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SHORT COMMUNICATIONS

Characterization of *Alpha*-Adrenergic Receptors in Human Platelets Using [^{3}H]Clonidine

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SUMMARY

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Pharmacological studies of human platelets indicate that epinephrine initiates platelet aggregation and secretion by interacting with *alpha*-adrenergic receptors on the platelet surface. Moreover, direct binding experiments using nonselective antagonists, such as [^{3}H]dihydroergocryptine, indicate a single class of platelet *alpha*-adrenergic receptors. Thus, we sought to examine the interaction of the *alpha*$_2$-selective compound, clonidine, with human platelets in both pharmacological and radioligand binding studies. Although clonidine is considered an agonist in some tissues, we found that this compound was a mixed agonist-antagonist for *alpha*-adrenergic responses in platelets. Furthermore, [^{3}H]clonidine bound to a single class of noncooperative binding sites on platelet membranes. Binding was rapid, reversible, of high affinity (K_d = 24.5 ± 2.1 nM) and low capacity (63.7 ± 4.2 fmoles/mg of protein). *Alpha*-adrenergic agonists and antagonists competed for [^{3}H]clonidine binding sites with a rank order of potency typical for interaction at an *alpha*$_2$-adrenergic receptor. The binding of [^{3}H]clonidine was enhanced in the presence of Mg^{2+} and decreased in the presence of GTP. The total number of [^{3}H]clonidine binding sites was only 18% of the number of [^{3}H]dihydroergocryptine binding sites in platelet membranes. These studies demonstrate that [^{3}H]clonidine, a mixed agonist-antagonist, can be used to identify high-affinity *alpha*$_2$-adrenergic receptors on human platelets. Because [^{3}H]clonidine sites are regulated by Mg^{2+} and GTP, these *alpha*$_2$-adrenergic receptors are likely to be the sites mediating *alpha*-adrenergic agonist responses in platelets.

Human platelets offer a convenient system for the study of a variety of membrane-active compounds. One such class of compounds, the catecholamines, interacts with *alpha*-adrenergic receptors on the platelet surface (1). The platelet responses triggered by *alpha*-adrenergic agonists include aggregation and secretion, potentiation of the aggregation and secretion induced by nonadrenergic agonists (e.g. ADP, thrombin), and inhibition of adenylate cyclase (1–7). Recently, studies of *alpha*-adrenergic action in platelets have been extended by the identification and characterization of *alpha*-adrenergic receptors on platelet membranes or intact platelets using radiolabeled *alpha*-adrenergic antagonists, such as [^{3}H]DHE[2] or [^{3}H]phentolamine (4–8).

On the basis of the relative potencies of various *alpha*-adrenergic agonists and antagonists in pharmacological and radioligand binding studies in other tissues, two subclasses of *alpha*-adrenergic receptors (*alpha*$_1$ and *alpha*$_2$) have been defined (9–11). Thus, *alpha*$_1$-adrenergic receptors exhibit a greater affinity for the agonist methoxamine than for the agonist clonidine, and a greater affinity for the antagonist prazosin than for the

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[2] The abbreviation used is: DHE, dihydroergocryptine.

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antagonist yohimbine. In contrast, *alpha*$_2$-adrenergic receptors demonstrate greater affinities for clonidine and yohimbine than for methoxamine and prazosin. Certain agonists, including epinephrine and norepinephrine, and certain antagonists, such as DHE and phentolamine, are nonselective in that they interact similarly with both *alpha*$_1$ and *alpha*$_2$ receptors.

Attempts to define the nature of the platelet *alpha*-adrenergic receptor have yielded contrasting results. Although studies of platelet aggregation suggested the presence of both *alpha*$_1$ and *alpha*$_2$ receptors (2, 3, 12), radioligand binding studies with [^{3}H]DHE suggested that platelets contain *alpha*$_2$-receptors exclusively (13). In order to clarify the nature of *alpha*-receptors on human platelets, we examined the interaction of platelets with the selective *alpha*$_2$ agent, clonidine, in both pharmacological and radioligand binding studies.

