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PROTEOLYSIS OF RAT LIVER PLASMA MEMBRANES CANCELS THE GUANINE NUCLEOTIDE SENSITIVITY OF AGONIST BINDING TO THE ALPHA-ADRENOCEPTOR

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SUMMARY

Incubation of rat liver plasma membranes with a variety of proteinases causes a time and dose-dependent 2- to 3-fold increase in (-)-[^{3}H]norepinephrine binding to the alpha-adrenergic receptor. Scatchard analysis of binding data shows that both the number of sites and the affinity of the ligand are increased. Following proteolysis, the association and dissociation rates of binding of the hormone are increased. Guanine nucleotides decrease the affinity of the agonist for the alpha-adrenoceptor in untreated membranes, but not in alpha-chymotrypsin-treated membranes. Thus, limited proteolysis of liver membranes results in drastic modifications, not only on the binding characteristics of catecholamines to the alpha-adrenoceptor, but also on its regulation by guanine nucleotides.

INTRODUCTION

Using tritiated catecholamines to label alpha-adrenergic receptor sites, El-Refai et al (1) showed that catecholamines manifested heterogenous binding in rat liver membranes. One type of binding sites exhibited high affinity for alpha-adrenergic agonists and sensitivity to guanine nucleotides, while the other type exhibited low affinity for agonists. More recently (2), they reported that treatment of liver plasma membranes with tryspin at low concentrations caused an increase in alpha-specific-high affinity agonist binding which correlated with a loss of antagonist binding. In the present communication, we report that, in rat liver plasma membranes, (-)-[^{3}H]norepinephrine binds to a single class of alpha-adrenergic binding sites exhibiting high affinity for agonists and sensitivity to guanine nucleotides; following proteolysis, tritiated norepinephrine binding is enhanced, but is insensitive to guanine nucleotides.

MATERIALS AND METHODS

(-)7,8-[^{3}H]Norepinephrine (20-25 Ci/mmol) was from New England Nuclear. Alpha-chymotrypsin (EC 3.4.21.1, 53 U/mg, batch n° CDI, 8LK), trypsin (EC 3.4.21.4, 194 U/mg, batch n° TRL 36C 876), collagenase (EC 3.4.24.3, 136 U/mg, batch n° 4194 CLS 49 M 127 P) and papain (EC 3.4.22.2, 18.9 U/mg, batch n° PAP 37 D 784) were supplied by Worthington. Subtilisin BPN', type VII from Bacillus amyloliquefaciens (EC 3.4.21.14, 8.5 U/mg, batch n° 113 C-3510) was obtained from Sigma. Soybean trypsin inhibitor was from Worthington or Sigma. Sources of other materials have been previously described (3). Hepatic

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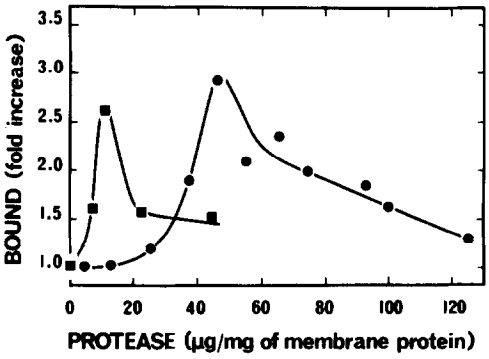

Fig. 1. Effects of trypsin and alpha-chymotrypsin on tritiated norepinephrine binding to rat liver plasma membranes. Rat liver membranes were incubated for 5 min at 25°C with trypsin (■) or alpha-chymotrypsin (●) at the indicated concentrations. Proteolysis was stopped by addition of soybean trypsin inhibitor, as described in "Methods". Membranes were then used directly for binding assays with 300 nM tritiated norepinephrine. 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 together, averaged 200 fmol/mg protein.

plasma membranes were prepared from female, albino Wistar rats (100 g to 200 g body weight) according to the method of Neville (4) up to step 11, and were stored in liquid nitrogen until use. Protein concentrations were determined by the method of Lowry (5) using bovine serum albumin as a standard.

Unless otherwise specified, proteolytic treatment of membranes was performed as follows: liver membranes containing 400 to 800 µg of protein were incubated with 20 to 50 µg of alpha-chymotrypsin in 33 mM Tris-HCl, 6.6 mM $MgCl_2$, pH 7,4 at 25°C. After 5 min, 200 to 500 µg of soybean trypsin inhibitor (10-fold excess over alpha-chymotrypsin) were added and the incubation was continued for another 2 min at 25°C, 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 used directly for binding assays. Alpha-adrenergic receptor binding was assayed according to El-Refai et al (1), with slight modifications. Unless otherwise stated, liver membranes containing 400 to 800 µg of protein were incubated with, 100 nM (-)-[^{3}H]norepinephrine 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°C. 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). Specific binding (displaceable by 10 µM phentolamine) represented 40-60 % of total binding. All results refer to "specific" binding.

