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EFFECT OF PROTEASES AND PHOSPHOLIPASES ON [^{3}H]YOHIMBINE BINDING TO HUMAN PLATELET MEMBRANES*

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Abstract—Trypsin and chymotrypsin inactivated specific [^{3}H]yohimbine binding sites in the partially purified human platelet membranes in a concentration- and time-dependent fashion. The maximal inactivation (70–80% of control) was incomplete regardless of the concentrations of the proteases used or the incubation time. Scatchard analysis of the binding data showed that the total number of binding sites was reduced, but the affinity of the receptor to the ligand remained unaffected. Pretreatment of the membranes with unlabeled yohimbine or epinephrine produced a 20–30% increase in the specific [^{3}H]yohimbine binding; however, this treatment offered only a slight protection (10–15%) against trypsin-induced inactivation of [^{3}H]yohimbine binding. Pretreatment with phospholipase A_2 produced a complete inhibition, while pretreatment with phospholipase C resulted in only a partial (70–80% of control) reduction in [^{3}H]yohimbine binding. The inhibitory effects were not reversed when the specific binding of [^{3}H]yohimbine was carried out with membranes treated with phospholipases and subsequently washed with defatted bovine serum albumin, suggesting that products released from phospholipolysis were not involved in the inhibition of [^{3}H]yohimbine binding. These results suggest that the integrity of the receptor proteins and phospholipids is necessary for the specific binding of the ligand to the α_2-adrenoreceptor proteins of the human platelet membranes.

Agents known to disrupt the cell membrane organization affect the specific binding of a variety of ligands as well as the activity of many membrane-bound enzymes. Thus, treatment of the hepatic plasma and synaptic membranes with trypsin increases the specific binding of [^{3}H]epinephrine and [^{3}H]glutamate respectively [1–3]. Treatment of the rat brain homogenates with trypsin produces a reduction in the opiate receptor binding sites [4]. When the microsomal fraction of ileal or synaptic membranes is incubated with trypsin, it causes a reduction in the apparent affinity of an agonist but not that of an antagonist for the muscarinic cholinergic receptors [5]. Among the membrane-bound enzymes, adenylate cyclase activity of plasma membranes from rat ovaries is increased in the presence of trypsin [6].

Phospholipids form a major component of the biomembranes. They, in addition to providing a fluid matrix for protein movement, strongly interact with membrane proteins, and are crucial for the activity of membrane-bound enzymes as well as for the ligand–receptor interactions, possibly serving as the specific receptors for the ligand, as modulators for the ligand–receptor interactions, and/or as cofactors for the functional receptors [7]. Pretreatment with phospholipases inhibits the binding of ligands to various receptors such as the opiate [4], muscarinic cholinergic [8], glucagon [9], and β-adrenoreceptors [10]. In this study we describe the effects of the proteases and phospholipases on the specific [^{3}H]yohimbine binding to α_2-adrenoreceptors of the human platelet membranes.

MATERIALS AND METHODS

The following chemicals and enzymes were purchased: phospholipase A_2 (bee venom), trypsin (bovine pancreas), chymotrypsin (bovine pancreas), trypsin inhibitor (soybean), fatty acid free bovine albumin, 5′-guanylylimidodiphosphate (Sigma); phospholipase C (*Bacillus cereus*) (Calbiochem), and [O-*methyl*-^{3}H]yohimbine (90 Ci/mmole; Amersham).

