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Characterization of the Human Platelet α-Adrenergic Receptor: *CORRELATION OF [^{3}H] DIHYDROERGOCRYPTINE BINDING WITH AGGREGATION AND ADENYLATE CYCLASE INHIBITION*

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Human platelets aggregate and undergo a release reaction when incubated with catecholamines. Indirect evidence indicates that these events are mediated through α-adrenergic receptors. We used [^{3}H]dihydroergocryptine, an α-adrenergic antagonist, to identify binding sites on platelets that have the characteristics of α-adrenergic receptors. Catecholamines compete for the binding sites in a stereo-specific manner with the potency series of (–) epinephrine > (–) norepinephrine > (±) phenylephrine > (–) isoproterenol. The dissociation constant (K_d) of (–) epinephrine is 0.34 μM. Binding is saturable using a platelet particulate fraction but not with intact platelets. There are 0.130 pmol binding sites per milligram protein in fresh platelet membranes. This number represents approximately 100 binding sites per platelet. The K_d for [^{3}H]-dihydroergocryptine was 0.003–0.01 μM. The α-adrenergic antagonist phentolamine (K_d = 0.0069 μM) was much more potent than the β-adrenergic antagonist (±) propranolol (K_d = 27 μM) in competing for the binding sites. The binding data were correlated with catecholamine-induced platelet aggregation and inhibition of basal and prostaglandin E_1-stimulated adenylate cyclase. (–) Epinephrine was more potent than (–) norepinephrine in producing aggregation whereas (–) isoproterenol was ineffective. The threshold dose for inducing aggregation by (–) epinephrine was 0.46 μM. Phentolamine and dihydroergocyrptine blocked this response, whereas (±) propranolol had no effect. (–) Epinephrine and (–) norepinephrine inhibited basal and prostaglandin E_1-stimulated adenylate cyclase in a […]

Characterization of the Human Platelet α-Adrenergic Receptor

CORRELATION OF [^{3}H]DIHYDROERGOCRYPTINE BINDING WITH AGGREGATION AND ADENYLATE CYCLASE INHIBITION

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ABSTRACT Human platelets aggregate and undergo a release reaction when incubated with catecholamines. Indirect evidence indicates that these events are mediated through α-adrenergic receptors. We used [^{3}H]dihydroergocryptine, an α-adrenergic antagonist, to identify binding sites on platelets that have the characteristics of α-adrenergic receptors. Catecholamines compete for the binding sites in a stereo-specific manner with the potency series of (−) epinephrine > (−) norepinephrine > (±) phenylephrine > (−) isoproterenol. The dissociation constant (K_d) of (−) epinephrine is 0.34 μM. Binding is saturable using a platelet particulate fraction but not with intact platelets. There are 0.130 pmol binding sites per milligram protein in fresh platelet membranes. This number represents approximately 100 binding sites per platelet. The K_d for [^{3}H]-dihydroergocryptine was 0.003–0.01 μM. The α-adrenergic antagonist phentolamine (K_d = 0.0069 μM) was much more potent than the β-adrenergic antagonist (±) propranolol (K_d = 27 μM) in competing for the binding sites. The binding data were correlated with catecholamine-induced platelet aggregation and inhibition of basal and prostaglandin E_1-stimulated adenylate cyclase. (−) Epinephrine was more potent than (−) norepinephrine in producing aggregation whereas (−) isoproterenol was ineffective. The threshold dose for inducing aggregation by (−) epinephrine was 0.46 μM. Phentolamine and dihydroergocyrptine blocked this response, whereas (±) propranolol had no effect. (−) Epinephrine and (−) norepinephrine inhibited basal and prostaglandin E_1-stimulated adenylate cyclase in a dose-related manner. The concentration of (−) epinephrine inhibiting adenylate cyclase 50% was 0.7 μM. This inhibition was also blocked by phentolamine and dihydroergocryptine but not by (±) propranolol. The specificity of binding and the close correlation with α-adrenergic receptor-mediated biochemical and physiological responses suggest that the [^{3}H]dihydroergocryptine binding site represents, or is closely related to, the human platelet α-adrenergic receptor. The ability to assay this receptor directly and to correlate these data with independently measured sequelae of receptor activation should facilitate increased understanding of the physiology and pathophysiology of the human platelet α-adrenergic receptor.

INTRODUCTION

Human platelets will aggregate and undergo a release reaction when incubated with catecholamines. Catecholamine receptors have been classified as α-adrenergic, β-adrenergic, or dopaminergic according to the potency of various catecholamines in inducing a response. Thus, β-adrenergic receptors have the potency series (−) isoproterenol > (−) epinephrine ≥ (−) norepinephrine, whereas α-adrenergic receptors respond to catecholamines with the order of potency (−) epinephrine > (−) norepinephrine > (−) isoproterenol (1). Indirect evidence suggests that catecholamine-induced aggregation of human platelets is mediated through an α-adrenergic receptor: (*a*) (−) Epinephrine is most potent whereas (−) isoproterenol is virtually ineffective in inducing aggregation (2); (*b*) aggregation by epinephrine or norepinephrine is blocked by the α-adrenergic an-

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tagonists phentolamine or dihydroergotamine (2, 3); (*c*) inhibition of adenylate cyclase activity in platelet lysates by epinephrine is blocked by α-adrenergic antagonists (4); (*d*) the ability of epinephrine to prevent the rise in platelet cyclic AMP induced by prostaglandin E_1, an inhibitor of aggregation, is also blocked by α-adrenergic antagonists (5, 6).