RESULTS AND DISCUSSION

As shown in Fig. 1, binding of (-)-[^{3}H]norepinephrine was enhanced 2.5 to 3 times by preliminary treatment of the liver membranes with trypsin or alpha-

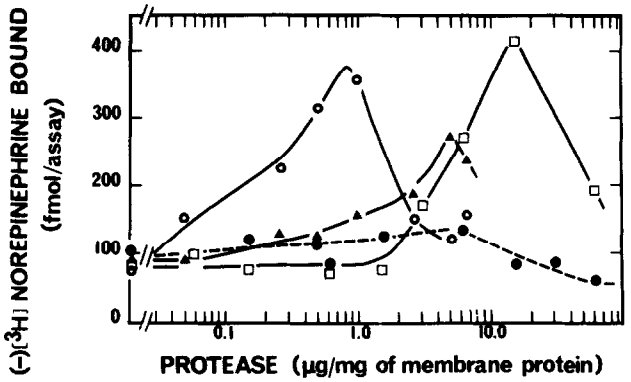

Fig. 2. Effect of various proteases on tritiated norepinephrine binding to rat liver plasma membranes. Liver membranes (500 to 800 µg protein) were incubated for 30 min at 25°C with 120 nM tritiated norepinephrine, 0.75 mM ascorbate, 3 mM catechol, 25 µM iproniazide, 10 µM (-)propranolol and 50 mM Tris-HCl/10 mM $MgCl_2$, pH 7.4, in the presence of various concentrations of the indicated proteases. Points shown are the mean value of duplicate determinations from two experiments. Alpha-chymotrypsin (○), papain (▲), subtilisin (□), collagenase (●).

chymotrypsin. Incubation of membranes with either no addition, or soybean trypsin inhibitor, or soybean trypsin inhibitor together with proteinase did not modify the extent of binding of tritiated norepinephrine to liver membranes. The effect was dose-dependent and biphasic, with enhancement of binding at low concentrations and reduction of binding at higher concentrations (Fig. 1). The increase in labeled hormone binding was not restricted to trypsin and alpha-chymotrypsin. Another serine proteinase, subtilisin, was also effective in enhancing the binding of tritiated norepinephrine (Fig. 2). In these experiments (Fig. 2), proteinases were added directly to the incubation medium used for binding assays and proteolysis occurred for 30 min at 25°C. Under these conditions, tritiated norepinephrine binding was enhanced 3 to 4 times by treatment with, either 1 µg of alpha-chymotrypsin/mg of membrane protein, or 15 µg of subtilisin/mg of membrane protein. The SH proteinase papain, though less effective, also enhanced labeled hormone binding (2-fold, with 4 to 5 µg of papain per mg of membrane protein) to rat liver plasma membranes. In contrast, the metalloproteinase collagenase, a widely used enzyme for the preparation of isolated hepatocytes, was nearly without effect within the range of 0 to 100 µg of proteinase/mg of membrane protein. In the following, we used alpha-chymotrypsin, as described in "Methods". The effect of alpha-chymotrypsin was time-dependent and maximal enhancement of tritiated norepinephrine binding was achieved in 5 min at 25°C with about 50 µg of alpha-chymotrypsin/mg of membrane protein (data not shown).

The binding of norepinephrine to control and alpha-chymotrypsin-treated membranes was studied as a function of radioligand concentration in the absence and presence of 100 µM guanyl-5'-yl imidodiphosphate[1]. Binding was saturable with increasing concentrations of ligand and Scatchard analysis of the data (Fig. 3) indicated a single component of binding in each case. In the absence of guanine nucleotides (closed symbols), apparent dissociation constants of 130 ± 60 nM and 55 ± 10 nM were observed (Fig. 3) and the calculated maximal numbers of binding sites (Bmax) were 340 ± 70 fmol/mg and 700 ± 100 fmol/mg, in untreated and treated membranes, respectively. Also shown in Fig. 3 is the effect of the non hydrolysable analog of GTP, Gpp(NH)p, on the affinity of the labeled hormone for its binding site: the number of binding sites is not changed but the dissociation constant is increased about 3 times (340 nM vs. 130 nM) in untreated membranes. The effect was rather specific since GTP and GDP, though less potent than Gpp(NH)p, were also active in reducing norepinephrine binding to rat liver membranes, while ATP, CTP, UTP were totally ineffective (Table I). Following proteolysis, the guanine nucleotide regulation of agonist binding was apparently lost (Fig. 3). These findings suggest that proteinases appear to act at any one of a series of steps involved in the linkage between receptors and guanine nucleotide binding proteins.

