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TWO DISTINCT α_1-ADRENERGIC RECEPTOR SITES IN RAT LIVER: DIFFERENTIAL BINDING OF (−)-[^{3}H]NOREPINEPHRINE, [^{3}H]PRAZOSIN AND [^{3}H]DIHYDROERGOCRYPTINE

EFFECTS OF GUANINE NUCLEOTIDES AND PROTEOLYSIS; IMPLICATIONS FOR A TWO-SITE MODEL OF α-RECEPTOR REGULATION

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Abstract—(−)-[^{3}H]Norepinephrine, [^{3}H]prazosin and [^{3}H]dihydroergocryptine bind to rat liver plasma membranes in a manner indicating a selective interaction with α_1-adrenergic receptors. All three ligands display monophasic saturation with a single component on Scatchard analysis. The binding capacities of (−)-[^{3}H]norepinephrine, [^{3}H]prazosin and [^{3}H]dihydroergocryptine are 340 ± 70 fmol/mg of protein 760 ± 40 fmol/mg of protein and 1200 ± 300 fmol/mg of protein, respectively. Differential drug potencies in competing for (−)-[^{3}H]norepinephrine and [^{3}H]prazosin binding sites suggest that these two ligands label two distinct binding sites at the α_1-adrenergic receptor, while [^{3}H]dihydroergocryptine labels both sites. Guanine nucleotides lower the apparent affinity of (−)-[^{3}H]norepinephrine for its binding site, without affecting the number of sites or the binding of [^{3}H]prazosin and [^{3}H]dihydroergocryptine. Incubation of membranes with α-chymotrypsin slightly reduces the binding of [^{3}H]prazosin and [^{3}H]dihydroergocryptine, but causes a 2-fold increase in (−)-[^{3}H]norepinephrine binding. Both the number of (−)-[^{3}H]norepinephrine binding sites and the affinity are increased. Following proteolysis, (−)-[^{3}H]norepinephrine binding still occurs to a single class of sites, but is no longer affected by guanine nucleotides. The effect of α-chymotrypsin is abolished by pretreatment of membranes with the irreversible α-adrenergic antagonist phenoxybenzamine. We propose that in rat liver, (−)-[^{3}H]norepinephrine labels the physiologically active form of the α-adrenergic receptor while [^{3}H]prazosin binds to a precursor form of the active receptor, and/or to an α_1-adrenergic binding site not coupled to an effector system.

In the past few years, a great deal of effort has been focused on the development of a means of studying the α-adrenergic receptor by direct radioligand binding techniques. To date, radiolabeled antagonists, as well as agonists, mixed agonist–antagonists and irreversible α-adrenergic blocking agents have been used to label α-adrenergic receptors in various systems [1–3]. For rat liver plasma membranes, Guellaen *et al.* [4] and Clarke *et al.* [5] have used the ergot alkaloid [^{3}H]dihydroergocryptine† to identify and characterize binding sites which display properties characteristic of an α-adrenergic receptor. Both groups have reported that tritiated dihydroergocryptine binds to a single class of high affinity binding sites with a binding capacity of about 1500 fmol/mg of protein and a dissociation constant of 2–5 nM; a wide variety of adrenergic drugs that compete for the binding sites displays appropriate specificity and stereospecificity for an interaction with presumed α-adrenergic receptors. These results have been confirmed by the recent report of Guellaen *et al.* [2] who have demonstrated that the covalent α-adrenergic blocker [^{3}H]phenoxybenzamine labels irreversibly a protein with a binding capacity of about 1500 fmol/mg of protein and all the characteristics expected for an α-adrenergic receptor. It has been further established in our laboratory [6] that the activation of glycogen phosphorylase in isolated rat hepatocytes, is mediated by an α_1-adrenergic receptor and that the order of potencies of both adrenergic agonists and antagonists are similar in [^{3}H]DHE binding and glycogen phosphorylase experiments. However, El-Refai *et al.* [7] have recently shown that [^{3}H]catecholamines bind to two classes of binding sites in rat liver, one with a low capacity (about 150 fmol/mg or protein) and high affinity for natural α-adrenergic agonists (K_D = 50 nM), the other with a large capacity (about 500 fmol/mg of protein) and low affinity for natural α-adrenergic agonists (K_D = 350 nM). Nevertheless they have also found that [^{3}H]DHE binds to a single class of sites with characteristics similar to those reported by Guellaen *et al.* [4] and Clarke *et al.* [5]. According to El-Refai *et al.* [7], only the high affinity class of catecholamine binding sites would represent physiological α-adrenergic receptors, since α-adrenergic agonist binding

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† Abbreviations: [^{3}H]DHE, [^{3}H]dihydroergocryptine; [^{3}H]NOR, (−)-[^{3}H]norepinephrine; [^{3}H]PRA, [^{3}H]prazosin; POB, phenoxybenzamine, WB4101, 2-([2′,6′ - dimethoxy] - phenoxyethylamino)methylbenzodioxane; Gpp(NH)p, guanyl-5′-yl imidodiphosphate.

properties to this site fit well with the effects of catecholamines on phosphorylase activation and calcium efflux in isolated rat hepatocytes. Therefore, the results of studies on the hepatic α-adrenergic receptor using direct radioligand binding techniques have been conflicting and the group of Exton [7] have questioned the general validity of using [^{3}H]DHE to study the characteristics of physiological α-adrenergic receptors. In order to resolve the apparent contradictions concerning the α-adrenergic receptor of rat liver and to answer the questions concerning the validity of using non natural compounds to study α-adrenergic receptors, we decided to specifically label the α-adrenergic binding sites of rat liver plasma membranes with different compounds including a natural α-adrenergic agonist, (−)-[^{3}H]norepinephrine, an α_1-specific α-adrenergic antagonist, [^{3}H]prazosin and the non selective agent, [^{3}H]dihydroergocryptine. Our data support the view that two discrete populations of α_1-adrenergic binding sites are present in rat liver membranes; they appear to correspond to two distinct states of a single α_1-adrenergic receptor, one of which probably represents an inactive uncoupled state of the physiological α-adrenergic receptor. The two states can be converted into a unique type of α-adrenergic binding site by proteolysis.

EXPERIMENTAL PROCEDURES

Materials

Phentolamine (Ciba-Geigy), phenoxybenzamine (Smith, Kline and French), dihydroergocryptine (Sandoz), prazosin (Pfizer), yohimbine (Rousse-Uclaf), (−)-propranolol (Imperial Chemical Industries), (−)- and (+)-norepinephrine, (−)- and (+)-epinephrine, (−)-isoproterenol (Sterling-Winthrop), clonidine (Boehringer-Ingelheim), iproniazide, ascorbic acid (Hoffman-La Roche) were obtained as gifts; (−)-phenylephrine, catechol, dopamine, GTP, CTP, UTP (Sigma); GDP, Gpp(NH)p, ATP (Boehringer-Mannheim); soybean trypsin inhibitor (Worthington or Sigma); α-chymotrypsin (EC 3.4.21.1, 53 U/mg, batch No. CDI, 8LK) (Worthington) were from the commercial sources indicated. All other chemicals were from Merck (Darmstadt, West Germany) and of analytical grade. (−)-7,8-[^{3}H]Norepinephrine (20–25 Ci/mmol) and 9,10-[^{3}H]dihydroergocryptine (24–30 Ci/mmol) were supplied by New England Nuclear Co. (Boston, MA). [^{3}H]Prazosin (33 Ci/mmol) prepared at the Radiochemical Centre (Amersham, U.K.) by reduction of bromoprazosin with tritium gas, was a generous gift of Dr P. Hodges (Pfizer, Sandwich, U.K.). Radiochemical purity of [^{3}H]prazosin was greater than 98 per cent as determined by t.l.c. on silica-gel in ethyl acetate/methanol/diethylamine (80:20:1) Purities of (−)-[^{3}H]norepinephrine and [^{3}H]dihydroergocryptine were routinely checked by t.l.c. on cellulose F_{254} in *n*-butanol/water/acetic acid (125:75:45) and on silica gel in chloroform/benzene/ethanol (4:2:1), respectively. Purities were always greater than 95 per cent.