Preparation of the platelet membranes. Platelet-rich plasma (PRP) was obtained from the local blood bank. The PRP was layered on the lymphocyte separating medium (which contained 9.4 g sodium diatrizoate and 6.2 g Ficoll in 100 ml water) and centrifuged at 300 *g* for 30 min to remove residual erythrocytes and leukocytes. The layer above the lymphocyte separating medium containing the platelets was examined for contamination with erythrocytes and leukocytes using an automated Coulter counter, and had less than 2% erythrocytes and 0.05% leukocytes. The layer containing the platelet was carefully aspirated and centrifuged at 16,000 *g* for 10 min. The procedures described below were carried out at 4°. The platelets were washed twice in ice-cold 50 mM Tris–HCl buffer (pH 7.5) containing

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100 mM NaCl and 5 mM EDTA, and were lysed by exposure to 5 mM Tris–HCl (pH 7.5) containing 1 mM EDTA. The lysed platelets were homogenized using a glass homogenizer with a motor-driven Teflon pestle and centrifuged at 40,000 *g* for 30 min. This process of lysis, homogenization, and centrifugation was done twice. The resulting pellet (platelet membrane) was washed twice with buffer made up of 50 mM Tris–HCl (pH 7.5) plus 1 mM EDTA. The partially purified platelet membranes were suspended in Tris–EDTA buffer and frozen at −70°.

Ligand binding assay. All incubations were carried out at 23° for 40 min. Binding of [^{3}H]yohimbine to platelet membranes was carried out in a final volume of 0.5 ml containing 20 mM Tris–HCl (pH 7.5) plus 1 mM EDTA and 150–200 μg of membrane protein. Ascorbic acid (1 mM final concentration) was used as an antioxidant in experiments in which catecholamines were included. This concentration of ascorbic acid had no effect on the binding of [^{3}H]yohimbine. The binding was initiated by adding the membranes to the incubation medium and terminated by the addition of 5.0 ml of ice-cold assay buffer followed by vacuum filtration over glass fiber filters. The filter was then rapidly washed twice with 5 ml of ice-cold assay buffer. The radioactivity bound to the membrane trapped by the filter was extracted into a toluene/methoxyethanol/2,5-diphenyloxazole scintillation mixture and counted in a Beckman scintillation counter with an efficiency of 40%. Nonspecific binding was determined in each assay by running parallel samples containing 10^{-5} M unlabeled yohimbine. Specific binding was obtained by subtracting nonspecific from total binding.

Data analysis. K_d, the dissociation constant, and B_{max}, the maximum number of binding sites/mg protein, were determined from Scatchard analysis of saturation isotherms. A plot of B/F versus B was constructed where B is the amount of radioligand specifically bound and F is the free concentration of radioligand in the assay (the amount of radioligand added minus the amount of total binding). The K_d of the ligand was calculated by taking the negative reciprocal of the slope of the plot, and the B_{max} was given by the intercept with the abscissa. Data were fitted by straight lines using linear regression analysis.

Proteolytic degradation of the platelet membranes. Fresh solutions of trypsin, chymotrypsin, and trypsin inhibitor were prepared just before use. Trypsin was dissolved in 1 mM HCl while chymotrypsin and trypsin inhibitor were dissolved in water. The platelet membranes (0.9 to 1.2 mg protein) were incubated with or without proteases in a total volume of 1.0 ml buffer containing 25 mM Tris–HCl + 0.5 mM EDTA (pH 7.5) at 37° for 30 min. Proteolytic digestion was terminated by adding 2 parts of soybean trypsin inhibitor to 1 part of trypsin or chymotrypsin by weight. The platelet membranes were diluted to 4.0 ml with ice-cold buffer (25 mM Tris–HCl + 1 mM EDTA, pH 7.5) and centrifuged at 100,000 *g* for 30 min in order to collect the small fragments of the cell membrane after proteolytic digestion. The pellet was suspended in 4.0 ml of ice-cold buffer and centrifuged as above. The pellet was resuspended in 1.0 ml of 50 mM Tris–HCl + 1 mM EDTA (pH 7.5). An aliquot of the suspended membrane was used for binding assays.