Recent technical advances have made possible the direct study of α-adrenergic receptors using radioactive adrenergic antagonists which bind to the receptor site (7–9). In most previous studies, correlation of binding data with physiologic data has been achieved indirectly by comparing affinity constants for binding to membranes with published affinity constants for inhibition or stimulation of contraction in rabbit uterus (7) or rat vas deferens (9) by the same compound. Recently, a direct correlation between the potency of (−) epinephrine in displacing [^{3}H]dihydroergocryptine (DHEC)[1] from dissociated rat parotid cells and in stimulating K^+ release from these cells has been noted (10). We have characterized the human platelet α-adrenergic receptor by direct radioactive ligand binding assay using the α-adrenergic antagonist [^{3}H]DHEC as the ligand and have correlated the binding data with physiologic and biochemical manifestations of platelet α-adrenergic receptor activation.

METHODS

Materials. [^{3}H]DHEC (spec act 24.1 Ci/mmol) was obtained from New England Nuclear, Boston, Massachusetts. This product was greater than 98% pure on thin-layer chromatography in two solvent systems (chloroform:ethanol:glacial acetic acid 9:5:1 and chloroform:benzene:ethanol:ammonium hydroxide 4:2:1:0.1). [^{3}H]DHEC was stored in the dark in ethanol at −10°C. [α^{32}P]ATP (10–30 Ci/mmol) and [^{3}H]cAMP (3′5′-cyclic adenosine monophosphate; 25 Ci/mmol) were also obtained from New England Nuclear.

Other compounds used included (−) epinephrine bitartrate, (−) norepinephrine bitartrate, (−) isoproterenol bitartrate, (±) phenylephrine HCl, dopamine HCl, serotonin creatinine sulfate, normetanephrine, catechol, vanillyl-mandelic acid, adenosine triphosphate (ATP), cAMP (Sigma Chemical Co., St. Louis, Mo.); (+) epinephrine bitartrate, (+) norepinephrine bitartrate, (+) isoproterenol bitartrate, and (±) ethyl-norepinephrine (Sterling-Winthrop, New York); phentolamine HCl (CIBA Pharmaceutical Company, Summit, N. J.); dihydroergocryptine, dihydroergocornine, and dihydroergocristine (Sandoz Pharmaceuticals, East Hanover, N. J.); phenoxybenzamine and chlorpromazine (Smith, Kline & French Laboratories, Philadelphia, Pa.); azapatine (Hoffmann-LaRoche Inc., Nutley, N. J.); (±) propranolol (Ayerst Laboratories, New York); clonidine HCl (Boehringer Ingelheim Ltd., Elmsford, N. Y.); mephentermine (Wyeth Laboratories, Philadelphia, Pa.); methoxamine (Burroughs Wellcome Co., Research Triangle Park, N. C.); imidazole (Eastman Kodak Co., Rochester, N. Y.); L-tryptophan (Calbiochem, San Diego, Calif.); and yohimbine (Aldrich Chemical Co., Inc., Milwaukee, Wis.). Prostaglandin E_1 (PGE_1) was from The Upjohn Company (Kalamazoo, Mich.).

Platelet isolation and preparation of the particulate fraction. 250 ml of blood was obtained from each normal volunteer studied. 200 ml of blood was mixed with sufficient acid citrate dextrose anticoagulant (ACD; National Institutes of Health Formula A) to lower the pH to 6.5. The remaining 50 ml of blood was mixed with 5 ml 3.8% sodium citrate to achieve a final concentration of 15 mM citrate. Platelet-rich plasma (PRP) was prepared by centrifugation at 160 *g* for 10 min at 25°C in a Sorvall RC-3B centrifuge (Ivan Sorvall Inc., Norwalk, Conn.). The PRP collected in sodium citrate was aspirated and kept at room temperature for up to 1 h for aggregation studies outlined below. The remaining platelets collected in ACD anticoagulant were sedimented at 1,600 *g* in the Sorvall RC-3B centrifuge at 25°C and washed twice with a buffer containing 138 mM NaCl, 5 mM KCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 10 mM EDTA, pH 7.2 (phosphate-saline). Half of the platelet pellet was resuspended in a buffer containing 138 mM NaCl, 5 mM $MgCl_2$, 1 mM ethylene glyco-bis (β-aminoethyl ether) *N,N′*-tetraacetic acid (EGTA), 25 mM Tris HCl pH 7.55 (Tris-saline) at a protein concentration of 3–5 mg/ml.

The remaining half of the washed platelet pellet was subjected to two cycles of freezing on acetone-dry ice and thawing at room temperature followed by sonication for 10 s in a Blackstone BP-4 sonicator (Blackstone Ultrasonics, Inc., Jamestown, N. Y.). The particulate fraction was then collected by centrifugation at 230,000 *g* for 30 min in a Beckman L5-65B ultracentrifuge equipped with an SW 50.1 rotor (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.). The supernate was discarded and the particulate fraction resuspended in the Tris-saline buffer at a concentration of 3–5 mg/ml. The washed platelets and platelet particulate fraction derived from these same platelets were kept on melting ice for up to 2 h before measurement of [^{3}H]DHEC binding. Adenylate cyclase activity was measured on these same membranes immediately after the freeze-thawing step.

In some experiments, platelet concentrates were obtained within 72–96 h of collection from the Massachusetts Division of the American Red Cross. 20–40 concentrates were pooled and sufficient ACD added to lower the pH to 6.5. The platelets were then processed as described above, except that the platelet particulate fraction was sedimented in a Beckman type 40 rotor. These particulate fractions were stored at −80°C for up to 4 wk before use.

Binding assay. Immediately before assay, the washed platelets or the two different platelet particulate fractions were gently suspended with a vortex mixer. 100-μl aliquots of each preparation and about 40,000 cpm [^{3}H]DHEC were incubated in the dark with and without agonists or antagonists for 17 min at 25°C in a total volume of 2 ml Tris-saline buffer unless otherwise specified.