Methods

Liver plasma membranes. Liver plasma membranes were prepared from female, albino, Wistar rats (100–200 g body weight) according to the procedure devised by Neville [8] up to step 11. The membrane preparations were stored in liquid nitrogen until use. Membrane protein was estimated according to Lowry *et al.* [9] using bovine serum albumin as standard.

Proteolytic treatment of liver membranes. Unless otherwise specified, liver membranes containing 400–800 μg of protein were incubated with 20 to 50 μg of α-chymotrypsin in 33 mM Tris-HCl, 6.6 mM $MgCl_2$, pH 7.4, at 25°. After 5 min, 200–500 μg of soybean trypsin inhibitor (10-fold excess over α-chymotrypsin) were added and the incubation was continued for another 2 min at 25°, before placing the incubation tubes on ice. The final volume of the reaction was 100 or 200 μl in 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4. Treated membranes were directly used for binding assays.

Binding assays. α-Adrenergic receptor binding was assayed according to El-Refai *et al.* [7], with slight modifications. Unless otherwise stated, liver membranes containing 20–800 μg of protein were incubated with either 100 nM [^{3}H]NOR, 10 nM [^{3}H]DHE, or 0.5 nM [^{3}H]PRA in a final volume of 400 μl containing 0.75 mM ascorbate, 3 mM catechol, 10 μM (−)propranolol, 25 μM iproniazide and 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4, at 25°. The assay mixture was similar for all three ligands. At the end of the 30 min incubation, duplicate or triplicate 100 μl aliquots were diluted with 4 ml of ice-cold buffer and immediately filtered under vacuum through Whatman GF/C glass fiber filters presoaked in buffer containing 0.75 mM ascorbate and 3 mM catechol. Filters were rapidly washed with 15 ml of ice-cold buffer and counted in 10 ml of Ready-solv EP liquid scintillation mixture (Beckman). Dilution with 4 ml of ice-cold buffer did not modify specific binding in a detectable manner. Specific binding was defined as the difference between binding of the radioligand in the absence and in the presence of 10 μM phentolamine. In all the results expressed as [^{3}H]ligand bound, only the specific binding was considered. The amount of ligand bound in the assay was linearly related to the amount of membrane protein up to 2.0 mg/ml, final concentration. Under these conditions, specific binding represented 40–60 per cent, 75–80 per cent and 90–95 per cent of total radioactivity retained on the filters, at 100 nM [^{3}H]NOR, 10 nM [^{3}H]DHE and 0.5 nM [^{3}H]PRA, respectively. As reported by El-Refai *et al.* [7], ascorbate and $MgCl_2$ were added to binding assays to prevent ligand degradation and membrane aggregation, respectively. Thin layer chromatograms of [^{3}H]NOR after incubation for 30 min at 25° both in the absence and presence of membranes revealed that all of the radioactivity in the supernatant chromatographed in a single peak at the same location as unincubated ligand (not shown). As expected, catechol (0.1–3 mM) reduced total binding but did not inhibit specific binding. Furthermore, it was found that α-adrenergic receptors in rat liver could not be reliably labeled by [^{3}H]NOR in the absence of catechol. Catechol did not reduce the non-specific binding of [^{3}H]PRA and [^{3}H]DHE. Assays of untreated and α-chymotrypsin-treated membranes were always run in parallel.

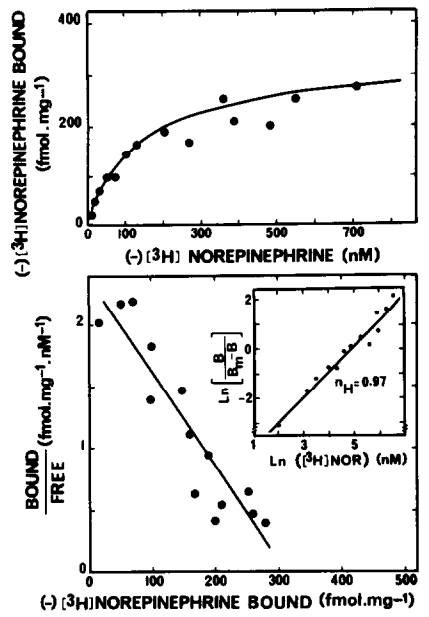

Fig. 1. (−)-[^{3}H]Norepinephrine binding to hepatic plasma membranes as a function of increasing concentrationsof (−)-[^{3}H]norepinephrine. Plasma membranes (760 μg/assay) were incubated at 25° for 30 min, as described in Methods, with various concentrations of [^{3}H]NOR, in the absence or presence of 10 μM phentolamine. Points shown are those obtained in a single experiment, performed in triplicate, which was replicated six times with different membrane preparations. The lower panel shows a Scatchard analysis of the data. Linear regression analysis yielded one straight line with a correlation coefficient of 0.92 ($P < 0.001$). Bound/free is expressed in fmol . mg^{-1}.nM^{-1} and bound is expressed in fmol . mg^{-1}. In this typical experiment, the receptor density (B_{max}) was 311 fmol/mg of protein and the K_D value was 130 nM. Average values are B_{max} = 340 ± 70 fmol/mg of protein and K_D = 130 ± 60 nM. Inset: Hill plot of the data with n_H = 0.97 (correlation coefficient 0.98; $P < 0.001$).

RESULTS

Equilibrium studies of (−)-[^{3}H]*norepinephrine binding*

Figure 1 shows the specific binding of [^{3}H]NOR to rat liver plasma membranes as a function of radioligand concentration. [^{3}H]NOR binding was saturable with increasing concentrations of ligand. Nonspecific binding measured in the presence of 10 μM phentolamine increased linearly between 0 and 600 nM[^{3}H]NOR (not shown). Specific binding appeared to plateau at about 300 nM. Half maximal binding occurred at about 120 nM. Scatchard analysis of the data (Fig. 1, lower panel) indicated a single component of binding with an apparent dissociation constant (K_D) of 130 nM. The mean ± standard error for six similar experiments was 130 ± 60 nM. The calculated maximal number of binding sites (B_{max}) from six experiments was 340 ± 70 fmol/mg of membrane protein. A plot of the data according to the Hill equation revealed a single straight line with a Hill coefficient (n_H) of 0.97, indicating the absence of heterogeneity and/or cooperative interactions.

