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UNCOUPLING BY PROTEOLYSIS OF ALPHA-ADRENERGIC RECEPTOR-MEDIATED INHIBITION OF ADENYLATE CYCLASE IN HUMAN PLATELETS

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Mild proteolytic treatment of human platelet membranes by alpha-chymotrypsin increased basal adenylate cyclase activity and abolished the inhibition induced by epinephrine in the presence of GTP. This treatment did not alter the total number of yohimbine binding sites, but markedly decreased the proportion of high affinity, GTP-sensitive sites for epinephrine as assessed from epinephrine competition studies. The effect of proteolysis was dose-dependent upon both adenylate cyclase inhibition and $alpha_2$-adrenergic binding sites, with a half-maximal effect occurring at similar alpha-chymotrypsin concentration. These results support the concept that only one protein is responsible for the GTP regulation of receptor and inhibition of cyclase.

INTRODUCTION

Over the past few years, we (1-3), and others (4-7), have demonstrated that various adenylate cyclase systems could be stimulated in vitro by mild proteolytic treatment. Depending on the system tested, this effect was found to be either independent of an action on the GTP-binding regulatory protein(s) (1, 2), or to be partially due to the prevention of the GTP-dependent inhibition of adenylate cyclase activity (4, 5). In human platelets, the alpha-adrenergic receptors have been well characterized and found to be exclusively of the $alpha_2$ subtype (8, 9). They are closely linked to a GTP-dependent inhibition of adenylate cyclase (10). Therefore this hormone-sensitive adenylate cyclase system constitutes a unique model for determining whether the proteolytic effects on adenylate cyclase inhibition are accompanied by changes in epinephrine binding and for further exploring the site of action of the protease.

MATERIALS AND METHODS

Materials. EGTA (Ethylene glycol bis(βamino ethyl ether)NNN'N' tetra acetic), l-epinephrine bitartrate, creatine phosphokinase, creatine phosphate, soybean trypsin inhibitor, dithiothreitol, GTP and ATP were obtained from Sigma Chemical Co. Cyclic AMP was from Calbiochem; l-propranolol was a gift from Imperial Chemical Industries; EDTA (ethylene diamine tetraacetic acid) was from Merck; alpha-chymotrypsin (E.C. 3.4.21.1, 53 U/mg) was from Worthington. [α-^{32}P] ATP (21.5 Ci/mmol) and tritiated yohimbine (82.6 Ci/mmol) were purchased from New England Nuclear. Cyclic [8-^{3}H] AMP (13 Ci/mmol) was obtained from the CEA (Saclay, France). All other chemicals were from Merck and of the highest degree of purity available.

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Platelet membrane preparation. Blood was obtained from healthy male human donors who had taken no drugs for two weeks. Sixty ml of venous blood were collected in a plastic syringe containing 3 ml of 100 mM EDTA, and diluted with 30 ml of washing medium containing 135 mM NaCl, 13 mM sodium citrate, 5 mM glucose and 1 mM EDTA, pH 6.5. Diluted platelet-rich plasma was obtained by centrifugation (500 x g, 10 min, room temperature), mixed with an equal volume of washing medium, and recentrifuged. Platelets were lysed by resuspending the pellet in 8 ml of cold hypotonic buffer (10 mM Tris-HCl pH 7.4, 1 mM DTT, 5 mM EGTA) followed by rapid freezing in liquid nitrogen and thawing at 20°C (cycle repeated three times). The homogenate was then treated with alpha-chymotrypsin as described below. Control and proteolysed platelets were then washed by 3 successive centrifugation steps (30000 x g, 15 min) in the hypotonic buffer. For adenylate cyclase assays, the final pellet was resuspended in the same buffer but containing 1 mM EGTA. For the binding assays, the pellet was resuspended in the incubation buffer (50 mM Tris-HCl, pH 7.4, 12 mM $MgCl_2$, 1.5 mM EGTA). Final protein concentration in the assays was determined according to Lowry et al (11) using bovine serum albumine as standard.

Proteolytic treatment. Lysed platelets (2.5 to 4 mg protein/ml) were incubated for 10 min at 25°C in the presence of the indicated concentrations of alpha-chymotrypsin. At the end of the incubation, soybean trypsin inhibitor (10 to 20 fold molar excess over alpha-chymotrypsin) was added in each fraction. Membranes were then prepared from the different fractions as described above.