The findings also confirm that agonist binding to the hepatic alpha-adrenoceptors (1) is susceptible to guanine nucleotide regulation in a manner apparently similar to that observed with other alpha-adrenergic receptors (6-8), beta-adrenergic receptors (9) and other adenylate cyclase-coupled receptors (10-12). We suggest that the mechanism by which hormones and neurotransmitters are envisionned to act, via the GTP-regulatory proteins, in order to interact with and control the activity of adenylate cyclase (13), might also apply to membrane signal transduction involving surface receptors apparently not coupled to adenylate cyclase.

Following proteolysis, the kinetics of association and dissociation of tritiated norepinephrine binding were drastically modified (Fig. 4A and B). While norepinephrine binding to untreated membranes reached equilibrium in about 30 min at 25°C, it took only 2.5 min at 25°C to reach equilibrium after proteolysis (Fig. 4A). Fig. 4B shows the time course of dissociation of bound tritiated norepinephrine by 200-fold dilution in incubation buffer at 25°C. Whereas the binding of tritiated norepinephrine to untreated membranes was slowly reversible in a monophasic manner with a mean half-time of 37 to 38 min, the binding to treated membranes was extremely rapidly reversible with as much as 90 % of dissociation occurring in 5 min at 25°C.

1. The abbreviation used is: Gpp(NH)p = guanyl-5'-yl imidodiphosphate.

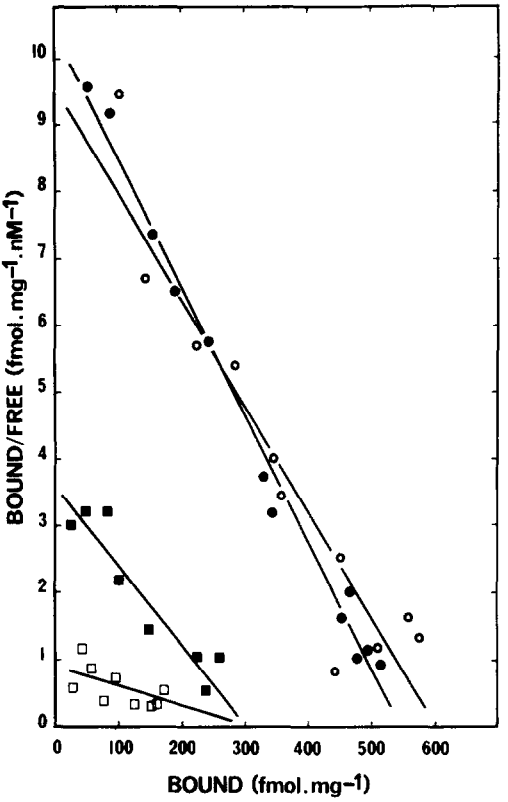

Fig. 3. Scatchard analysis of tritiated norepinephrine binding data obtained with untreated (■,□) and alpha-chymotrypsin-treated (●,○) rat liver plasma membranes, in the absence (closed symbols) or presence (open symbols) of Gpp(NH)p. Liver plasma membranes were incubated with or without alpha-chymotrypsin (57 µg of protease/mg of membrane protein) as described in "methods". Membranes were then incubated at 25°C for 30 min with various concentrations of tritiated norepinephrine, in the absence (closed symbols) or presence (open symbols) of 100 µM Gpp(NH)p. Points shown are those obtained in a single experiment performed in duplicate, which was replicated five to six times in the absence of Gpp(NH)p and twice in the presence of Gpp(NH)p. Bound/free is expressed in $fmol.mg^{-1}.nM^{-1}$ and bound is expressed in $fmol.mg^{-1}$. In this typical experiment, the receptor densities (Bmax) were 540 and 600 fmol/mg protein in treated membranes and 300 fmol/mg in untreated membranes; in the absence of Gpp(NH)p the K_D values were 85 nM and 52 nM in untreated and treated membranes respectively. In the presence of Gpp(NH)p, the K_D values were 340 nM and 63 nM in untreated and treated membranes respectively. Average values for untreated membranes were Bmax = 340 ± 70 fmol/mg and K_D = 130 ± 60 nM and 340 ± 100 nM in the absence and presence of Gpp(NH)p respectively. Average values for alpha-chymotrypsin-treated were Bmax = 700 ± 100 fmol/mg and K_D = 55 ± 10 nM.

The present study indicates that limited proteolytic digestion of rat liver membranes can cause considerable changes in the characteristics of binding of agonists to the alpha-adrenoceptor. In untreated membranes, binding of the agonist hormone displays rather slow kinetics of binding, slow disso-

Table I

Effect of increasing concentrations of various nucleotides on the specific binding of norepinephrine to rat liver plasma membranes.