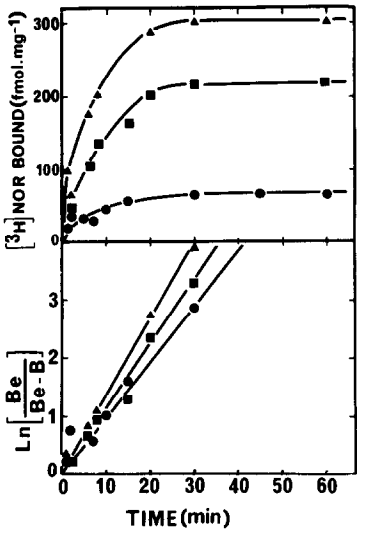

Fig. 2. Association time course of (−)-[^{3}H]norepinephrine binding to plasma membranes at 25°. Rat liver plasma membranes (about 700 μg/assay) were incubated at 25° with 60 nM (●), 112 nM (■) and 215 nM (▲) [^{3}H]NOR in the absence or presence of 10 μM phentolamine. Aliquots of 100 μl were removed at different times and specifically bound radioligand was determined as described in Methods. Values shown are averages of triplicate determinations. Each curve was performed with different membrane preparations. The lower panel shows the same data plotted as ln Be/(Be–B) vs time. Values for k_{ob} were 0.098, 0.108 and 0.128 min^{-1} for [^{3}H]NOR concentrations of 60, 112 and 215 nM, respectively.

Kinetics of (−)-[^{3}H]*norepinephrine binding*

The time course of association of [^{3}H]NOR to rat liver plasma membranes was studied at 25°, under pseudo-first order conditions, with three concentrations of ligand (Fig. 2). Specific binding reached equilibrium in about 30 min and remained stable at

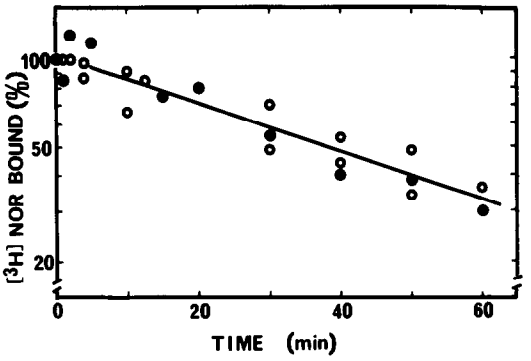

Fig. 3. Dissociation time course for (−)-[^{3}H]norepinephrine specifically bound to rat liver plasma membranes. [^{3}H]NOR at 115 nM was incubated at 25° for 30 min with membranes, under standard assay conditions before, either addition of 10 μM (−)-norepinephrine (●), or dilution of 50 μl-aliquots with 10 ml of buffer at 25° (○), at the indicated zero time. The dissociation of [^{3}H]NOR was followed at the indicated times by filtration of triplicate tubes, as described in Methods. The radioactivity bound to the membranes was expressed as a percentage of the radioactivity at time zero and plotted as a function of time elapsed after addition of (−)-norepinephrine or 200-fold dilution in buffer. Data shown are the averages of triplicate determinations from three experiments.

Table 1. Affinity of various drugs for (−)-[^{3}H]norepinephrine and [^{3}H]prazosin binding sites in rat liver plasma membranes*

Drug	(−)-[^{3}H]Norepinephrine binding [K_D (μM)]	[^{3}H]Prazosin binding [K_D (μM)]
(−)-Norepinephrine	0.17	12.6
(−)-Epinephrine	0.21	7.8
(−)-Phenylephrine	4.8	24.9
(−)-Isoproterenol	15.4	> 1000
(+)-Epinephrine	1.81	153
(+)-Norepinephrine	21.7	> 1000
Clonidine	0.55	1.25
Dihydroergocryptine	0.005	0.016
Phentolamine	0.099	0.108
Phenoxybenzamine	0.137	0.012
Prazosin	0.0166	0.00051
Yohimbine	0.311	0.992

* Incubations were performed at 25°, as described in Methods in the absence and presence of 6–10 concentrations of the indicated drugs. Values for inhibition of binding at each concentration were the mean of triplicate determinations. Dissociation constants (K_D) were calculated from IC_{50} values according to Cheng and Prusoff [10].

least until 60 min. Half maximal binding was obtained at about 5 min. The plots of ln(Be/Be–B) vs time (where B and Be are the amounts of radioligand specifically bound at time t and at equilibrium, respectively) gave slopes (equal to the pseudo-first order rate constant k_{ob}) of 0.098, 0.108 and 0.128 min^{-1} for ligand concentrations of 60, 112 and 215 nM, respectively (Fig. 2, lower panel). From these values, the second order rate constant (k_1) could be calculated and was 1.93×10^5 $M^{-1}.min^{-1}$.

Figure 3 shows the time course of dissociation of specifically bound [^{3}H]NOR (at 115 nM) by 10 μM (−)-norepinephrine at 25°. The binding of [^{3}H]NOR was reversible with monoexponential kinetics. Dissociation obtained by 200-fold dilution in incubation buffer yielded similar results (Fig. 3). This was not changed by inclusion of an excess of (−)-norepinephrine in the dilution buffer suggesting, once again, an absence of cooperativity between the binding sites. The mean half-time, from three experiments, for dissociation at 25° was 37–38 min and the dissociation rate constant (k_{-1}) was 0.0185 min^{-1}. The association and dissociation data yielded a K_D value of about 105 nM, which is in good agreement with the value obtained by equilibrium studies.

Specificity of (−)-[^{3}H]norepinephrine binding

Inhibition of [^{3}H]NOR binding to hepatic plasma membranes displayed a typical α-adrenergic order of potency (Table 1) with (−)-norepinephrine ≥ (−)-epinephrine > (−)-phenylephrine > (−)-isoproterenol. (−)-Norepinephrine was 80–90 times more potent than the β-adrenergic agonist (−)-isoproternol and (−)-isomers were 10–100 times more potent than the corresponding (+)-isomers. Clonidine, a mixed agonist-antagonist in liver [6] and an α_2-directed compound, was less potent than either (−)-norepinephrine or (−)-epinephrine in competing for the [^{3}H]NOR binding sites. Table 1 also shows that α-adrenergic antagonists competed for [^{3}H]NOR binding sites with an order of potency dihydroergocryptine > prazosin > phentolamine > yohimbine indicative of an α_1-adrenergic receptor subtype. Phenoxybenzamine, reported as more specific for α_1-sites, also displayed a high apparent affinity for [^{3}H]NOR binding sites (Table 1). The data support the contention that [^{3}H]NOR binds to an α_1-adrenergic receptor site in rat liver and agree with recent data of El-Refai and Exton [11].

Binding of [^{3}H]dihydroergocryptine

This laboratory [4] and others [1, 5, 7, 11] have clearly demonstrated that dihydroergocryptine binds to rat liver plasma membranes in a manner indicating a selective interaction with α-adrenergic receptors. The binding of [^{3}H]DHE was measured in the conditions of temperature, time and assay medium used to evaluate the binding of (−)norepinephrine in this study. The data indicated a single component of binding with a dissociation constant K_D of about 5–10 nM; the binding capacity was 1200 ± 300 fmol/mg (data not shown). These values are similar to those previously reported for DHE binding in rat liver [4, 5, 7]. Such studies have shown that DHE binds to an α_1-receptor subtype in rat liver, as indicated by the higher potency of prazosin than that of yohimbine to compete for [^{3}H]DHE binding sites [6, 11, 12].