Whole blood was obtained from normal donors, platelet-rich plasma and gel-filtered platelets were prepared, and aggregation studies were performed as described previously (14, 15). For radioligand binding studies, platelet particulates were prepared as follows: platelet-rich plasma was centrifuged at 16,000 × *g* for 10 min and the platelets were resuspended and washed twice in ice-cold buffer containing Tris-HCl, 50 mM, pH 7.4; NaCl, 100 mM; and EDTA, 5 mM. Washed platelets were resuspended in a hypotonic buffer (Tris-HCl, 5 mM, pH 7.5; EDTA, 5 mM) and then homogenized with 12 strokes of a motorized Teflon pestle in a "no clearance" glass tube (7, 16). The homogenate was centrifuged at 39,000 × *g* for 10 min, and the pellet was resuspended in the same buffer and centrifuged a second time. The resultant pellet was resuspended to 1–3 mg of protein per milliliter in an incubation buffer which, in preliminary studies, supported maximal specific binding of [^{3}H]clonidine (Tris-HCl, 70 mM, pH 7.5; EDTA, 0.5 mM; $MgCl_2$, 8 mM; sodium ascorbate, 0.8 mM). Platelet particulates were used immediately after preparation.

[^{3}H]Clonidine binding was determined by incubating platelet particulates with [^{3}H]clonidine (22.2 Ci/mmole; New England Nuclear Corporation, Boston,, Mass.) and various adrenergic compounds in a total volume of 0.25 ml of incubation buffer at 25°. Reactions were terminated by adding 5 ml of ice-cold incubation buffer and filtering samples immediately over glass fiber filters (Gelman A/E). Filters were washed rapidly (<20 sec) four times with ice-cold incubation buffer (5 ml each time) and then counted in a liquid scintillation system (7). In preliminary studies, these conditions were found to be optimal for separating free from membrane-bound [^{3}H]clonidine without reversing specifically bound counts. Nonspecific binding was determined by incubating platelet particulates with [^{3}H]clonidine in the presence of 10 μM phentolamine. When (−)-epinephrine (100 μM) was substituted for phentolamine, values for nonspecific binding were identical. Specific binding of [^{3}H]clonidine (total minus nonspecific binding) represented 40%–75% of total binding. Binding data in this report refer to specifically bound [^{3}H]clonidine. In a typical experiment run between concentrations of 2 and 100 nM [^{3}H]clonidine, specifically bound counts ranged from 100 to 1000 cpm. Binding of [^{3}H]DHE was measured as described previously (7) except that the platelet particulates were resuspended in the incubation buffer described above. K_i values in competition studies were calculated (17) after incubating particulates with various concentrations of unlabeled compounds in the presence of 20 nM [^{3}H]-clonidine or 4 nM [^{3}H]DHE. [^{3}H]Clonidine was chromatographed in a thin-layer system containing 1-butanol-acetic acid-water (4:1:5) (7).

All materials were from standard sources except for certain compounds obtained as gifts: clonidine, Boehringer Ingelheim Ltd., Ridgefield, Conn.; phentolamine, CIBA-Geigy Corporation, Summit, N. J.; DHE, Sandoz Pharmaceuticals, East Hanover, N. J.; (−) and (+)-epinephrine and norepinephrine, Sterling-Winthrop Laboratories, New York, N. Y.; prazosin, Pfizer Laboratories, New York, N. Y.; and (−)-propranolol, Ayerst Laboratories, New York, N. Y.

Clonidine is a mixed agonist-antagonist in studies of platelet aggregation. At concentrations up to 1 mM, clonidine by itself fails to stimulate platelet aggregation (refs. 2 and 3, and data not shown). However, it potentiates aggregation induced by nonadrenergic agonists (2, 3). At concentrations as low as 20 nM, clonidine potentiated ADP-induced primary and secondary aggregation of gel-filtered platelets (Fig. 1, *left panel*). This potentiation by clonidine could be prevented by the prior addition of the *alpha*-adrenergic antagonist, phentolamine (10 μM). In contrast to its enhancement of ADP-induced aggregation, clonidine inhibited primary and secondary aggregation induced by epinephrine (Fig. 1, *right panel*).