Nucleotide	Tritiated Norepinephrine Binding* (% of control)
ATP, CTP, UTP (1 mM)	100
GTP (0.01 mM)	96
" (0.1 mM)	60
" (1 mM)	40
GDP (0.01 mM)	98
" (0.1 mM)	80
" (1 mM)	45
Gpp(NH)p (0.1 μM)	100
" (1 μM)	60
" (0.01 mM)	44
" (0.1 mM)	23
" (1 mM)	0

*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 (about 200 fmol/mg protein) in the absence of nucleotides.

ciation rate, sensitivity to guanine nucleotides compatible with the existence of at least two states of affinity of the receptor (9) and limited binding capacity as compared to the binding capacity of antagonists (1, 3). Following proteolysis, binding of the radiolabeled hormone displays rapid kinetics of binding, rapid dissociation rate, insensitivity to GTP, larger binding capacity and higher affinity. In other words, following proteolysis binding of the hormone seems to exhibit all the binding properties shared by antagonists (3). That alpha-chymotrypsin treatment of liver membranes causes an increase of the binding capacity of the hormone can be the result of either an unmasking of spare receptors or of transformation of preexisting recognizable sites. After proteolysis, the binding of radiolabeled antagonists such as tritiated dihydro-

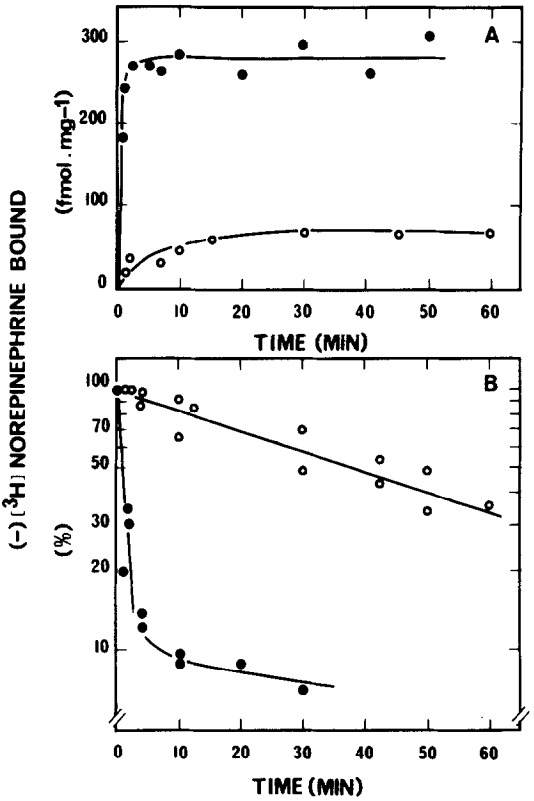

Fig. 4. Time course of association and dissociation of tritiated norepinephrine binding to untreated (open symbols) and alpha-chymotrypsin-treated (closed symbols) liver membranes. A) Rat liver plasma membranes were incubated with or without alpha-chymotrypsin (58 µg/mg of membrane protein) as described in "Methods". Association of tritiated norepinephrine was then measured at various time intervals following the addition of tritiated norepinephrine (60 and 70 nM with untreated and treated membranes respectively). Incubation conditions were as described in "Methods". Data shown are the averages of triplicate determinations from two experiments. B) Rat liver plasma membranes were incubated with or without alpha-chymotrypsin (45 µg/mg of membrane protein) as described in "Methods". Tritiated norepinephrine at 120 nM was then incubated with untreated and treated membranes under standard assay conditions. After 30 min at 25°C, 50 µl-aliquots were diluted 200-fold in a series of tubes containing buffer at 25°C. The dissociation of tritiated norepinephrine was followed at the indicated times by filtration of triplicate tubes. The radioactivity bound to the membranes was expressed as the 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.

ergocryptine (2) is impaired thus favoring a proteolytic transformation of the receptor rather than an unmasking of cryptic sites.

In our laboratory, treatment of plasma membranes with a variety of endopeptidases was demonstrated to cause increases of adenylate cyclase activities

(14-17) as well as guanylate cyclase activation and solubilisation (18, 19). In various systems, the regulatory effect of GTP on the adenylate cyclase was either unaffected or impaired (see review in ref. 17). In rat liver, Stengel et al. (17) have shown that the GTP regulation of the adenylate cyclase is unaffected by proteolysis. Therefore, the results reported in this communication may indicate that the GTP-regulation of liver alpha-adrenoceptors does not appear to involve the same type of interactions and/or the same GTP regulatory protein(s).

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