Treatment of platelet membranes with phospholipases. The platelet membranes containing 0.9 to 1.0 mg protein were incubated at 37° for 30 min (with gentle agitation) in 1.0 ml of the medium containing 25 mM Tris–HCl (pH 7.5) and 1 mM $CaCl_2$ with or without phospholipases. Thirty minutes later, the enzymatic reaction was terminated by adding ethyleneglycolbis-(amino-ethylether)tetra-acetate (EGTA, final concentration 10 mM). The samples were diluted to 4.0 ml with ice-cold buffer (25 mM Tris–HCl + 1 mM EDTA, pH 7.5) and centrifuged at 100,000 *g* for 30 min. The supernatant fluid was discarded, and the pellet was suspended in 4.0 ml of cold buffer and centrifuged as above. The pellet was resuspended in 1.0 ml of buffer containing 50 mM Tris–HCl + 1 mM EDTA (pH 7.5). An aliquot of the suspended membrane was used for binding assays as described above.

Washing of platelet membranes with defatted bovine serum albumin. The procedure for washing the membranes with defatted bovine serum albumin was followed as described by Azhar *et al.* [11]. Briefly, the platelet membranes treated with or without phospholipases A_2 or C were washed as described above and resuspended in 50 mM Tris–HCl (pH 7.5). Aliquots of these membranes were incubated with 1 or 2% (w/v) defatted bovine serum albumin at 0° for 30 min. After incubation, the samples were washed with buffer containing 1% (w/v) defatted bovine serum albumin. Finally, the membranes were washed twice with 50 mM Tris–HCl + 1 mM EDTA (pH 7.5) to remove adsorbed albumin. An aliquot of the washed membrane was then used for ligand binding assay.

Protein concentration was measured by the procedure of Lowry *et al.* [12] using bovine serum albumin as standard.

RESULTS

Proteolytic degradation of the platelet membranes. Results in a typical experiment where pretreatment with either trypsin or chymotrypsin reduced specific [^{3}H]yohimbine binding to the platelet membranes in a concentration-dependent manner are shown in Fig. 1. However, neither enzyme caused a complete inhibition of [^{3}H]yohimbine binding even in concentrations as large as 2 mg/mg membrane protein. No significant difference in the potency or overall inhibitory activity of trypsin or chymotrypsin was noted; however, there was a greater variability in the inhibitory response from one batch of platelet membrane to another at low concentrations of the enzyme (1–2 μg/mg membrane protein). The reduction in [^{3}H]yohimbine binding after trypsin digestion of platelet membranes was due to a reduction in the number of binding sites without an effect on the affinity of the receptor sites for the ligand (Fig. 2). Similar results were obtained when the specific binding was checked in the presence of 2 μg chymotrypsin/mg membrane protein.

Time course of the proteolytic degradation. In these experiments, [^{3}H]yohimbine binding was carried out in membranes that were exposed to the proteolytic

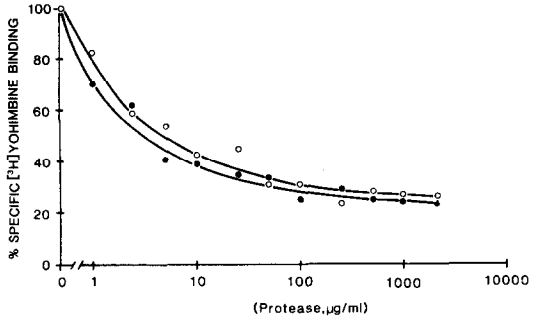

Fig. 1. Effect of various concentrations of proteases on specific binding of [^{3}H]yohimbine to human platelet membranes. Platelet membranes were incubated for 30 min at 37° with or without proteases (○—○, chymotrypsin; ●—●, trypsin) in a total volume of 1.0 ml buffer. Proteolytic activity was terminated with tryspin inhibitor. After washing the membranes, binding was carried out in the presence of 5 nM [^{3}H]yohimbine as described in the text.

