there is also evidence that these states are at least partially interconvertible. Finally there is no indication suggesting the existence of types of angiotensin receptors respectively involved in Ca^{2+}-dependent activation of phosphorylase and in inhibition of adenylate cyclase.

In conclusion, both purified liver plasma membranes and isolated hepatocytes possess specific receptors for angiotensin that are responsible for hormone binding and very likely mediate the cyclic AMP-independent control of liver glycogenolysis by angiotensin. It seems therefore that the hepatocytes can provide a model to further study the initial step(s) in the cellular action of angiotensin not involving cyclic AMP.

We thank Dr. S. Fermandjian for the generous supply of the angiotensin analogues; G. Cumps for skilful technical assistance, M. Coppens for careful typing of the manuscript and J. Gilliard for drawing the illustrations. This work was supported by Institut National de la Santé et de la Recherche Médicale (F.R.A. 29), Centre National de la Recherche Scientifique (L.A. 219), Fondation pour la Recherche Médicale (France) and Fonds voor Geneeskundig Wetenschappelijk Onderzoek (Belgium).

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