The final concentration of [^{3}H]DHEC was approximately 0.8 nM. At the end of the incubation, 1.8 ml was added to 3 ml Tris-saline buffer at room temperature and rapidly filtered through a Whatman GF/C glass filter (Whatman, Inc., Clifton, N. J.) under reduced pressure. The assay tube and filter were then washed with four 5-ml portions of Tris-saline buffer. The filter was then dried and counted in 10 ml of Packard Instagel in a Packard liquid scintillation counter (Packard Instrument Co. Inc., Downers Grove, Ill.) with an efficiency of 45%. All drug solutions were freshly prepared in distilled water immediately before use. The catecholamine and serotonin solutions also contained 0.1% ascorbic acid. Ergot alkaloids were dissolved in 50% ethanol before dilution in distilled H_2O. The

[1] *Abbreviations used in this paper*: cAMP, 3′,5′-cyclic adenosine monophosphate; DHEC, dihydroergocryptine; EGTA, ethylene glycol-bis (β-amino-ethyl ether) *N,N′*-tetra-acetic acid, IC_{50}, half-maximal inhibition; K_d, dissociation constant; PGE_1, prostaglandin E_1; PRP, platelet-rich plasma.

highest concentration of ethanol (0.5%) did not affect binding. The [^{3}H]DHEC in ethanol was added to assay buffer immediately before use.

"Specific binding" was defined as the difference between radioactivity bound in the presence and absence of 100 μM phentolamine and is referred to in all figures and tables. Specific binding amounted to 65–80% of total bound counts. Total bound counts were approximately 3–4% of added radioactivity. The filter blank was 0.5% of added radioactivity. Separate experiments demonstrated that the agonists and antagonists used did not affect the radioactivity bound to the filters.

Adenylate cyclase assay. Enzyme activity was measured by the method of Salomon et al. (11) as previously modified (12) using α-[^{32}P]ATP as substrate and directly measuring the α[^{32}P]cAMP product. Assay mixtures contained 1.5×10^6 dpm α[^{32}P]ATP; 1.0 mM ATP; 25 mM Tris-HCl, pH 7.4; 5 mM $MgCl_2$; 2 mM cAMP; 0.1% albumin; 10 mM theophylline; 1 mM EGTA; and an ATP-regenerating system consisting of 20 mM creatine phosphate and 1 mg/ml creatine kinase. The addition of EGTA increased basal and prostaglandin-sensitive enzyme activity by 20%. Reactions were initiated by the addition of 100 μl of platelet suspension containing 50–80 μg protein and incubated for 10 min at 37°C. Enzyme activity was linear for at least 20 min at protein concentrations up to 80 μg. Data were expressed as nanomoles cAMP per milligrams protein per 10 min.

Platelet aggregation. Platelet aggregation was carried out using the standard nephelometric technique (13). Platelet aggregation was initiated by adding various catecholamines in 0.1 ml buffer made up of 15 mM Tris-HCl and 138 mM NaCl, pH 7.6, to 0.4 ml stirred PRP using a chrono-log aggregometer (Chrono-Log Corp., Havertown, Pa.). The aggregometer was adjusted so that PRP sample allowed 10% light transmission and platelet-poor plasma from the same donor permitted 90% light transmission. The PRP was stirred for 2 min at 37°C before addition of catecholamine and, in some cases, the platelets were incubated for an additional 5–20 min at room temperature with α- or β-adrenergic antagonists before the addition of the catecholamines. The aggregation tracings were analyzed for the presence of primary and secondary waves of aggregation. The threshold dose of a given aggregating agent is defined as the lowest dose that produces a discernible secondary wave of aggregation. A change in light transmission of at least 10% after the addition of a catecholamine was arbitrarily defined as a primary wave.

Platelet counts were performed on PRP using an electronic particle counter (Coulter model F, Coulter Electronics Inc., Hialeah, Fla.). Protein determinations were carried out by the technique of Lowry et al. (14) using bovine serum albumin as a standard. The experimental protocol has been reviewed and approved by the Human Subjects Committee of the Peter Bent Brigham Hospital.

Calculations. The interaction of [^{3}H]DHEC with platelets and platelet membrane particles was directly analyzed by the method of Scatchard (15). The K_d for the nonradioactive agonists and antagonists was calculated from the concentration required to inhibit [^{3}H]DHEC binding by 50% (IC_{50}) by the method of Cheng and Prusoff (16) according to the relationship: K_d [1 + [ligand]/K_d ligand] = IC_{50}. The K_d of the ligand ([^{3}H]DHEC) is determined independently. This assumes competitive inhibition of a first order ligand substrate interaction. Each K_d value was determined from the average of two or three experiments in which triplicate dose-response curves were generated.

The K_d for DHEC was also calculated from kinetic data (7). The binding reaction was considered to be pseudo-first order because it is reversible and because ligand concentration (1 nM) was much greater than receptor concentration (0.05 nM). The second order rate constant, k_1, was calculated from the relationship $k_1 = (K_{ob} - K_2)/[\text{DHEC}]$. The observed forward rate constant, k_{ob}, is determined from $k_{ob} \cdot t = (\ln B_{max}/[B_{max} - B])$, where B_{max} equals total specific binding at equilibrium and B equals binding at time t. The first order rate constant, k_2, is calculated from the relationship $k_2 \cdot t = \ln (B/B_{max})$. Then $K_d = k_2/k_1$.

RESULTS

Saturability and affinity of [^{3}H]DHEC binding sites. In these experiments, increasing concentrations of [^{3}H]DHEC were incubated with intact platelets, the particulate fraction derived from these platelets, or a particulate fraction prepared from platelets 72–96 h after collection. Fig. 1 depicts the binding of [^{3}H]DHEC to intact, washed platelets. Phentolamine-displaceable binding was linear and nonsaturable over the range 1–40 nM [^{3}H]DHEC.

The pattern of [^{3}H]DHEC binding by the particulate fraction derived from these same platelets is shown in Fig. 2. In contrast with the whole platelets, saturation occurred with 0.13 pmol ligand bound/mg protein. At a ligand concentration of approximately 4 nM, half the binding sites were occupied. This value gives an estimate of the dissociation constant (K_d) of [^{3}H]DHEC for the binding sites. Analysis of the data by Scatchard plot (Fig. 2, *inset*) demonstrated a single class of binding sites with a K_d of 10.3 nM. This analysis gave an estimate of 0.173 pmol ligand bound/mg protein which represents approximately 100 binding sites per platelet.