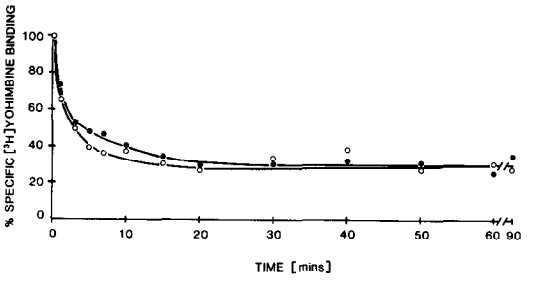

Fig. 3. Inactivation of specific [^{3}H]yohimbine binding to human platelet membranes by proteases as a function of time. Platelet membranes were incubated at 37° with 500 μg of proteases (○—○, chymotrypsin; ●—●, trypsin), and the proteolytic activity was terminated with trypsin inhibitor at the indicated times. The membranes were washed and binding was carried out in the presence of 5 nM [^{3}H]yohimbine as described under Materials and Methods.

enzymes for different time intervals. Specific [^{3}H]yohimbine binding of the membrane exposed to the enzymes as a function of time showed that the inactivation of the binding occurred within minutes with a maximum decrease of 70–80% occurring within 20 min of exposure to the enzyme. Longer incubation time had no further effect on [^{3}H]yohimbine binding (Fig. 3).

Effects of pretreatment of the membranes with monovalent cations or with Mg^{2+} *+ GTP analogue on the inhibition of [^{3}H]yohimbine binding by trypsin.* The effects of pretreatment of the membranes with Mg^{2+} + 5′-guanylylimidodiphosphate (GMP–PNP), sodium chloride, sodium acetate and sulfate, and chlorides of lithium or potassium were examined by adding these substances to the incubation medium for 30 min. *N*-Methyl-D-glucamine hydrochloride was also used to study the effect of ionic perturbation produced by these salts. Table 1 summarizes the results obtained in this series of experiments which again showed that trypsin alone produced approximately 60% inactivation of [^{3}H]yohimbine binding. Pretreatment of the membranes with Mg^{2+} + GMP–PNP did not protect this action of trypsin. However, salts of sodium, potassium, and lithium offered protection against trypsin inhibition. The antagonism of trypsin effect by these salts appeared to be nonspecific since *N*-methyl-D-glucamine also had a similar effect. Furthermore, sodium sulfate, which would produce a higher ionic strength in the solution, offered a greater degree of protection than the other salts, and this was confirmed by a smaller inhibition when the concentration of NaCl was increased to 100 mM (Table 1).

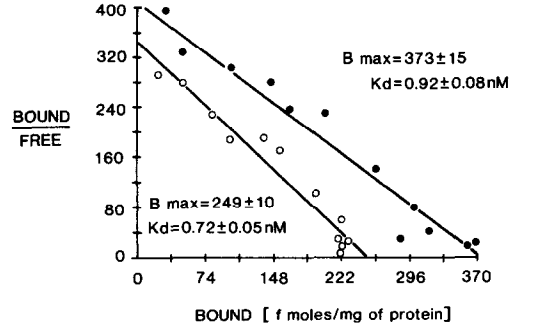

Fig. 2. Scatchard analysis of specific binding of [^{3}H]yohimbine to human platelet membranes treated with (○—○) and without (●—●) trypsin. Platelet membranes (200–250 μg protein) were incubated with or without 2 μg of trypsin at 37° for 30 min. The reaction was stopped by adding trypsin inhibitor. After allowing the membranes to attain room temperature (23°), binding was carried out in the presence of various concentrations of [^{3}H]yohimbine.

The effect of pretreatment of the membranes with adrenergic ligands on the inhibition of [^{3}H]yohimbine binding by trypsin. The effects of pretreatment of the membranes with α-adrenergic agonist (epinephrine) and an antagonist (yohimbine) were tested on the inactivation of [^{3}H]yohimbine binding to the receptor by trypsin. As shown in Table 2, pretreatment of the membranes with either saturating concentrations of unlabeled yohimbine or epinephrine produced a 20–35% increase in the specific binding of the radioactive ligand, and yohimbine was consistently more potent than epinephrine in this respect. The mechanism of increased binding induced by these drugs is not clear and is currently being investigated. The inactivation of [^{3}H]yohimbine binding by trypsin was not influenced by pretreatment with either epinephrine or yohimbine (Table 2). The apparent difference between 40% binding in the presence of trypsin alone and 65–73% binding in the presence of yohimbine or epinephrine plus trypsin may be explained on the basis of an increased specific ligand binding with yohimbine or epinephrine alone since the absolute change after trypsin under the three different experimental conditions remains the same.