Binding of [^{3}H]prazosin

Since dihydroergocryptine is considered as a nonselective agent of α-adrenergic receptor subtypes, the binding of the α_1-specific antagonist [^{3}H]PRA was measured in rat liver plasma membranes. Under the conditions of temperature, time and assay medium used in this study, [^{3}H]PRA bound to a single class of binding sites (Fig. 4). The binding capacity averaged 760 ± 40 fmol/mg of membrane protein and the dissociation constant (K_D) was 0.150 ± 0.025 nM.

Specificity of [^{3}H]prazosin binding

Inhibition of [^{3}H]PRA binding to hepatic plasma membranes displayed an α-adrenergic order of potency (Table 1) with (−)-epinephrine ≥ (−)-norepinephrine > (−)-phenylephrine > (−)-isoproterenol. (−)-Epinephrine and (−)-norepinephrine were at least 100-fold more potent than the β-adre-

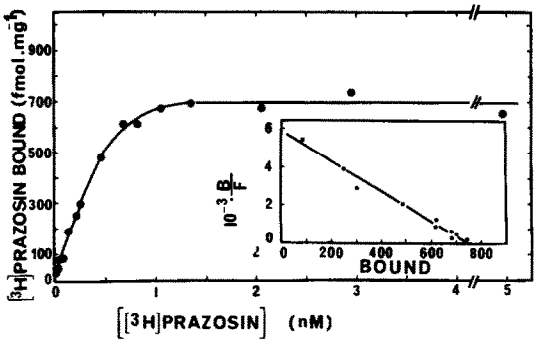

Fig. 4. [3H]Prazosin binding to rat liver membranes as a function of increasing concentrations of [3H]prazosin. Membranes (20 μg/assay) were incubated at 25° for 30 min as described in Methods, with various concentrations of [3H]PRA, in the absence or presence of 10 μM phentolamine. Points shown are those obtained in a single experiment, performed in duplicate, which was replicated twice with different membrane preparations. Inset shows a Scatchard analysis of the data. Linear regression analysis yielded one straight line with a correlation coefficient of 0.98 ($P < 0.001$). Bound/free (B/F) is expressed, in fmol. mg^{-1}. nM^{-1} and bound is expressed in fmol. mg^{-1}. In this experiment, the receptor density (B_{max}) was 730 fmol/mg of protein and the K_D value was 0.125 nM. Average values are B_{max} = 760 ± 40 fmol/mg and K_D = 0.150 ± 0.025 nM.

nergic agonist (−)-isoproterenol and (−)-isomers were 10 to more than 100 times more potent than the corresponding (+)-isomers. α-Adrenergic antagonists potently competed for [3H]PRA binding sites with an order of potency prazosin > dihydroergocryptine > phentolamine > yohimbine indicative of an α_1-adrenergic receptor subtype (Table 1). The data are in agreement with the contention that the observed binding of [3H]PRA occurs at the level of an α_1-adrenergic receptor site in rat liver.

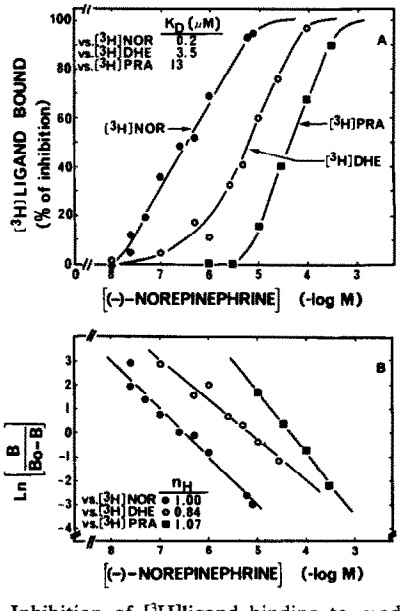

Fig. 5. Inhibition of [3H]ligand binding to α-adrenergic receptors by (−)-norepinephrine. [3H]ligand binding assays were performed as described in Methods with 77 nM [3H]NOR (●), 10 nM [3H]DHE (○) and 0.37 nM [3H]PRA (■). Data shown are the means of triplicate determinations in a single experiment. K_D values were calculated from IC_{50} values according to Cheng and Prusoff [10]. (A) Displacement of [3H]ligands by (−)-norepinephrine. (B) Hill plots of (−)-norepinephrine displacement curves.

Displacement of 3H-ligands by (−)-norepinephrine

If the three mentioned tritiated ligands label the same population of receptors, then (−)-norepinephrine should have the same potency and displacement slope in competing for all tritiated ligands (Fig. 5). However (−)-norepinephrine was almost 65 times more potent in reducing [3H]NOR than [3H]PRA binding (Fig. 5A). Hill coefficients for (−)-norepinephrine inhibition of these two 3H-ligands were close to 1.0 (Fig. 5B). (−)-Norepinephrine inhibited [3H]DHE binding with a potency intermediate to that observed for [3H]NOR or [3H]PRA binding. The Hill plot of (−)-norepinephrine displacement of [3H]DHE was shallow yielding a Hill coefficient of 0.84 (Fig. 5A and B). Furthermore, displacements of [3H]DHE by various other drugs, agonists or even antagonists, were found to be shallow with pseudo-Hill coefficients significantly lower than 1.0 (data not shown).

All three tritiated ligands displayed monophasic saturation with a single component on Scatchard analysis. The maximal number of [3H]NOR and [3H]PRA binding sites (Figs 1 and 4) were 340 ± 70 fmol/mg and 760 ± 40 fmol/mg, respectively. The amount of [3H]DHE bound was 1200 ± 300 fmol/mg and roughly correlated with the sum of tritiated norepinephrine and prazosin binding sites. Therefore, the data suggested that in rat liver plasma membranes the agonist and the antagonist bound to two discrete binding sites at the α_1-adrenergic receptor with respective high and low affinities for agonists.

Effect of guanine nucleotides on binding of 3H-ligands

Guanine nucleotides decreased the binding of the agonist [3H]NOR to α-receptor sites (Fig. 6). Gpp(NH)p was the most potent in reducing specific binding of [3H]NOR while GTP and GDP were less active. By contrast, ATP, CTP and UTP were unable

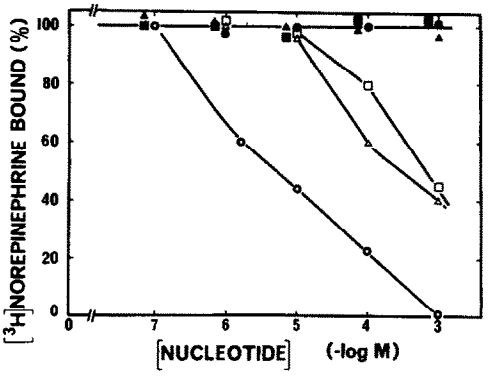

Fig. 6. Effect of increasing concentrations of Gpp(NH)p (○), GTP (△), GDP (□), ATP (▲), CTP (●) and UTP (■) on the specific binding of [3H]NOR to rat liver plasma membranes at 25°. Specific binding in the presence or absence of nucleotides was determined under standard assay conditions (see Methods). Values are expressed as percentage of the specific binding obtained in the absence of nucleotides.