A similar study performed with the particulate frac-

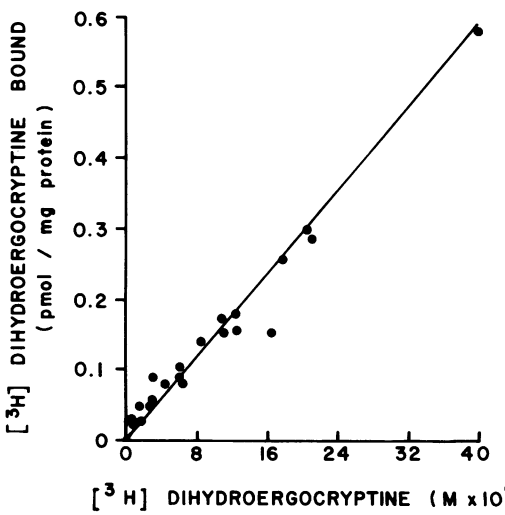

FIGURE 1 The binding of [^{3}H]DHEC to intact platelets is depicted. Mean values of triplicate determinations from five separate experiments are shown. In these experiments increasing quantities of [^{3}H]DHEC were added to a fixed number of platelets (approximately 5×10^8). The samples were incubated for 20 min at 25°C. Specific binding is plotted and defined as the radioactivity displaceable by 100 μM phentolamine.

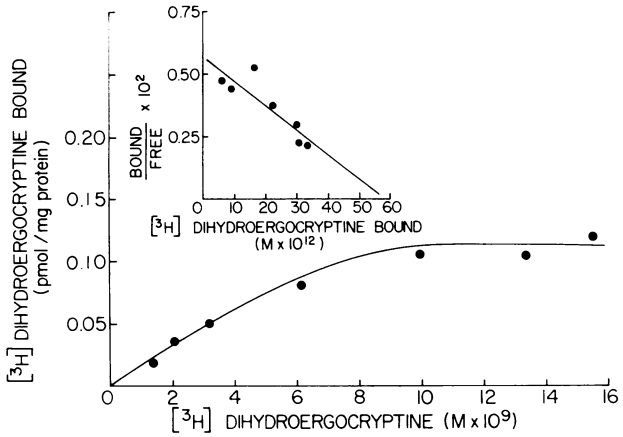

FIGURE 2 The binding of [³H]DHEC to a particulate fraction derived from fresh platelets is depicted. Mean values from three separate experiments with each point run in triplicate are shown. Assay conditions are identical to those described in Fig. 1. Protein concentrations averaged 0.35 mg/ml. The *inset* shows the Scatchard analysis of this data. The line represents the least squares fit of the data points and has a slope of -9.7×10^7 M ($r = 0.88$). The reciprocal of the slope, the K_d, equals 10.3 nM. The intercept of the line with the ordinate gives an estimate of 0.173 pmol receptor/mg protein.

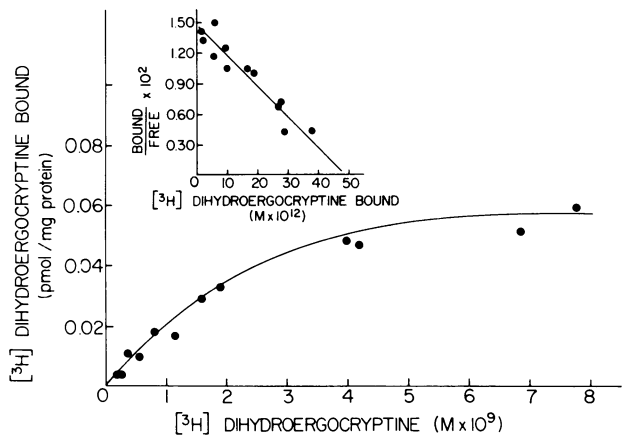

FIGURE 3 The binding of [³H]DHEC to a platelet particulate fraction derived from stored platelets is depicted. Conditions are identical to those described in Figs. 1 and 2. Values from two experiments are shown. Protein concentration was 0.4 mg/ml. The slope of the Scatchard plot is -3.0×10^8 M ($r = 0.97$) and the K_d equals 3.3 nM. There are 0.06 pmol receptor/mg protein.

tion derived from stored platelets is shown in Fig. 3. The binding sites for [³H]DHEC were saturable with half the sites occupied at about 2 nM. The Scatchard plot again shows a single class of binding sites with a K_d of 3.3 nM and 60 fmol ligand bound/mg protein. This value represents 33 binding sites per platelet, suggesting that a loss of binding sites may occur during storage.

Specificity of [³H]DHEC binding. The ability of various catecholamines to compete for binding sites on the intact platelets or their membrane fraction is illustrated in Fig. 4. The potency series for the three (−) stereoisomers tested was (−) epinephrine > (−) norepinephrine ≫ (−) isoproterenol which, as previously noted, is characteristic of an α-adrenergic response. The dose-response curves for intact platelets were shifted to the right, and the maximum [³H]DHEC displacement by catecholamines was decreased compared with the platelet particulate fraction. (−) Epinephrine-displaceable counts in intact platelets were 30–60% of that displaceable by 100 μM phentolamine over the range 3–40 nM [³H]DHEC (data not shown). The K_ds for (−) epinephrine, (−) norepinephrine, and (−) isoproterenol were 0.41 μM, 2.5 μM, and 25.4 μM, respectively, in the particulate fraction. The apparent differences in the affinities of catecholamines for the binding sites in the two preparations probably reflect the discrepancy between the radioactivity displaceable by (−) epinephrine and by phentolamine in the intact platelet. Thus, if specific binding is redefined in the intact platelet as that displaceable by 100 μM (−) epinephrine, then the IC_{50} is approximately 1 μM, which is not strikingly dissimilar from that in the particulate fraction.