Effect of pretreatment of the platelet membranes with the phospholipases on [^{3}H]yohimbine binding. The effects of the two phospholipases on [^{3}H]yohimbine binding to the platelet membranes are shown in Fig. 4. Phospholipase A_2 produced a concentration-dependent decrease in [^{3}H]yohimbine binding, and a complete inhibition was observed

Table 1. Effects of Mg^{2+} + GMP–PNP and monovalent cations on the trypsin-induced inactivation of [^{3}H]yohimbine binding to platelet membranes

Treatment	Specific [^{3}H]yohimbine binding (% of control)
Control	100
Trypsin 100 μg	39 ± 4
Trypsin 100 μg + 5 mM $MgCl_2$ + 0.1 mM GMP–PNP	42 ± 3
Trypsin 100 μg + 50 mM NMDG HCl	54 ± 5
Trypsin 100 μg + 50 mM NaCl	59 ± 6
Trypsin 100 μg + 100 mM NaCl	85 ± 5
Trypsin 100 μg + 50 mM sodium acetate	55 ± 4
Trypsin 100 μg + 50 mM Na_2SO_4	82 ± 3
Trypsin 100 μg + 50 mM KCl	62 ± 5
Trypsin 100 μg + 50 mM LiCl	57 ± 3

Platelet membranes (0.9 to 1.1 mg) were incubated with or without trypsin in a total volume of 1.0 ml buffer containing 25 mM Tris–HCl + 0.5 mM EDTA (pH 7.5) at 37° for 30 min. Tryptic digestion was terminated by adding 200 μg of trypsin inhibitor. The tubes were centrifuged at 100,000 *g* for 30 min. Each pellet was resuspended in the above buffer and centrifuged again. The pellets were finally suspended in 1.0 ml of 50 mM Tris–HCl + 1 mM EDTA (pH 7.5). An aliquot of the suspended membrane was used for binding, and the binding was carried out in the presence of 5 nM [^{3}H]yohimbine. The membranes treated with various electrolytes in the absence of trypsin were washed and used for binding. In all cases, the amount of binding was between 99 and 100% of control. Values are means ± S.E., N = 5. NMDG = *N*-methyl-D-glucamine; all other abbreviations are explained in the text.

Table 2. Effect of trypsin on pre-bound ligand–receptor complex

Treatment	Specific [^{3}H]yohimbine binding (% of control)
Control	100
Membrane + 100 μg trypsin	40 ± 5
Membrane + 10 μM yohimbine	132 ± 3
Membrane + 100 μM epinephrine	123 ± 5
Membrane + 10 μM yohimbine + 100 μg trypsin	73 ± 6
Membrane + 100 μM epinephrine + 100 μg trypsin	65 ± 4

Platelet membranes (0.9 to 1.1 mg) were incubated with or without ligand in a total volume of 1.0 ml buffer containing 25 mM Tris–HCl + 0.5 mM EDTA (pH 7.5) at 37° for 30 min. Tryptic digestion was initiated by adding trypsin. Proteolysis was terminated 30 min later by adding 200 μg of trypsin inhibitor. The tubes were diluted to 4.0 ml with buffer and centrifuged at 100,000 *g* for 30 min. Each pellet was resuspended in the above buffer and centrifuged again. This process of resuspension and centrifugation was repeated three more times. The pellets were finally suspended in 1.0 ml of 50 mM Tris–HCl + 1 mM EDTA (pH 7.5). An aliquot of the suspended membrane was used for binding, and binding was carried out in the presence of 5 nM [^{3}H]yohimbine. Values are means ± S.E., N = 5.