Adenylate cyclase assay. Adenylate cyclase activity was determined as previously described (1). Unless otherwise stated, the reaction mixture contained 50 mM Tris-HCl, pH 7.6, 0.1 mM [α-^{32}P] ATP (0.5 µCi), 5 mM $MgCl_2$, 250 µM EGTA, 1 mM cyclic tritiated AMP, 10 µM propranolol, and an ATP-regenerating system consisting of 25 mM phosphocreatine and 1 mg/ml of creatine phosphokinase, in a final volume of 60 µl. Incubation was initiated by the addition of platelet membranes (10-30 µg membrane protein) and continued at 30° for 10 min. Reaction was terminated as previously described (12). The results obtained from triplicate determinations agreed within ± 5 %.

Binding assay. Control or alpha-chymotrypsin-treated membranes (15-50 µg of membrane protein) were incubated for 30 min at 25°C with tritiated yohimbine (7-8 nM) in a final volume of 250 µl containing 50 mM Tris-HCl pH 7.4, 12 mM $MgCl_2$, 1.5 mM EGTA and various concentrations of (-)epinephrine. At the end of the incubation time, each sample was diluted with 10 ml of incubation buffer and immediately filtered through Whatman GF/C glass fiber filters under vacuum. Each filter was quickly rinsed with 10 ml of the same buffer and counted in 10 ml of Ready-Solve E-P scintillation mixture. Non-specific binding was determined in the presence of 10 µM phentolamine and represented approximately 30 % of total binding. All values shown in the figures and text refer to specific binding and are the mean of triplicate determinations. K_D values for epinephrine determined from tritiated yohimbine displacement experiment were calculated according to Cheng and Prusoff (13).

RESULTS

In Fig. 1A is depicted the effect of increasing concentrations of GTP on adenylate cyclase activity of control and alpha-chymotrypsin-treated platelet membranes, assayed in the absence or presence of 30 µM epinephrine. In control as well as in the protease-treated membranes, GTP alone caused a slight, transient activation of the enzyme at low concentration (Fig. 1A). However, addition of epinephrine caused a marked, GTP-dependent inhibition which reached 55 % of the cyclase activity in control membranes, but not in the protease-treated membranes (Fig. 1A). This is shown more clearly in Fig. 1B where percent inhibitions are depicted. These data clearly demonstrate that the marked inhibition of cyclase induced by GTP in the presence of epinephrine no longer occurred after proteolytic treatment.

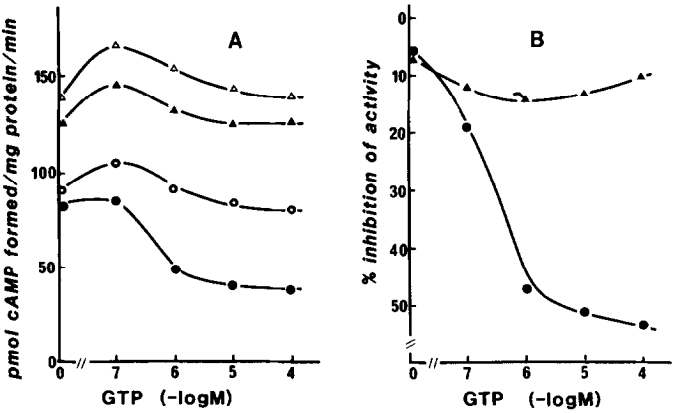

Fig. 1. *Dose-response pattern of GTP on the epinephrine inhibition of adenylate cyclase in control and alpha-chymotrypsin-treated platelets.*
Panel A. Control (○,●) and alpha-chymotrypsin-treated (▲,△) platelets (3.2 µg alpha-chymotrypsin/mg protein) were incubated as described in "Methods" in the absence (open symbols) or presence (closed symbols) of 30 µM epinephrine, with varying concentrations of GTP.
Panel B represents the percent inhibition induced by epinephrine as a function of GTP concentration, with (▲) and without (●) proteolytic treatment.

The effect of alpha-chymotrypsin is dose-dependent. Fig. 2A depicts the effect of increasing concentrations of alpha-chymotrypsin on basal and epinephrine inhibited adenylate cyclase activity. Basal activity was maximaly enhanced (1.7 fold) by 4 µg protease/mg platelet protein. In the presence of GTP and epinephrine and in the absence of protease, cyclase activity was 40 % of control value; addition of increasing amounts of alpha-chymotrypsin markedly enhanced (2.5 fold)