In radioligand binding studies, [^{3}H]clonidine bound rapidly to platelet membranes, with equilibrium being reached within 10 min at 1 nM [^{3}H]clonidine and within 3 min at 150 nM [^{3}H]clonidine at 25° (data not shown). Once equilibrium was reached, the amount of [^{3}H]clonidine bound remained constant for at least 1 hr. Specific binding was linear with platelet concentration (1–3 mg of protein per milliliter) and was reversed fully by the

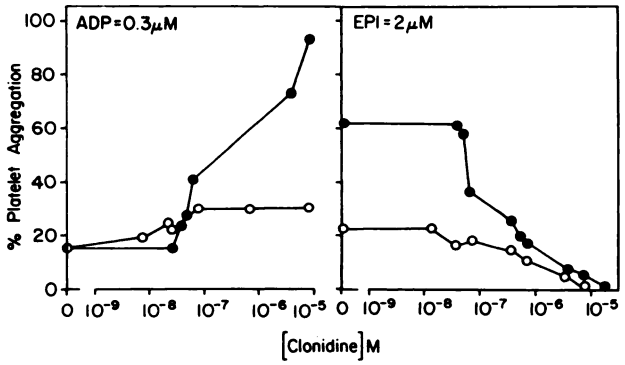

FIG. 1. *The effect of clonidine on ADP- and epinephrine-induced aggregation of gel-filtered platelets*

Various amounts of clonidine were incubated with platelets for 2 min at 37° before the addition of either ADP, 0.3 μM (*left panel*) or epinephrine, 2 μM (*right panel*). Data are presented as the percentage of platelet aggregation 4 min after the addition of ADP or epinephrine (●). To examine primary platelet aggregation, 1 mM acetylsalicylic acid was incubated with platelet-rich plasma for 30 min at 37° prior to gel filtration in order to inhibit platelet cyclooxygenase and secondary platelet aggregation (○). The experiments shown are representative of four so performed.

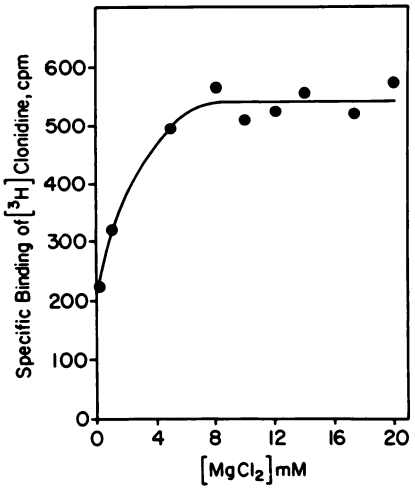

FIG. 2. *Effect of magnesium on the specific binding of [^{3}H]clonidine to platelet particulates*

Platelet particulates in incubation buffer were incubated for 30 min at 25° with 17 nM [^{3}H]clonidine in the presence of various concentrations of $MgCl_2$, and the specific binding of [^{3}H]clonidine was determined as described in the text. This experiment is representative of four so performed. Because specific binding was maximal in the presence of 8 mM $MgCl_2$, this concentration of $MgCl_2$ was used in all further binding studies.

addition of 10 μM phentolamine or by dilution of the sample 100-fold with buffer. Moreover, [^{3}H]clonidine that bound to platelets migrated in a pattern identical with that of native [^{3}H]clonidine on thin-layer chromatography. The inclusion of 8 mM $MgCl_2$ in the binding assays resulted in enhanced specific binding of [^{3}H]clonidine, as is shown in Fig. 2.

Unlabeled *alpha*-adrenergic compounds competed for [^{3}H]clonidine binding sites stereoselectively and with a rank order of potency typical for binding to an *alpha*$_2$-adrenergic receptor (Table 1). Levo-enantiomers of epinephrine and norepinephrine were 36- and 13-fold more potent in competing for [^{3}H]clonidine binding sites than were their respective dextro-enantiomers. The *alpha*$_2$-selective antagonist yohimbine was approximately 600-fold more potent in competing for [^{3}H]clonidine sites than was the *alpha*$_1$-selective antagonist prazosin. The K_i values shown in Table 1 for these adrenergic compounds are similar to values obtained in previous studies with [^{3}H]DHE and [^{3}H]phentolamine (5–8).