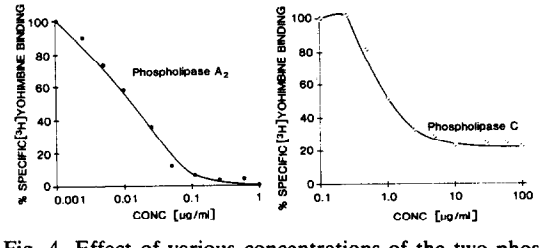

Fig. 4. Effect of various concentrations of the two phospholipases on specific binding of [^{3}H]yohimbine to human platelet membranes. The membranes were treated with the phospholipase, as described in the text, and washed; binding was carried out in the presence of 5 nM [^{3}H]yohimbine.

with 1.0 μg of the enzyme/mg membrane protein. Phospholipase C also produced an inhibition of [^{3}H]yohimbine binding; however, the inhibition was incomplete with a maximal decrease of 70–80% of control even with concentrations as high as 100 μg/ml (Fig. 4). Similar results were obtained when phospholipase A_2 or phospholipase C was heated at 90° for 5 min prior to digestion of platelet membranes. It is interesting to note that the effect of phospholipase C on [^{3}H]yohimbine binding was similar to that produced by the proteolytic enyzmes. The specificity of the action of the phospholipases A_2 and C was also investigated by studying their effect on [^{3}H]yohimbine binding in the absence of Ca^{2+}, since it is well known that both these enzymes require

Table 3. Effect of calcium ion on phospholipase-induced changes in [^{3}H]yohimbine binding to human platelet membranes

Treatment	Specific binding of [^{3}H]yohimbine (fmoles/mg protein)
Membrane alone	203.1 ± 10.8
Membrane + 1 mM $CaCl_2$	205.2 ± 7.5
Membrane + 10 mM EGTA	201.3 ± 6.8
Membrane + 0.1 μg phospholipase A_2	210.5 ± 15.2
Membrane + 0.1 μg phospholipase A_2 + 1 mM $CaCl_2$	27.48 ± 3.5
Membrane + 10 μg phospholipase C	208.53 ± 8.5
Membrane + 10 μg phospholipase C + 1 mM $CaCl_2$	54.07 ± 4.8

Membrane preparations (1 mg) were incubated for 30 min at 37° in 1.0 ml buffer with phospholipase A_2 or phospholipase C in the presence or absence of 1 mM $CaCl_2$. The incubation was terminated by the addition of EGTA to a final concentration of 10 mM. After washing the membranes, binding was carried out with 5 nM [^{3}H]yohimbine as described under Materials and Methods. Values are means ± S.E., N = 5.

Ca^{2+} for their activity. As shown in Table 3, the effects of both phospholipase A_2 and C were lost completely in the absence of Ca^{2+} in the medium. The results show that the decrease in [^{3}H]yohimbine binding produced by phospholipase A_2 or phospholipase C was due to intrinsic activity of these enzymes.

Effect of washings of phospholipase A_2 or phospholipase C platelet membranes with defatted bovine serum albumin on [^{3}H]yohimbine binding. Since the phospholipid hydrolysis products have been shown to inhibit ligand binding to receptor proteins [13], experiments were carried out to test the possibility of hydrolytic products having inhibitory effects on [^{3}H]yohimbine binding. Since defatted bovine serum albumin has been reported to effectively remove fatty acids and lysophosphatides from solutions or from membranes [11], phospholipase A_2 or phospholipase C treated membranes were washed with and without defatted bovine serum albumin, and the binding activity of the washed membranes was determined. As shown in Table 4, washings of platelet membranes digested with phospholipase A_2 or phospholipase C with increasing concentrations of defatted serum albumin did not restore the [^{3}H]yohimbine binding, suggesting that the decrease in [^{3}H]yohimbine binding produced by phospholipase A_2 or phospholipase C was not due to hydrolytic products of phospholipids.