Although the total number of [³H]DHEC binding sites was diminished when a particulate fraction was prepared from stored platelets, the affinity for catecholamines was not affected. The K_ds for (−) epinephrine,

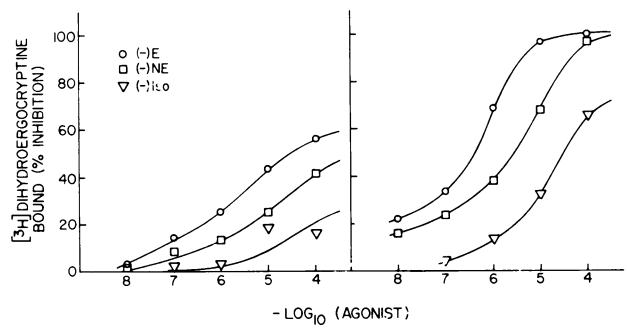

FIGURE 4 The inhibition of [³H]DHEC binding by (−) epinephrine (○), (−) norepinephrine (□), and (−) isoproterenol (▽) is depicted. The left-hand panel depicts the response of intact platelets and the right-hand panel a platelet particulate fraction prepared from the same platelets. When studying the intact platelets, 1 μM pargyline and 0.3 μM imipramine were included in the assay mixture to minimize uptake and metabolism of added catecholamines. At this concentration, neither compound reduced specific binding. In these experiments, either 5×10^8 platelets or 300 μg of particle protein were incubated with ~0.8 nM [³H]DHEC and varying concentrations of the three agonists. The reactions were carried out in Tris-saline buffer for 17 min at 25°C and terminated by filtration onto glass fiber filters. The percent displacement of [³H]DHEC vs. the concentration of each agonist is plotted. Each point from three experiments was determined in triplicate and the actual K_d calculated from the IC_{50} as described in the text.

(−) norepinephrine, and (−) isoproterenol (0.34, 2.5, and 51.0 μM, respectively) were virtually identical with the values obtained with freshly prepared platelet membranes. Competition for the binding sites is stereospecific because the (+) isomers are approximately 10-fold less potent than are the (−) isomers. The K_ds for the (+) isomers of epinephrine, norepinephrine, and isoproterenol were 4.5, 18.0, and 430.0 μM, respectively.

Inasmuch as the binding characteristics of the particulate fraction from these platelets were quite similar to those of the fresh material and were available in much larger quantities, more extensive analysis of [^{3}H]-DHEC binding was carried out exclusively with this material. The inhibition of [^{3}H]DHEC binding by a variety of α-adrenergic antagonists is illustrated in Fig. 5. The indolealkylamine alkaloid yohimbine is most potent (K_d = 0.0015 μM) and the dibenzazepine derivative, azapetine, the least potent inhibitor (K_d = 0.95 μM). The dihydrogenated ergotoxine alkaloids (K_d = 0.014 μM) and the 2-substituted imidazoline phentolamine (K_d = 0.0069 μM) are more potent than any of the catecholamine agonists. For comparison the K_d for (±) propranolol, a well-recognized β-adrenergic antagonist, was 27 μM (Table I).

Table I also summarizes the effects on [^{3}H]DHEC binding of some additional agonists and of several non-catecholamine compounds. Serotonin competes for binding sites at relatively high concentration (K_d = 8.7 μM) whereas catecholamine metabolites like normetanephrine or vanillylmandelic acid were less active. In addition, very high concentrations of imidazole, catechol, and L-tryptophan did not block binding.

Binding kinetics. [^{3}H]DHEC binding to platelet

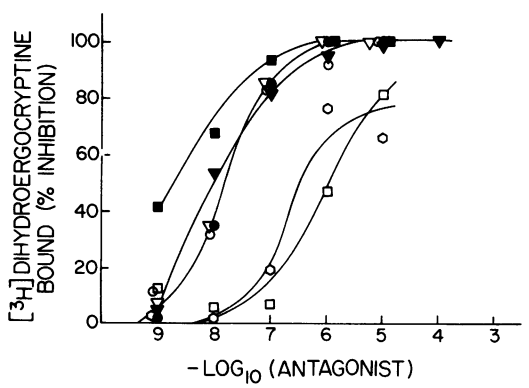

FIGURE 5 The inhibition of [^{3}H]DHEC binding by various antagonists is depicted. Conditions were identical to those in Fig. 4. Each curve represents mean values from two to three experiments with each point run in triplicate. Yohimbine (■), K_d 0.0015 μM; phentolamine (▼), K_d 0.0069 μM; phenoxybenzamine (0), K_d 0.26 μM; azapetine (□), K_d 0.95 μM; dihydroergocryptine (●), K_d 0.014 μM; dihydroergocristine (○), K_d 0.014 μM; dihydroergocornine (▽), K_d 0.014 μM.

TABLE I
Effect of Additional Agonists and Miscellaneous Compounds on [^{3}H]DHEC Binding

	K_d(μM)
Antagonists	
Chlorpromazine	0.30
(±) Propranolol	27.0
Agonists	
Clonidine	0.097
(±) Phenylephrine	3.8
Dopamine	4.3
Mephentermine	7.0
(±) Ethyl-norepinephrine	9.5
Methoxamine	27.0
Miscellaneous	
Serotonin	8.7
Normetanephrine	33.0
Vanillylmandelic acid	*
Imidazole	*
Catecohol	NI‡
L-tryptophan	NI

* <50% inhibition at 0.1 mM.
‡ NI, no inhibition (<1.0% at 10^{-4} M).

fragments reached equilibrium at approximately 20 min with $t_{1/2}$ of 8.5 min, and binding was stable for as long as 60 min (Fig. 6). The observed forward rate constant was 0.120 min^{-1} giving a second order rate constant (k_1) of 1.03 × 10^8 min^{-1} M^{-1}. At equilibrium, addition of the α-adrenergic antagonist phentolamine (0.5 mM) resulted in slow displacement of [^{3}H]DHEC for up to 40 min (Fig. 7). This slow reverse rate is compatible with the observations that α-adrenergic blockade with ergot alkaloids, although competitive, is quite persistent relative to substituted imidazoline antagonists such as phentolamine (17). Because of evidence that the [^{3}H]DHEC was not chemically stable for longer than 60 min under the assay conditions, it was not meaningful to extend the incubation. The first order rate constant (k_2) for the reversal of [^{3}H]DHEC binding was 0.017 min^{-1}. The K_d calculated from the reverse rate constant was 0.2 nM which is an order of magnitude less than the K_d estimated from equilibrium data (2–10 nM).