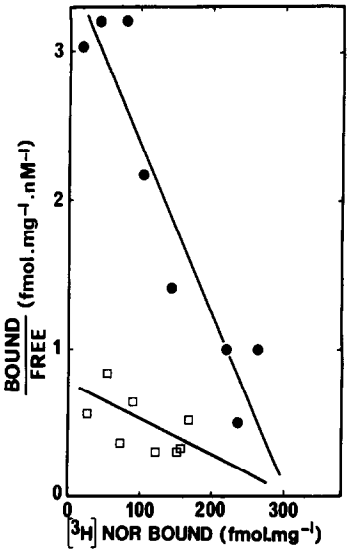

Fig. 7. Effect of Gpp(NH)p on (−)-[³H]norepinephrine binding to α-adrenergic receptors as a function of increasing concentrations of (−)-[³H]norepinephrine. Plasma membranes were incubated at 25° for 30 min as described in Methods with various concentrations of [³H]NOR, in the absence (●) or presence of 100 μM Gpp(NH)p (□), and with or without 10 μM phentolamine. Points shown are those obtained in a single experiment performed in duplicate which was replicated once with a different membrane preparation. Bound/free is expressed in fmol.mg^{-1}.nM^{-1} and bound is expressed in fmol.mg^{-1}.

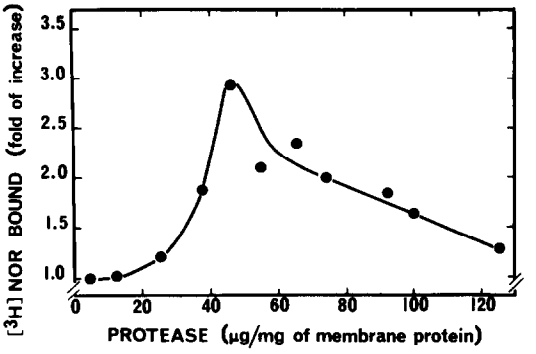

Fig. 8. Effects of α-chymotrypsin on (−)-[³H]norepinephrine binding to rat liver plasma membranes. Rat liver membranes were incubated for 5 min at 25° with α-chymotrypsin at the indicated concentrations. Proteolysis was stopped by addition of soybean trypsin inhibitor, as described in Methods. Membranes were then directly used for binding assays with 300 nM [³H]NOR in the absence or presence of 10 μM phentolamine, as described in Methods. Points shown are those obtained in two identical experiments performed in triplicate. Specific binding in original membranes, membranes plus soybean trypsin inhibitor and membranes plus soybean trypsin inhibitor and protease added at the same time, averaged 200 fmol/mg.

to affect the binding of tritiated norepinephrine (Fig. 6). To ascertain the mechanism whereby guanine nucleotides influence the binding of catecholamines to α-receptors, we evaluated the saturation parameters of [³H]NOR binding to rat liver membranes in the presence of 100 μM Gpp(NH)p (Fig. 7). As observed in the absence of Gpp(NH)p, specific [³H]NOR binding was a saturable process. Half maximal binding (K_D) occurred at 340 nM with a binding capacity of 300 fmol/mg. Scatchard analysis still revealed a single population of binding sites, but an increase in the apparent K_D of (−)-norepinephrine and no change in the B_{max} value. Contrary to the marked influence of Gpp(NH)p upon binding of the tritiated agonist, guanine nucelotides did not influence the binding of either tritiated dihydroergocryptine [4] or prazosin (data not shown). Similarly, El-Refai *et al.* and Guellaen *et al.* [4, 7] have found that guanine nucleotides do not influence the ability of agonists to displace the binding of [³H]DHE, in rat liver. Neither do guanine nucleotides influence the ability of agonists to displace the binding of [³H]DHE and [³H]-labeled WB 4101 in calf brain [13].

Effects of proteases on (−)-[³H]*norepinephrine binding to rat liver plasma membranes*

Rat liver plasma membranes were incubated at 25° for 5 min with α-chymotrypsin and proteolysis was stopped by addition of an excess of soybean trypsin inhibitor, as described in Methods. Membranes were then used directly for binding assays. As shown in Fig. 8, specific binding of [³H]NOR was enhanced 2.5–3 times by preliminary treatment of the liver membranes with α-chymotrypsin. Incubation of membranes at 25° with either no addition, or addition of soybean trypsin inhibitor, or soybean trypsin inhibitor together with protease, did not modify the extent of specific binding of [³H]NOR to liver membranes. The effect of α-chymotrypsin was dose- and time-dependent with maximal activation promoted in 5 min at 25° by 45 to 50 μg of α-chymotrypsin per mg of membrane protein (data not shown). The effect of α-chymotrypsin was biphasic with enhancement of binding at low concentrations and decrease of binding at high concentrations. In the following, further data on the binding of [³H]NOR to α-chymotrypsin-treated membranes are described but only the enhancement phase of the binding will be considered.

Inhibition of [³H]NOR binding to α-chymotrypsin-treated membranes exhibited a typical α-adrenergic order of potency with prazosin (K_D = 0.05 nM) being 10,000-fold more potent than yohimbine (K_D = 0.5 μM).

Equilibrium studies of (−)-[³H]*norepinephrine binding to α-chymotrypsin-treated membranes*

Figure 9 shows the specific binding of [³H]NOR to α-chymotrypsin-treated membranes as a function of radioligand concentration. Binding was saturable with increasing concentrations of ligand. Specific binding appeared to plateau at 300 nM with half maximal binding at about 50 nM. Scatchard analysis of the data (Fig. 9, lower panel) indicated a single component of binding with an apparent dissociation constant (K_D) of 61 nM. The mean ± standard error for five similar experiments was 55 ± 10 nM. The calculated maximal number of binding sites from five experiments (B_{max}) was 700 ± 100 fmol/mg of membrane protein. A plot of the data according to the Hill equation revealed a single straight line with a Hill coefficient (n_H) of 0.94, indicating the absence

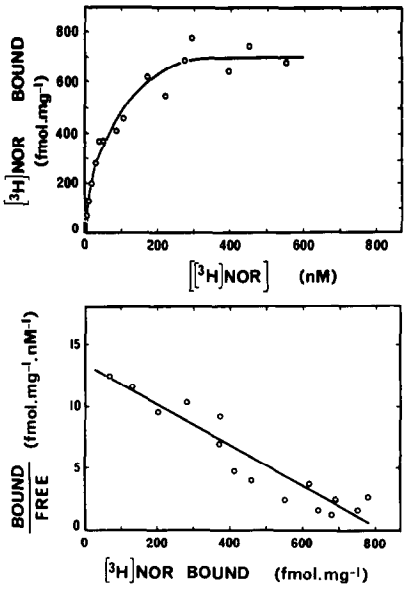

Fig. 9. (−)-[^{3}H]Norepinephrine binding to α-chymotrypsin-treated plasma membranes as a function of increasing concentrations of (−)-[^{3}H]norepinephrine. Liver plasma membranes were incubated at 25° for 5 min with α-chymotrypsin (50 μg/mg of membrane protein). Proteolysis was stopped by addition of soybean trypsin inhibitor, as described in Methods. Proteolytic treated membranes were then directly incubated for 30 min at 25° with various concentrations of [^{3}H]NOR in the absence or presence of 10 μM phentolamine. Points shown are those obtained in a single experiment, performed in triplicate, which was replicated five times with different membrane preparations. The lower panel shows a Scatchard analysis of the data. Linear regression analysis yielded one straight line with a correlation coefficient of 0.94 ($P < 0.001$). Bound/free is expressed in fmol . mg^{-1} . nM^{-1} and bound is expressed in fmol . mg^{-1}. In this typical experiment the receptor density (B_{max}) was 813 fmol/mg of protein and the K_D value was 61 nM. Average values are B_{max} = 700 ± 100 fmol/mg and K_D = 55 ± 10 nM.