TABLE 1

Competition for alpha-adrenergic binding sites on platelet membranes by adrenergic agents

	K_i				
	[^{3}H]Clonidine	[^{3}H]Dihydroergocryptine			[^{3}H]-Phentolamine
	nM				
Agonists					
(±)-Oxymetazoline	9				
(±)-Clonidine	19	180	17	100	20
(−)-Epinephrine	50	130	260	410	140
(−)-Norepinephrine	205	250	850	2,500	440
(+)-Epinephrine	1,800		2,300		2,180
(+)-Norepinephrine	2,700	8,000	17,000		
(−)-Isoproterenol	15,800	>10,000	142,000	25,400	26,400
Antagonists					
(±)-Yohimbine	6	2	2	2	
(±)-Dihydroergocryptine	10	59	11	14	35
(±)-Phentolamine	26	12	14	7	45
(±)-Prazosin	3,700				
(−)-Propranolol	6,300	>1,000	12,000		>10,000
Reference	This study	4	5	6	8

[^{3}H]Clonidine bound at equilibrium to a single class of binding sites of high affinity (Kd = 24.5 ± 2.1 nM) and low capacity (63.7 ± 4.2 fmoles/mg of protein) (mean ± SEM, n = 14) (Fig. 3). A Hill plot of the data had a slope of 1.0, indicating that [^{3}H]clonidine was noncooperatively bound. In five experiments, the addition of 100 μM GTP decreased the binding affinity for [^{3}H]clonidine 2-fold ($p < 0.001$) and decreased the maximal binding capacity by 20% ($p < 0.05$).

The binding capacity for [^{3}H]clonidine was considerably lower than that which we and others have observed for the number of [^{3}H]DHE or [^{3}H]phentolamine binding sites in platelets (4–8). In order to characterize more precisely the relationship between *alpha*-receptor sites defined using the nonselective *alpha*-agent [^{3}H]DHE and those defined using the selective *alpha*$_2$ agent [^{3}H]-clonidine, we performed seven parallel experiments with these two radioligands. The maximal number of binding sites identified by [^{3}H]clonidine was only 18% of the number of sites identified by [^{3}H]DHE (Table 2). In additional studies we compared unlabeled clonidine and DHE in their ability to compete for [^{3}H]DHE binding sites (Fig. 4). The competition curve for clonidine had two components, one of which represented approximately 20% of the sites and had an affinity similar to that obtained in the [^{3}H]clonidine studies. Clonidine competed for the remaining 80% of the [^{3}H]DHE sites with a much lower affinity. In contrast, DHE did not discriminate between two receptor classes.

These data indicate that clonidine is a mixed *alpha*-adrenergic agonist-antagonist for human platelets and that [^{3}H]clonidine can be used to identify *alpha*$_2$-adrenergic receptors on platelet membranes. Whether the observed effects on platelet aggregation occur when clonidine is administered to patients is unknown, although therapeutic plasma levels of clonidine (<10 nM) are less

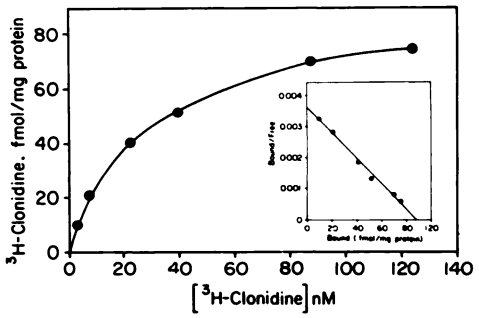

FIG. 3. *Equilibrium binding of [^{3}H]clonidine to platelet particulates*

Particulates were incubated with various concentrations of [^{3}H]-clonidine for 30 min at 25°, and specific binding was assayed as described in the text. The *inset* is a Scatchard analysis of the binding data. This experiment is representative of 14 so performed, and the results are summarized in Table 2. In this single experiment, the K_d of [^{3}H]clonidine was 25 nM and the maximal binding of [^{3}H]clonidine was 88 fmoles/mg of protein.