Platelet aggregation. Based on the study of 10 normal subjects, threshold doses that caused visible aggregation were 0.46 μM for (−) epinephrine and 1.95 μM for (−) norepinephrine, a fourfold difference in concentration. In each case, as the concentration of the two catecholamines was increased, the magnitude of the primary aggregation wave increased and the time to the onset of secondary aggregation decreased. Maximal aggregation, as measured by the final change in light transmission, was achieved with 0.93 μM epinephrine and 3.9 μM norepinephrine, also a fourfold difference in dose. (−) Isoproterenol and methoxamine did not in-

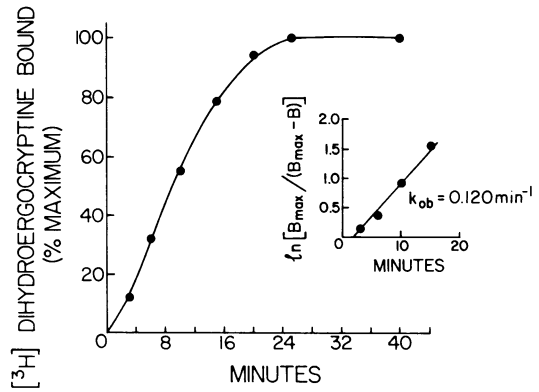

FIGURE 6 Rate of "specific" binding of [^{3}H]DHEC to platelet particles at 25°C is depicted. The protein concentration was 0.4 mg/ml. The reaction was initiated by adding membranes to two tubes containing 1.0 nm [^{3}H]DHEC. One tube contained 100 μM phentolamine. Each tube was sampled at the times indicated. Each point represents the mean of two experiments with duplicate determinations at each point. The *inset* shows the plot for calculating the observed forward rate constant. B_{max} refers to maximum binding at equilibrium.

duce aggregation at any concentration studied up to a maximum of 0.35 mM.

(−) Epinephrine- and (−) norepinephrine-induced aggregation were blocked by preincubation with phentolamine but not with (±) propranolol. As shown in Fig. 8, increasing concentrations of DHEC could also inhibit (−) epinephrine-induced aggregation. Aggregation induced by collagen or ADP was not inhibited by 1 μM DHEC.

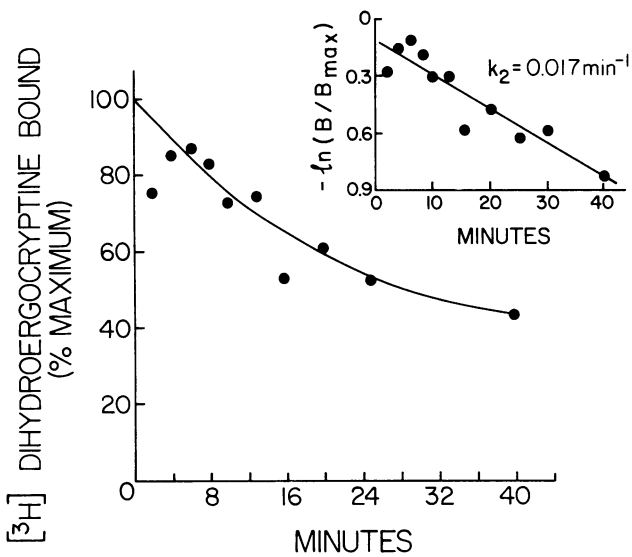

FIGURE 7 Rate of dissociation of [^{3}H]DHEC from platelet particles at 25°C. Platelet particles were incubated with 1.0 nM [^{3}H]DHEC for 20 min, and at time zero 500 μM phentolamine was added, and aliquots were taken at the times indicated. Each point represents the mean of points determined in duplicate from three separate experiments. The *inset* shows the plot for determining the first order rate constant. The line represents the least squares fit (r = 0.9).

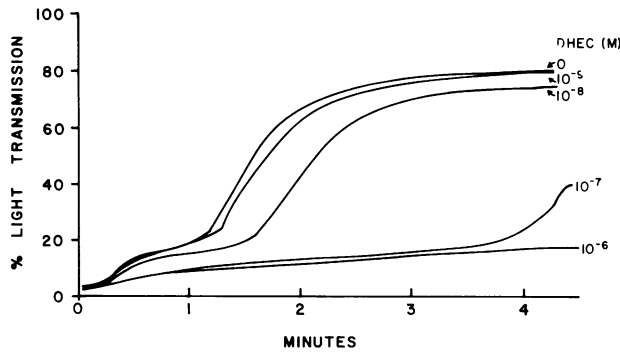

FIGURE 8 The inhibition of (−) epinephrine-induced platelet aggregation by increasing concentrations of DHEC is depicted. PRP was incubated with 0.1–1 μM DHEC for 20 min at room temperature in the dark. 0.4-ml portions were then transferred to the aggregometer, stirred at 1,000 rpm at 37°C for 2 min and the reaction initiated by addition of 5 μM (−) epinephrine. The change on the percentage of light transmission was continuously recorded for 5 min.