DISCUSSION

Trypsin and chymotrypsin have been utilized to probe the mechanism of hormonal and guanine nucleotide regulation of the adenylate cyclase of the human platelet membranes. Ferry *et al.* [14] and Stiles and Lefkowitz [15] reported that neither trypsin nor chymotrypsin reduces specific [^{3}H]yohimbine binding to human platelet membranes whereas both enzymes inhibit the effect of epinephrine on adenylate cyclase. However, these investigators had used only low concentrations of the proteases, concentrations which apparently uncoupled the adrenoreceptor-mediated inhibition of adenylate cyclase activity, but had no effect on the specific binding of the ligand to the receptor proteins. In the present study, we used a wide range of the trypsin and chymotrypsin concentrations and found a concentration- as well as a time-dependent inhibition of specific [^{3}H]yohimbine binding by these enzymes. Further characterization of their effect showed that they were able to reduce the specific binding only by a maximum of 70–80% of control, and this effect was prevented nonspecifically by pretreatment of the

Table 4. Specific [^{3}H]yohimbine binding to platelet membranes digested with phospholipase A_2 or phospholipase C followed by washing the membranes with different concentrations of defatted bovine serum albumin

Treatment	[^{3}H]Yohimbine binding to platelet membranes (fmoles/mg protein)		
	Control	1% (w/v) BSA	2% (w/v) BSA
Membrane alone	252.5 ± 8	245.3 ± 10	248.2 ± 12
Membrane + 0.1 μg phospholipase A_2	30.8 ± 6	34.2 ± 9	38.4 ± 5
Membrane + 10 μg phospholipase C	62.3 ± 5	65.6 ± 4	75.3 ± 6

Platelet membranes were treated with phospholipases A_2 and C as described under Materials and Methods. The hydrolysis products of phospholipids were removed by washing with two different concentrations of defatted bovine serum albumin (BSA). The washing procedures are described under Materials and Methods. Aliquots of the washed membranes were used to study specific binding in the presence of 5 nM [^{3}H]yohimbine. Values are means ± S.E., N = 5.

membranes by monovalent ions. Saturation binding data showed that both trypsin and chymotrypsin reduced the total number of the membrane receptors without altering their affinity for [^{3}H]yohimbine. These observations can be explained by the possibility that part of the receptor protein is located within the cell membrane but is resistant to complete proteolysis because of their inaccessibility to the enzymes. Alternatively, a smaller number of fragments after cleavage of the receptor protein by the enzyme are still capable to specific binding with [^{3}H]yohimbine. These possibilities are currently being investigated.

It has been demonstrated that the phospholipids form an important component of the receptor proteins of the opioid [4], glucagon [9], β-adrenergic [10], benzodiazepine [16], and serotonin [17] receptors. Phospholipases are known to remove the nonpolar (by phospholipase A_2) or polar (by phospholipase C or D) moieties of phospholipids [18]. In the present study with the α_2-adrenoreceptors of the human platelet membranes, phospholipase A_2 produced a complete and total inactivation of specific [^{3}H]yohimbine binding, whereas phospholipase C produced only a partial inhibition of [^{3}H]yohimbine binding. It is intriguing that maximal concentrations of phospholipase C, like proteases, were able to inhibit only 70–80% of [^{3}H]yohimbine binding. The effects of phospholipase A_2 or phospholipase C on [^{3}H]yohimbine binding suggest that both hydrophobic and hydrophilic groups may be involved in the binding of [^{3}H]yohimbine to its receptor or the stabilization of receptor activity. The inhibitory effect of phospholipases on ligand binding to the receptor proteins may be due either to the disruption of the membrane-organization by their own intrinsic activity or via the destabilizing effect of its hydrolytic products such as lysophospholipids and fatty acids [19]. Lin *et al.* [13] reported that the inhibition of opiate binding activity by phospholipase A was due to the products resulting from phospholipolysis. Azhar *et al.* [11] showed that the effect of phospholipase A on choriogonadotropin binding to plasma membranes of bovine corpus luteum was due to the accumulation of end products, whereas the effect of phospholipase C was the result of alteration of the phospholipid environment. In the present study, the inhibitory effects of phospholipase A_2 or phospholipase C were re-examined after eliminating the by-products formed during phospholipolysis by repeated washing with defatted bovine serum albumin. As shown in Table 4, defatted bovine serum albumin did not restore the specific [^{3}H]yohimbine binding. We conclude from these findings that the effect of phospholipase A_2 or phospholipase C on specific binding of [^{3}H]yohimbine to the receptor was directly due to their action on the membrane and not to the accumulation of the end products. It is evident from these results that membrane phospholipids play an important role in either the α_2-adrenoreceptor activity or the formation of hormone–receptor complex. It is also conceivable that the α_2-adrenoreceptor activity may be regulated through an increase or decrease in the phospholipase A_2 activity. This hypothesis is consistent with the recent demonstration of the presence of phospholipase A_2 activity in the plasma membrane of human platelets [20, 21]. In conclusion, the results of the present study show that a disruption of the lipid or protein environment of the platelet membranes reduced the α_2-adrenoreceptor ligand binding. Further, these results suggest that an intact lipid and protein environment is necessary in order for the receptor protein to maintain its ability to bind [^{3}H]yohimbine.