TABLE 2

Quantitation of platelet alpha-adrenergic receptors using [^{3}H]-clonidine and [^{3}H]dihydroergocryptine

Equilibrium binding of [^{3}H]clonidine was measured after incubation for 30 min at 25° as detailed in the text. Equilibrium binding of [^{3}H]-DHE was measured as previously described (7), except that platelet particulates were suspended in the same incubation buffer as that used in the [^{3}H]clonidine binding studies. Paired [^{3}H]clonidine and [^{3}H]-DHE binding studies were performed on the same day using identical platelet particulate preparations. The data shown are means ± standard error of the mean of seven experiments.

Ligand	Dissociation constant	No. of binding sites
	nM	*fmoles/mg protein*
[^{3}H]Clonidine	28.8 ± 2.3	59.4 ± 5.6
[^{3}H]DHE	6.6 ± 1.6	337.0 ± 38.0

than those shown here to affect platelet aggregation (18).

The binding sites identified with [^{3}H]clonidine in platelet membranes are sites with properties that one would expect for interaction of this radioligand with *alpha*$_2$-adrenergic receptors: Binding of [^{3}H]clonidine is rapid, reversible, saturable, and is competed for more potently by *alpha*$_2$-selective adrenergic compounds than by *alpha*$_1$-selective compounds. Because the percentage of [^{3}H]DHE binding sites for which low concentrations of unlabeled clonidine compete (Fig. 4) is comparable to the 18% of [^{3}H]DHE sites detected by [^{3}H]-clonidine (Table 2), it would appear that these two qualitatively different experiments (using unlabeled and radiolabeled clonidine respectively) are examining the same population of *alpha*$_2$-adrenergic receptors on platelets. By contrast, the binding of [^{3}H]clonidine appears to give little, if any, information about the much lower affinity sites observed when concentrations greater than 100 nM clonidine compete for [^{3}H]DHE sites (Fig. 4).

It should be emphasized that the particulate membrane preparation used in the present studies contains not only platelet surface membranes, but internal membranes as well, such as dense-tubular membranes and granule membranes. Therefore, it is likely that the receptor density observed here for [^{3}H]clonidine and [^{3}H]-DHE are underestimates of the number of sites per milligram of protein in the surface membrane. Although it is difficult to isolate pure platelet surface membranes, in preliminary studies we have examined *alpha*-adrenergic receptors in a platelet membrane preparation free of contaminating granule membranes (16). The receptor density determined with [^{3}H]clonidine and [^{3}H]DHE were 3-fold greater than those observed with the particulate preparation used in the present study. However, binding affinities for these radioligands and the relative proportion of [^{3}H]clonidine sites to [^{3}H]DHE sites were similar to those in the present study.[3]

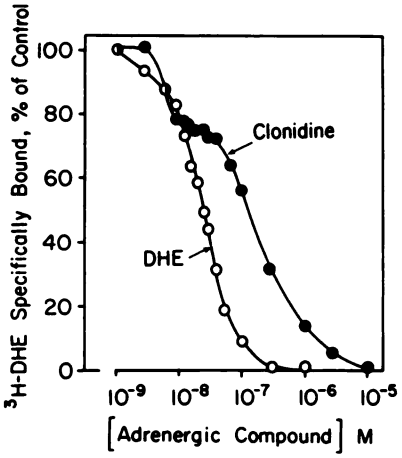

FIG. 4. *Competition for [^{3}H]DHE binding sites in platelet particulates by unlabeled clonidine or DHE*

Platelet particulates were incubated with 4 nM [^{3}H]DHE, and varying concentrations of either unlabeled clonidine (●) or unlabeled DHE (○) as described in the text. Data are plotted as the percentage of specifically bound [^{3}H]DHE. This experiment is representative of five so performed.