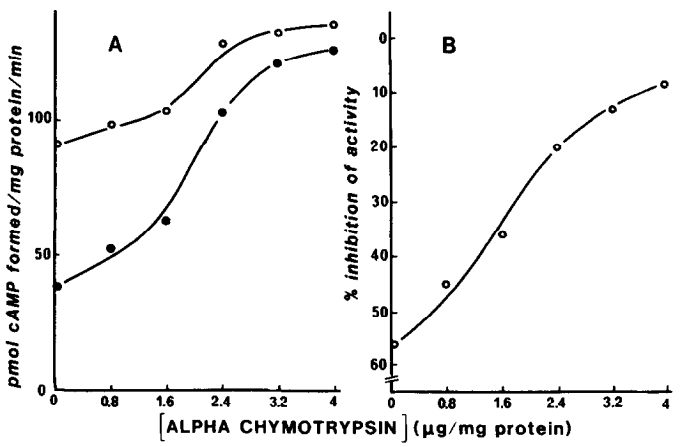

Fig. 2. *Dose-response pattern of alpha-chymotrypsin on the epinephrine inhibition of adenylate cyclase.*
Panel A shows the effect of alpha-chymotrypsin pretreatment on the adenylate cyclase activity assayed in the absence (○) or presence (●) of 0.1 mM GTP and 30 µM epinephrine.
Panel B depicts the inhibition of adenylate cyclase induced by epinephrine plus GTP expressed as percent of the corresponding value without GTP, and as a function of alpha-chymotrypsin concentration.

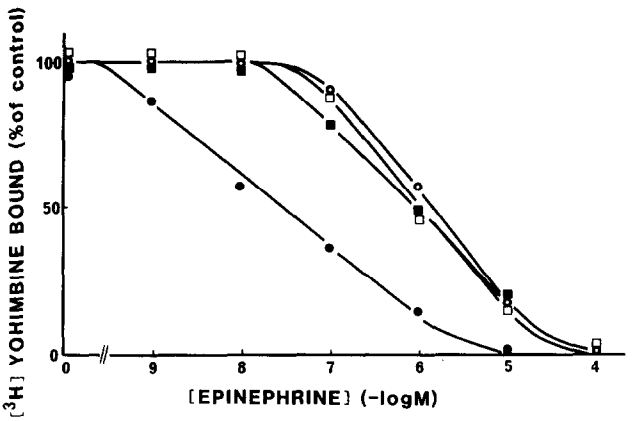

Fig. 3. *Displacement of tritiated yohimbine binding by epinephrine in control and treated membranes.*
Platelets membranes (15 to 50 µg protein) pretreated (■,□) or not (●,○) with alpha-chymotrypsin (3.2 µg/mg protein), were incubated with tritiated yohimbine (7 nM) as described in "Methods" in the absence (closed symbols) or presence (open symbols) of 100 µM GTP. Values are the mean of triplicate determination and represents the percent of maximal specific tritiated yohimbine binding as a function of epinephrine concentration. Control value (100 %) represents 230 fmol of tritiated yohimbine bound per mg of protein and was not modified by the proteolytic treatment.

this activity, to reach values similar to the control ones. As a consequence, the percent inhibition of cyclase due to epinephrine plus GTP decreased as a function of protease concentration (Fig. 2B) with a half-maximal effect at 2 µg protease per mg of membrane protein.

As shown in Fig. 3, epinephrine displaced tritiated yohimbine binding in control platelet membranes with a K_D value of 7 nM. Addition of 100 µM GTP resulted in a 60-fold decrease in the affinity of epinephrine (K_D = 410 nM). After proteolytic treatment, the number of yohimbine binding sites (230 fmol/mg membrane protein) was not changed. However, the affinity of epinephrine was markedly decreased (K_D = 120 nM) and could no longer be significantly modified by the further addition of GTP (K_D = 220 nM).

This effect of alpha-chymotrypsin appeared to be dose-dependent. In Fig. 4 is depicted the effect of increasing concentrations of protease on the displacement of tritiated yohimbine by epinephrine. This is plotted as the difference between the specific binding measured with GTP and that measured without GTP, expressed as the percent of the maximal displacement due to GTP. It appears that alpha-chymotrypsin markedly reduced the GTP shift with a half-maximal effect at 5 µg/mg protein.