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REFERENCES

1. P. Geynet, A. Borsodi, N. Ferry and J. Hanoune, *Biochem. biophys. Res. Commun.* **97**, 947 (1980).
2. M. F. El-Refai and J. H. Exton, *J. biol. Chem.* **255**, 5853 (1980).
3. M. Baudry, E. Smith and G. Lynch, *Molec. Pharmac.* **20**, 280 (1981).
4. G. W. Pasternak and S. H. Snyder, *Molec. Pharmac.* **10**, 183 (1974).
5. K. Matsumoto, S. Uchida, K. Takeyasu, H. Higuchi and H. Yoishida, *Life Sci.* **31**, 211 (1982).
6. H. Neurath and K. A. Walsh, *Proc. natn. Acad. Sci. U.S.A.* **73**, 3825 (1976).
7. H. H. Loh and P. Y. Law, *A. Rev. Pharmac. Toxic.* **20**, 201 (1980).
8. R. S. Aronstam, L. G. Abood and J. Baumgold, *Biochem. Pharmac.* **26**, 1689 (1977).
9. S. L. Pohl, M. J. Drans, V. Kozyreff, L. Birnbaumer and M. Rodbell, *J. biol. Chem.* **246**, 4447 (1971).
10. L. E. Limbird and R. J. Lefkowitz, *Molec. Pharmac.* **12**, 559 (1976).
11. S. Azhar, A. K. Hajra and K. M. Jayaram Menon, *J. biol. Chem.* **251**, 7408 (1976).
12. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
13. H. K. Lin, M. C. Holland and E. J. Simon, *J. Pharmac. exp. Ther.* **216**, 149 (1981).
14. N. Ferry, S. Adnot, A. Borsodi, M. L. LaCombae, G. Guellaen and J. Hanoune, *Biochem. biophys. Res. Commun.* **108**, 708 (1982).
15. G. L. Stiles and R. J. Lefkowitz, *J. biol. Chem.* **257**, 6287 (1982).
16. E. Veno and K. Kuriyama, *Neuropharmacology* **20**, 1169 (1981).
17. S. Yoshikawa and R. Ishitani, *Life Sci.* **36**, 485 (1985).
18. A. L. Lehninger, *Biochemistry*, p. 279. Worth Publishers, New York (1971).
19. B. Rubalcava and M. Rodbell, *J. biol. Chem.* **248**, 3831 (1973).
20. R. L. Jesse and R. C. Franson, *Biochim. biophys. Acta* **575**, 467 (1979).
21. R. Apitz-Castro, M. Cruz, M. Mas and M. K. Jain, *Thromb. Res.* **23**, 347 (1981).