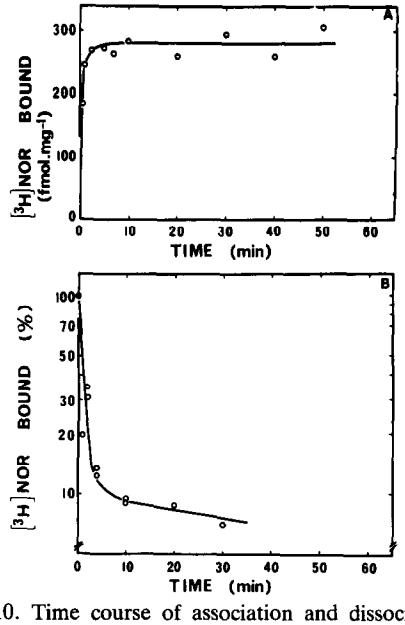

Fig. 10. Time course of association and dissociation of (−)-[^{3}H]norepinephrine binding to α-chymotrypsin-treated liver membranes. (A) Rat liver plasma membranes were incubated with α-chymotrypsin (58 μg/mg of membrane protein) as described in Methods; association of [^{3}H]NOR specific binding at 25° was then measured at various time intervals following the addition of 70 nM [^{3}H]NOR. Incubation conditions were as described in Methods. Points shown are those from a single experiment performed in triplicate, which was replicated twice. (B) Rat liver plasma membranes were incubated with α-chymotrypsin (45 μg/mg of membrane protein) as described in Methods. [^{3}H]NOR at 120 nM was then incubated with treated membranes, under standard assay conditions. After 30 min at 25°, 50 μl-aliquots were diluted 200-fold in a series of tubes containing buffer at 25°. The dissociation of [^{3}H]NOR was followed at the indicated times by filtration of triplicate tubes as described in Methods. The radioactivity bound to the membranes was expressed as a percentage of the radioactivity at time zero and plotted as a function of time elapsed after dilution. Time zero refers to the samples filtered immediately after 1:200 dilution. Data shown are the averages of triplicate determinations from two experiments.

of heterogeneity and/or cooperative interactions between binding sites, after proteolysis. One of the major changes in the binding of [^{3}H]NOR following proteolysis was that the effect of guanine nucleotides was no longer apparent (not shown). Thus, it appears that mild proteolytic digestion of liver membranes results in an enhanced binding capacity of the tritiated agonist associated with an increased affinity of the ligand for its own binding site (2 to 3-fold) as well as a loss of influence of guanine nucleotides. Inhibition of [^{3}H]NOR binding to α-chymotrypsin-treated membranes still displayed an α_1-adrenergic order of potency with prazosin > yohimbine (not shown).

Kinetics of (−)-[^{3}H]*norepinephrine binding to α-chymotrypsin-treated membranes*

Specific binding of [^{3}H]NOR to α-chymotrypsin-treated membranes was very rapid (Fig. 10A), reached equilibrium within 2.5 min at 25° as compared to 30 min for untreated membranes (Fig. 2) and remained stable for at least 50 min. Half maximal binding was attained at about 0.5 min. Figure 10(B) shows the time course of dissociation of [^{3}H]NOR (at 120 nM) specifically bound to protease-treated membranes obtained by 200-fold dilution in incubation buffer at 25°. While the binding of [^{3}H]NOR to control membranes was slowly reversible in a monophasic manner with a mean half-time of 37–38 min at 25° (see Fig. 3), the binding to α-chymotrypsin-treated membranes was extremely rapidly reversible in a biphasic manner with as much as 90 per cent of the radioligand which dissociated monoexponentially in 5 min at 25°.

Binding of [^{3}H]*prazosin and* [^{3}H]*dihydroergocryptine to α-chymotrypsin-treated membranes*

After treatment of rat liver plasma membranes by α-chymotrypsin (40–50 μg of protease/mg of membrane protein), the specific binding of [^{3}H]PRA and [^{3}H]DHE were studied as a function of radioligand concentration. Binding of [^{3}H]PRA was saturable with increasing concentrations of ligand. Scatchard analysis of the data still indicated a single component of binding with an apparent dissociation constant of 0.225 nM. The calculated maximal number of binding sites was 550 fmol/mg. These data indicated that

proteolysis of rat liver membranes resulted in a reduction of both the binding capacity (from 760 to 550 fmol/mg) and the apparent affinity of the tritiated prazosin for its own binding site. Under these conditions, the extent of binding of [^{3}H]DHE to protease-treated membranes was slightly reduced. Binding capacity averaged 900 fmol/mg. The dissociation constant K_D was not significantly changed and binding still occurred to one class of binding sites.

Effect of proteolysis on (−)-[^{3}H]norepinephrine binding to phenoxybenzamine-treated membranes

In order to ascertain the origin of the "new" binding sites labeled by [^{3}H]NOR after proteolysis, rat liver membranes were first incubated with the irreversible α-adrenergic antagonist phenoxybenzamine to block all the available receptors [2] and then treated with α-chymotrypsin under usual conditions. If the proteolytically enhanced binding capacity of the tritiated agonists were generated from preexisting recognizable binding sites, previous exposure of membranes to POB should not result in a greater degree of binding of [^{3}H]NOR to the POB-α-chymotrypsin-treated membranes. Should this hypothesis prove to be wrong, and proteolysis activate cryptic receptor binding sites, then the binding capacity of [^{3}H]NOR would still be enhanced by proteolysis even after POB treatment. Incubations of membranes with POB were carried out at 25° for 10 min, in a final volume of 1 ml, as described by Williams and Lefkowitz [14]. At the end of the incubation, membranes were washed as follows. The incubation medium was diluted with 40 ml of 20° incubation buffer and allowed to stand for 10 min at room temperature before centrifugation at 28,000 × g for 10 min. The pellet was resuspended and similarly washed once more in buffer at 20° then a third time at 4°. The final pellet was resuspended in incubation buffer and treated by α-chymotrypsin, as described in Methods. When membranes were exposed to 10 μM POB and washed as described above, all of the [^{3}H]NOR binding was inhibited (Fig. 11). Likewise exposure of membranes to POB, followed by several washes and proteolysis (with 50–70 μg of α-chymotrypsin/mg of membrane protein) did not result in measurable specific binding of [^{3}H]NOR (Fig. 11). However the control untreated (incubated in the absence of POB) membranes exhibited a specific binding of [^{3}H]NOR which could be enhanced 3–4 times by proteolysis (Fig. 11). These experiments argue against an unmasking of cryptic receptors. Thus, "new agonist" binding sites, arising after proteolytic treatment of the membranes, did not appear to originate from spare, hitherto unrecognized receptors, but rather from the preexisting pool of α-adrenergic binding sites labeled by [^{3}H]DHE.