Adenylate cyclase. The effect of (−) epinephrine on basal adenylate cyclase activity is depicted in Fig. 9. In these experiments, the fresh platelet lysates from the same platelets on which binding studies were performed were incubated with varying concentrations of the catecholamine, and adenylate cyclase activity was determined. In five experiments, (−) epinephrine lowered basal adenylate cyclase by as much as 30% ($P > 0.01$). Half-maximal inhibition, the IC_{50}, occurred at 0.7 μM (−) epinephrine, whereas maximal inhibition required 10 μM (−) epinephrine. To increase total adenylate cyclase activity, the membrane fractions

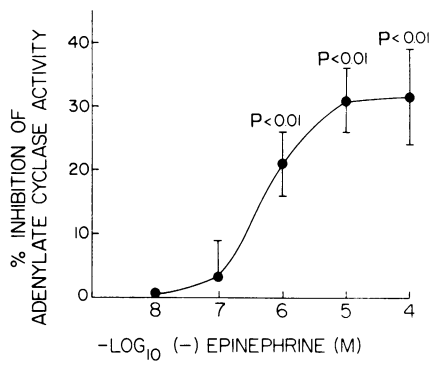

FIGURE 9 Inhibition of basal adenylate cyclase activity by (−) epinephrine is depicted. In these experiments, 60–80 μg of platelet protein was incubated for 10 min at 37°C in the presence of increasing concentrations of (−) epinephrine. Adenylate cyclase activity was determined as described in Methods. Mean values (±SEM) from five experiments with each point determined in duplicate are shown. The P values refer to comparison using Student's t test between basal adenylate cyclase activity and experimental points with (−) epinephrine added.

were incubated with 5 μM PGE_1. This concentration of PGE_1 stimulated enzyme activity an average of 23-fold over base line. The effects of several adrenergic agonists and antagonists on this PGE_1-stimulated adenylate cyclase activity are outlined in Table II. PGE_1 increased basal activity from 0.28 to 6.17 nmol cAMP/mg protein per 10 min of incubation. The addition of 10 μM (−) epinephrine decreased PGE_1-stimulated adenylate cyclase by 70% to 2.85 nmol/mg protein per 10 min. A similar inhibitory effect was noted after incubation with 10 μM (−) norepinephrine, whereas 10 μM (−) isoproterenol decreased adenylate cyclase activity by only 10%. Phentolamine completely blocked the inhibitory effects of (−) epinephrine and (−) norepinephrine. DHEC also blocked inhibition by (−) epinephrine in a dose-dependent manner.

Table III summarizes and compares the effects of catecholamines on [^{3}H]DHEC binding, adenylate cyclase inhibition, and aggregation threshold. The concentrations of (−) epinephrine and (−) norepinephrine required to initiate aggregation and to achieve half-maximal inhibition of adenylate cyclase are closely comparable to the K_d for inhibiting [^{3}H]DHEC binding for each compound. (−) Isoproterenol is much less potent than the other two catecholamines in inhibiting binding and adenylate cyclase and in promoting aggregation. Thus, each independently measured manifestation of catecholamine function exhibited the α-adrenergic potency series of (−) epinephrine > (−) norepinephrine > (−) isoproterenol. Furthermore, the doses required to inhibit binding or adenylate cyclase and to initiate aggregation are quite similar.

TABLE II

Effect of Adrenergic Agonists and Antagonists on PGE_1-Stimulated Adenylate Cyclase

Addition	Adenylate cyclase activity
	nmol cAMP/mg protein/10 min
None	0.28
5 μM PGE_1 plus:	6.17
10 μM (−) Epinephrine	2.85
10 μM (−) Epinephrine, 2 μM phentolamine	6.17
10 μM (−) Norepinephrine	2.92
10 μM (−) Norepinephrine, 2 μM phentolamine	6.03
10 μM (−) Isoproterenol	5.19
5 μM PGE_1, 10 μM (−) epinephrine plus:	
10^{-9} M DHEC*	3.99
10^{-8} M DHEC	4.17
10^{-7} M DHEC	6.00
10^{-6} M DHEC	6.12

* For these experiments, intact platelets were incubated with the indicated concentration of DHEC, then centrifuged. The plasma was discarded and the pellet disrupted by a freeze-thaw cycle. An aliquot was then added to the adenylate cyclase assay buffer containing 5 μM PGE_1 and 10 μM epinephrine to initiate the reaction.

TABLE III

Comparison of [^{3}H]DHEC Binding and Physiologic and Biochemical Responses

Agonist	K_d*	IC_{50} Adenylate cyclase‡	Aggregation threshold dose§
	μM	μM	μM
(−) Epinephrine	0.34	0.7	0.5
(−) Norepinephrine	2.5	4.96	1.93
(−) Isoproterenol	51.0	>100	>300

* K_d was calculated from IC_{50} for binding using freshly prepared membrane as described in the text.

‡ IC_{50}, the concentration of catecholamine inhibiting PGE_1 (5 μM)-stimulated platelet adenylate cyclase 50%.

§ Threshold dose, lowest concentration of catecholamine producing a biphasic aggregation pattern when added to stirred PRP.

DISCUSSION

Our data suggest that [^{3}H]DHEC, an α-adrenergic antagonist, identifies binding sites in human platelets with characteristics of α-adrenergic receptors. First, the catecholamine potency series for displacing [^{3}H]-DHEC is appropriate, and α-adrenergic antagonists are much more potent than is the β-adrenergic antagonist propranolol. The binding site exhibits stereospecificity with respect to the competition of catecholamines for binding, and binding is saturable in disrupted platelet particulate fractions. Furthermore, inhibition of binding and adenylate cyclase and induction of aggregation require the same concentrations of catecholamine. The characteristics of the binding and the close correlation with α-adrenergic receptor-mediated biochemical and physiological responses within the platelet suggest that the [^{3}H]DHEC binding site represents, or is closely related to, the α-adrenergic receptor.