What do our results tell us about the nature of platelet *alpha*-adrenergic receptors as recognized by [^{3}H]DHE and [^{3}H]clonidine? If, as has been hypothesized, all platelet *alpha*-adrenergic receptors are of the *alpha*$_2$ subclass and exist in interconvertible, high and low affinity states, then a labeled *alpha*$_2$-agonist might preferentially recognize the high affinity sites (13, 19). This is, in fact, what we observe for [^{3}H]clonidine. However, this interpretation of our results ignores the pharmacological evidence that clonidine is not a pure agonist, but rather a mixed agonist-antagonist with respect to platelets (Fig. 1). Thus, one must consider whether the high affinity sites detected with [^{3}H]clonidine are a subset of *alpha*$_2$-adrenergic receptors that mediate either agonist or antagonist (or both) responses in platelets.

We find that GTP decreases the affinity and maximal binding capacity of platelet *alpha*$_2$-adrenergic receptors for [^{3}H]clonidine, whereas Mg^{2+} increases the specific binding of [^{3}H]clonidine. Similar results with [^{3}H]clonidine were observed with *alpha*-adrenergic receptors in brain membranes (20). Recent studies of several neurotransmitter and hormone receptors in a variety of tissues have demonstrated that GTP and other guanyl nucleotides decrease the affinity of agonists, but not the affinity of antagonists, for receptors (21). Tsai and Lefkowitz (22) previously reported a decrease in the affinity of agonists for [^{3}H]DHE sites in platelet membranes in the presence of guanyl nucleotides. Conversely, Mg^{2+} increases the affinity of agonists but not antagonists in several *alpha*-adrenergic systems, including platelet membranes (23). Therefore, the effects of GTP and Mg^{2+} on the binding of [^{3}H]clonidine to platelet membranes observed in the present study suggest that at least a portion of the sites detected by this radioligand are *alpha*$_2$-adrenergic receptors linked to agonist responses. Such high-affinity *alpha*$_2$-adrenergic receptors may be linked to any or all of the platelet's functional responses to epinephrine: aggregation, secretion, and inhibition of adenylate cyclase.

Although [^{3}H]clonidine recognizes platelet *alpha*$_2$-adrenergic receptors, our data do not clarify the identity of the 82% of [^{3}H]DHE sites on platelet membranes that apparently are not detected by [^{3}H]clonidine (Table 2). Current evidence suggests that these represent "low affinity" *alpha*$_2$-adrenergic receptors (13, 19). However, these remaining [^{3}H]DHE sites might also include *al-*

[3] S. J. Shattil, unpublished observations.

pha$_1$-adrenergic receptors, serotonergic, or dopaminergic receptors. In addition to being a nonselective radioligand for *alpha*-adrenergic receptors, [^{3}H]DHE binds to serotonin and dopamine receptors in other tissues (24, 25), and there is suggestive evidence for the presence of receptors for both serotonin and dopamine on platelets (26, 27). Evidence against these remaining [^{3}H]DHE sites being other than "low affinity" *alpha*$_2$-adrenergic receptors is the fact that *alpha*$_1$ selective agents, dopamine, and serotonin compete relatively poorly for [^{3}H]DHE sites in platelet membranes (5, 6, 13). However, before excluding the possibility that [^{3}H]DHE binds to receptors other than *alpha*$_2$-adrenergic receptors on platelets, further direct binding studies with selective radioligands for *alpha*$_1$-adrenergic, dopamine, and serotonin receptors must be performed.

We and others have used [^{3}H]DHE to characterize platelet *alpha*-adrenergic receptors in a variety of settings characterized by altered platelet responsiveness to epinephrine. These include essential thrombocythemia (28), modification of platelet membrane cholesterol content (7), carbenicillin-induced platelet dysfunction (15), and agonist-induced refractoriness (29). The present study indicates that [^{3}H]clonidine may give direct information about platelet *alpha*$_2$-adrenergic receptors that are linked to agonist-induced platelet function. Therefore, it may be advantageous in further studies of platelets with altered *alpha*-adrenergic responsiveness to characterize *alpha*-adrenergic receptors by using [^{3}H]-clonidine in addition to nonselective, antagonist radioligands.

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