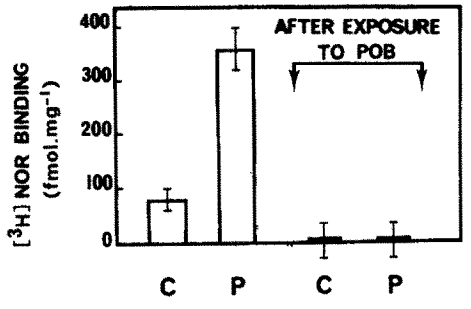

Fig. 11. Effect of exposure of rat liver membranes to phenoxybenzamine (POB) followed by proteolysis, on (−)-[^{3}H]norepinephrine binding. Rat liver plasma membranes (4–5 mg of protein) were first incubated at 25° for 10 min in the presence or absence of 10 μM POB. At the end of the incubation, membranes were washed three times as follows. The incubation medium (1 ml) was diluted with 40 ml of 20° incubation buffer and allowed to stand for 10 min at room temperature before centrifugation at 28,000 × g for 10 min. The pellet was resuspended and similarly washed once more in buffer at 20°, and a third time in buffer at 4°. The final pellet was incubated at 25° for 5 min with (P) or without (C) α-chymotrypsin (50–70 μg/mg of membrane protein) and proteolysis was stopped by addition of soybean trypsin inhibitor, as described in Methods. Membranes were then directly used in binding assays, as described in Methods.

DISCUSSION

One of the major findings of the present study is that (−)-[^{3}H]norepinephrine and [^{3}H]prazosin label distinct binding sites at the α_1-adrenergic receptor, while [^{3}H]dihydroergocryptine appears to label both of these sites. Evidence supporting this conclusion includes the following.

(1) [^{3}H]NOR binds to a finite number of high affinity catecholamine binding sites in a manner indicating a selective interaction with an α_1-adrenergic receptor subtype. Equilibrium binding studies yield one class of binding sites with a binding capacity of about 340 fmol/mg of protein and a dissociation constant of about 130 nM. Hormone binding is relatively rapid and slowly reversible; dissociation of the radioligand using either unlabeled norepinephrine or dilution with a large volume of buffer has identical characteristics and is monophasic, consistent with the presence of only one class of sites. These binding sites exhibit stereospecificity as well as high affinities for agonists, and rather low affinities for antagonists. Displacement of [^{3}H]NOR with adrenergic drugs yields the potency series expected for α_1-adrenergic receptors. The present findings confirm the reports of El-Refai *et al.* [7, 11] that catecholamines bind to a class of α_1-adrenergic binding sites exhibiting high affinity for natural α-adrenergic agonists in rat liver membranes. According to these authors, this high affinity class of sites would represent the physiologically relevant α-adrenergic receptor. In our hands, norepinephrine binding did not reveal an agonist, low affinity binding site, as evidenced by El-Refai *et al.* [7], probably because of its too weak affinity for the agonist.

(2) The α_1-specific antagonist [^{3}H]PRA binds to a finite number of high affinity binding sites in rat liver plasma membranes. Equilibrium binding studies yield one class of binding sites with a binding capacity of about 760 fmol/mg of protein and a dissociation constant of about 0.15 nM. The binding sites exhibit stereospecificity as well as high affinity for antagonists, and low affinity for agonists. Displacement of [^{3}H]PRA with adrenergic drugs yield the potency series expected for α_1-adrenergic receptors.

(3) The ergot alkaloid [^{3}H]DHE also binds specifically to one class of sites in plasma membranes from rat liver in a manner indicating a selective interaction with α_1-adrenergic receptor sites [4, 5, 11]. Equilibrium binding studies yield a binding capacity of about 1200 fmol/mg of protein and a dissociation constant of about 5–10 nM. Guellaen *et al.* [4] and Aggerbeck *et al.* [6, 15] have shown that DHE binding sites exhibit high affinity for antagonists and intermediate affinity for agonists as compared to their affinity for [^{3}H]NOR and [^{3}H]PRA binding sites (Fig. 8, Table 1).

(4) The number of [^{3}H]DHE binding sites is approximately equal to the sum of [^{3}H]NOR and [^{3}H]PRA binding sites.

(5) Potencies of (−)-norepinephrine differ by a factor of up to 65-fold in competing differentially with [^{3}H]NOR and [^{3}H]PRA binding. Accordingly, potencies of prazosin differ by a factor of up to 100-fold in competing differentially with [^{3}H]NOR and [^{3}H]PRA binding. Potencies of dihydroergocryptine itself do not differ significantly in competing with [^{3}H]NOR, [^{3}H]PRA or [^{3}H]DHE binding.

(6) [^{3}H]NOR and [^{3}H]PRA binding sites may be further differentiated by the action of guanine nucleotides. Thus, guanine nucleotides lower the affinity of agonists for [^{3}H]NOR, but not [^{3}H]PRA or [^{3}H]DHE binding sites.

On the basis of these findings we propose the existence of two discrete populations of binding sites with respective high and low affinities for agonists; both sites are α-adrenergic receptors and appear to be mainly, if not totally, of the α_1-subtype. Indeed, although the sites labeled by all the tritiated ligands exhibit α_1-selectivity (prazosin > yohimbine), our data do not exclude the existence of a small amount of α_2-adrenergic receptors in rat liver. In this respect, Hoffman *et al.* [16] have recently shown that very low concentrations (1–10 nM) of tritiated catecholamines preferentially label α_2-adrenergic receptors and that the proportion of these sites amounts to 10–20 per cent of total α-adrenergic receptors in rat liver. Thus, if one cannot exclude the possibility that our data arise from the presence of both α_1- and α_2-adrenergic receptors in rat liver, we think this explanation unlikely for the following reasons: (1) [^{3}H]NOR binds to only one type of binding sites and the affinity is much lower than that currently reported for the binding of an agonist at the α_2-adrenergic receptor (K_D = 100–200 nM vs 2–5 nM in ref. [16]); (2) if we suppose that [^{3}H]NOR binds preferentially to α_2-adrenergic binding sites in rat liver membranes, then we would have to admit that contamination by a small amount of α_1-adrenergic binding sites is sufficient to give an overall α_1-selectivity to the agonist binding; (3) norepinephrine would recognize α_2-adrenergic receptors in liver membranes when it activates glycogen phosphorylase in isolated rat hepatocytes through α_1-adrenergic receptors with similar affinities [6, 7, 11, 15]; (4) the displacement of [^{3}H]NOR binding by prazosin and yohimbine were monophasic with pseudo-Hill coefficients of 0.95–1.0 (data not shown), a result which does not fit with the idea that [^{3}H]NOR binds to both α_2- and α_1-adrenergic receptors in the conditions used in this study. We would rather suggest that only the population of sites displaying high affinities for agonists is capable of coupling to the "effector" through interactions with guanine nucleotide binding protein(s) (Fig. 12), while the other population of sites represents α-adrenergic receptors not coupled to the regulatory nucleotide binding protein(s). That the binding of norepinephrine to α_1-adrenergic binding sites is sensitive to guanine nucleotides supports the concept that the regulatory role of GTP in membrane receptor activity might not be restricted to receptors mediating adenylate cyclase activation; recently, for example, Cantau *et al.* [17] have shown that GTP exerts a regulatory role on the hepatic vasopressin receptor, a receptor for which the physiological responses are known to be totally independent of adenylate cyclase, in rat liver. By applying radioligand binding techniques to the study of the β-adrenergic receptor-adenylate cyclase complex, the group of Lefkowitz [18, 19] has proposed a model to explain the guanine nucleotide dependent coupling between receptors and adenylate cyclase. The model proposes that the β-adrenergic receptor can exist in either a high or a low affinity state for the hormone. This model includes the induction of a high affinity slowly dissociable complex between the receptor and the hormone. Formation of this tight complex is thought to facilitate the activation of the enzyme by regulatory nucleotides and leads to a decrease in affinity of the receptor for the hormone, thus resulting in dissociation of the hormone-receptor complex into free hormone and receptor. We propose that this kind of general model might also apply to the α-adrenergic receptor-"effector" complex.