DISCUSSION

In the present paper, we demonstrate that proteolytic treatment exerts multiple effects on adenylate cyclase activity and the $alpha_2$-adrenergic receptor of human platelet membranes: activation of basal adenylate cyclase, suppression

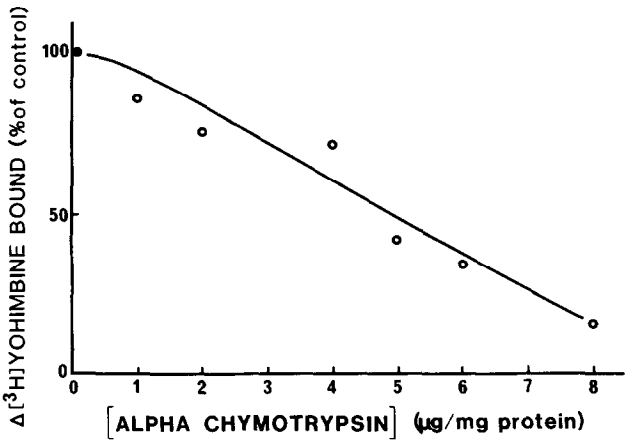

Fig. 4. *Dose-response pattern of alpha-chymotrypsin on the GTP-induced shift in the displacement curves of tritiated yohimbine by epinephrine.* Platelet membranes (25 µg protein) were pretreated with the various concentrations of alpha-chymotrypsin indicated and incubated afterwards with tritiated yohimbine (7 nM) and 0.1 µM epinephrine, with or without 100 µM GTP. Each value represents the difference between the specific binding measured with GTP and that measured without GTP, expressed as the percent of the maximal displacement due to GTP (when no protease was added), as a function of alpha-chymotrypsin concentration.

of the GTP-mediated, epinephrine-induced adenylate cyclase inhibition, and shift of the epinephrine displacement of yohimbine binding towards a low affinity state. The question therefore arises as to whether alpha-chymotrypsin acts through multiple sites or via a unique regulatory component. The epinephrine inhibition of human platelet adenylate cyclase is known to be dependent upon the presence of GTP (14, 15) acting via a GTP-binding inhibitory subunit (usually called Ni (16)). Since the effect of GTP is completely abolished after proteolytic treatment (Fig. 1) in a dose-dependent manner (Fig. 2), Ni is the likely site of action of alpha-chymotrypsin.

It has also now been well demonstrated that human platelet $alpha_2$-adrenergic receptors exist in two different states varying by their affinity for agonists (9, 17), one of high affinity for agonists, coupled with a GTP-regulatory component, the other of low affinity, supposedly independent of GTP control. Proteolytic treatment of human platelet membranes can irreversibly uncouple the $alpha_2$-adrenergic receptor from the GTP regulatory subunit as evidenced by the low affinity state for agonist obtained after alpha-chymotrypsin treatment (Fig. 3).

One of the unanswered questions concerning the GTP-controlled inhibitory regulation of adenylate cyclase (recently reviewed by Cooper (15) and Jakobs (18)) deals with the apparent dual effects of GTP on agonist binding and adenylate cyclase, and with the possible identity of Ni with the regulatory component Ns mediating activation of cyclase. Hoffman and coworkers (19) have reported that low concentrations of manganese could uncouple the $alpha_2$-

receptor-mediated inhibition of adenylate cyclase in human platelets without affecting either the GTP sensitivity of agonists binding to the receptor, or the PGE_1 activation of cyclase.

More recently, Jakobs and coworkers (20) reported that pretreatment of intact platelets or platelet membranes with the sulfhydryl reagent, N-ethylmaleimide abolished the inhibition of the adenylate cyclase and the concomitant stimulation of the GTPase by epinephrine, without affecting the sensitivity of adenylate cyclase to stimulatory agents. It therefore appears likely that Ni and Ns are different entities. Whether or not the Ni protein mediating the GTP inhibition of adenylate cyclase and the GTP-binding component regulating agonist affinity for the $alpha_2$-adrenergic receptor are the same protein is still an open question. From our own results, it appears that alpha-chymotrypsin suppresses both the GTP-dependent epinephrine inhibition of adenylate cyclase and the high affinity sites for epinephrine within the same concentration range, suggesting that it acts on a common site. This supports the concept that only one protein is responsible for the GTP regulation of $alpha_2$-receptor and inhibition of cyclase. Furthermore, our data strongly suggest that the high affinity binding sites sensitive to proteolysis are directly responsible for the $alpha_2$-adrenergic regulation of platelet adenylate cyclase. Along the same line, it should be noted that proteolytic treatment of liver plasma membrane also alters the regulation by GTP of the $alpha_1$-adrenergic receptor, a receptor not linked to adenylate cyclase (21-24).

The loss of epinephrine inhibition of cyclase in aged platelets (25) may be explained by the sensitivity of Ni to proteolytic activity. However, that Ni is uniquely sensitive to protease cannot be responsible for the stimulatory effect of protease in most other systems tested (3). In these cases, where no Ni is usually functional, another mechanism of action is probably involved.

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