The other major finding of this study is that the two discrete populations of binding sites may correspond to different states of the same α_1-receptor molecule whose balance can be changed by proteo-

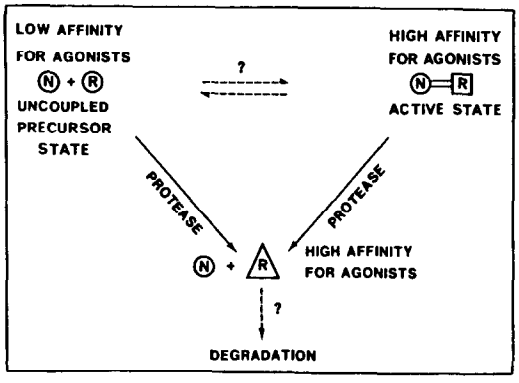

Fig. 12. Speculative model of α-receptor regulation. Ⓝ represents the GTP-binding protein(s) through which hormonal receptors appear to be coupled to the "effector". Ⓡ represents the uncoupled precursor state of the receptor. R represents the active state of the receptor coupled to Ⓝ. R represents the high affinity catecholamine site generated by the action of exogeneous proteases on rat liver membranes. This model does not deal with the specific problem of the receptor–"effector" coupling, i.e. with the influence that guanine nucleotides and possibly the "effector" itself might exert on the affinity states of the active receptor sites. Models for receptor–effector coupling are presented and discussed in Ref. [21, 22 and 25].

lysis. Evidence supporting this statement originates from the differential action of proteases on ^{3}H-ligand binding to the α-adrenergic receptor sites from rat liver membranes and is based on the following grounds.

Firstly, treatment of liver membranes with low concentrations of proteases results in an increase of both the capacity and affinity of binding of [^{3}H]NOR to proteolysed membranes. Equilibrium binding studies yield one class of binding sites with a binding capacity of about 700 fmol/mg of protein and a dissociation constant of 55 nM. Hormone binding is very rapid (equilibrium is reached in 2.5 min at 25°) and dissociation is nearly complete in 5 min at 25°. As a result or cause of the considerable changes observed in the kinetics of binding of tritiated norepinephrine, guanine nucleotides no longer modulate the ability of agonists to bind to the catecholamine site after proteolysis. These results suggest that very high affinity catecholamine binding sites have been generated by proteolysis. These findings also suggest that proteolytically-generated binding sites are uncoupled from guanine nucleotide binding protein(s).

A second line of evidence comes from experiments performed after exposure of liver membranes to the irreversible α-blocker phenoxybenzamine. When all the measurable [^{3}H]DHE binding sites are blocked by phenoxybenzamine [4], subsequent proteolytic treatment of liver membranes does not result in the generation of agonist binding sites. This result argues against an unmasking of cryptic receptor sites and for a DHE binding site origin.

A third line of evidence comes from [^{3}H]PRA binding studies. Under the conditions in which the binding of agonist is maximally enhanced, the binding of [^{3}H]PRA is lowered to 550 fmol/mg of protein and the dissociation constant is increased from 0.150 to 0.225 nM. Taken together, these findings are compatible with the possibility that the [^{3}H]PRA binding sites may have been transformed into agonist high affinity binding sites by proteolysis. Thus proteolysis appears to cause a transformation of the preexisting heterogeneous population of binding sites into a single type of binding site displaying high affinity for agonists (Fig. 12). We believe that this implies that the two populations of binding sites correspond, in fact, to two distinct states of a single α_1-adrenergic receptor in rat liver.

Finally, El-Refai and Exton [20] have also recently reported that trypsin treatment of rat liver membranes results in an enhancement of tritiated catecholamine binding together with a concomitant decrease in [^{3}H]dihydroergocryptine binding to the α-adrenergic receptor.

If the binding data obtained with different radioligands were to be explained in the use of α_1- and α_2-adrenergic receptors, then the results that we have obtained by proteolysis would imply that α_1-adrenergic binding sites are transformed into α_2-adrenergic binding sites by proteolysis. We think this interpretation of the data inconsistent with the facts that: (1) the guanine nucleotide regulation of the radiolabeled agonist binding is lost after proteolysis; and (2) not only is preserved the order of potency prazosin > yohimbine, but this typical α_1-adrenergic order of affinity is amplified since prazosin is 10,000-fold more potent than yohimbine, after proteolysis. In fact, if any contamination by α_2-adrenergic binding sites occurred in the binding of [^{3}H]NOR to the α_1-adrenergic receptor in untreated rat liver membranes, it would have been totally eliminated by proteolysis.

In the present study, and others, dihydroergocryptine appeared to be a useful ligand for α-adrenergic receptor studies. Indeed it actually represents the unique radiolabeled α-adrenergic compound that can equipotently label all the states and subtypes of α-receptors in a given system [1, 11, 12]. However, selective compounds, whether natural or not, are demonstrated to be absolutely necessary since they allow one to discriminate between different subtypes and states of the receptors under study.

To date, one can only speculate about the biological role and significance of these findings. Other membrane-bound receptors for neurotransmitters such as the β-adrenergic receptors [18, 19], the muscarinic receptors [21, 22], the dopaminergic receptors [23], the serotonimergic receptors [24], and the opiate receptors [25], are known to exist under distinct states in membrane fractions. Our findings with the hepatic α-adrenergic receptors led us to formulate the following working hypothesis represented pictorially in Fig. 12. Antagonist-preferring states of the α-adrenergic receptors might represent uncoupled precursor forms of physiologically-active receptors. Functional binding sites could be induced from the uncoupled pool of precursor binding sites under the influence of one or several biological signals. Whether or not the precursor uncoupled form of the receptor is in equilibrium with the physiologically active form of the receptor is an open question. The results presented here do not permit the determination of a definitive model of α-receptor regulation. As far as membrane fractions are concerned, guanine nucleotides appear to lower the affinity of the active receptor state for the agonist hormone, indicating that regulatory nucleotides may mediate a transition of the high affinity state of the receptor to a lower affinity state, in a manner similar to that described for the β-adrenergic receptor [18, 19]. However, as long as the membrane-bound "effector" of the α-adrenergic receptor complex is unknown, one cannot expect to fully demonstrate this point. In this respect, the activation of the glycogen phosphorylase is not suitable since it has to be studied in isolated hepatocytes in presumption of survival (i.e. in the presence of a large excess of GTP).

We have obtained evidence indicating that treatment of hepatic plasma membranes with an exogeneous protease may cause the transformation of both the precursor and the functional forms of the receptors into a state of very high affinity for agonists which is insensitive to guanine nucleotides. The mechanism by which the protease is acting is unknown. One might postulate that it acts at any one of a series of steps involved in the linkage between receptor and guanine nucleotide binding protein(s). The proteolytically-generated state of the receptor is envisaged as a first step towards degradation and/or internalization (Fig. 12). These conclusions are in general agreement with the conclu-

sions of El-Refai and Exton [20] and suggest a mechanism by which proteolytic enzymes might play a regulatory role in the actions of certain hormones. Whether this effect of proteases is to be compared or related to the well-documented effects of proteases on adenylate and guanylate cyclases in this system [26–29], is an open question.

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