Ideally, it would be desirable to characterize fully the α-adrenergic receptor in intact platelets. This was not practical because of the lack of saturability of [^{3}H]-DHEC binding sites in whole platelets. These findings are not unexpected because similar findings have been reported for intact oxygenated rat parotid cells (10). Uptake and internalization of the triated ligand by intact cells may be the explanation for these observations. In spite of these difficulties, a catecholamine potency series appropriate for the α-adrenergic receptors was obtained even in the intact platelet (Fig. 4). The apparent rightward shift of the dose-response curves may reflect a lack of accessibility of the ligand for displace-

ment by catecholamines under these circumstances rather than a true change in the affinity.

Because [^{3}H]DHEC binding is much slower than the onset of a catecholamine-induced physiologic response, this ligand cannot be used for kinetic studies of agonist-receptor interactions. In addition, the kinetic studies reported here give a K_d which differs from the K_d calculated from equilibrium studies. Three previous studies have reported kinetically derived K_ds that were consistently two- to threefold less than the K_d derived from equilibrium data in rabbit uterus (7), rat parotid cells (10), and rat brain (18). In view of these observations and of the present data, the possibility must be considered that [^{3}H]DHEC binding is more complex than a simple bimolecular reaction with the α-adrenergic receptor.

The potency of the primary catecholamine agonists in displacing binding, presented in Fig. 4, reflects the known potency of these compounds in stimulating platelet aggregation and release (2, 3). The binding data do not explain why α-adrenergic agonists such as phenylephrine and dopamine do not induce aggregation since they are similar to (−) norepinephrine in potency. The data do not support the suggestion that the human platelet α-adrenergic receptor may be unique in not interacting with such agonists (19). Dopamine and phenylephrine potentiate aggregation to other agents through an α-adrenergic mechanism (20). Thus, measurement of catecholamine-induced aggregation and adenylate cyclase inhibition may not be appropriate to assess intrinsic activity in this sytem.

Of the antagonists tested, all except the β-adrenergic antagonist (±) propranolol have the pharmacologic properties of α-adrenergic antagonists (17). The K_ds reported here are in reasonable agreement with previously published binding data (7, 9) except for yohimbine which appears to be a more potent antagonist in the human platelet than in uterine (7) or vas deferens membranes (9). However, yohimbine has also been reported to be more potent than phentolamine in inhibiting platelet aggregation (2). (±) Propranolol is almost four orders of magnitude less potent than is phentolamine. The K_d of 27 μM is identical with that previously reported for inhibition of [^{3}H]DHEC binding in rabbit uterine membranes (7).

The nature of the interactions between the α-adrenergic receptor and adenylate cyclase in various tissues is unclear (1). It has been speculated that there is a reciprocal relationship between α- and β-adrenergic receptors and the catalytic subunit of adenylate cyclase (21). Although the β-adrenergic receptor clearly stimulates adenylate cyclase and increases intracellular cAMP, the general physiologic significance of a decrease in cAMP mediated by α-adrenergic agonists is not clear (1). In fact, α-adrenergic receptor-mediated metabolic events in rat liver have been demonstrated recently which are not accompanied by changes in cAMP (22).

The present data and those of another recent report clearly demonstrate that catecholamines can inhibit basal platelet adenylate cyclase activity (4). It is possible that this inhibition is due to a direct link between the platelet α-adrenergic receptor and the catalytic subunit of adenylate cyclase. It is also tenable that α-adrenergic receptor activation alters membrane conformation in a more general way and hence leads to inhibition of this membrane-bound enzyme.

Although the concentration of catecholamines usually found in the circulation may not be sufficient to cause aggregation, catecholamines may sensitize the platelet to the effects of other aggregating substances (23–25). They may be responsible for the increased aggregation seen with cigarette smoking and in animals and humans subjected to psychologic and physical stress (26–28). Platelets obtained from patients with hyperbetalipoproteinemia aggregate with very low doses of (−) epinephrine (29, 30). This hyperaggregability may be a postreceptor event related to the increased cholesterol and increased microviscosity of the platelet membrane or may be due to alteration in the platelet α-adrenergic receptor. Thus, the platelet α-adrenergic receptor may be important in modulating events in a number of pathologic states. The ability to assay this receptor directly in human platelets using binding techniques and to correlate these data with well-defined biochemical and physiological correlates of receptor activation should facilitate our understanding of the α-adrenergic receptor and its role in human platelet physiology and pathophysiology.

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REFERENCES

1. Moran, N. C. 1975. Adrenergic receptors. *Handb. Physiol.* **6:** 447–472.
2. Barthel, W., and F. Markwardt. 1974. Aggregation of blood platelets by biogenic amines and its inhibition by antiadrenergic and antiserotoninergic agents. *Biochem. Pharmacol.* **24:** 37–46.
3. O'Brien, J. R. 1963. Some effects of adrenaline and antiadrenaline compounds on platelets *in vitro* and *in vivo*. *Nature (Lond.).* **200:** 763–765.
4. Jakobs, K. H., W. Saur, and G. Schultz. 1976. Reduction of adenylate cyclase activity in lysates of human platelets by the alpha-adrenergic component of epinephrine. *J. Cyclic Nucleotide Res.* **2:** 381–392.
5. Salzman, E. W., and L. L. Neri. 1969. Cyclic 3′,5′-adenosine monophosphate in human blood platelets. *Nature (Lond.).* **224:** 609–612.

6. Robison, G. A., A. Arnold, and R. C. Hartmann. 1969. Divergent effects of epinephrine and prostaglandin E_1 on level of cyclic AMP in human blood platelets. *Pharmacol. Res. Commun.* **1**: 325–334.
7. Williams, L. T., D. Mullikin, and R. J. Lefkowitz. 1976. Identification of α-adrenergic receptors in uterine smooth muscle membranes by [^{3}H]dihydroergocryptine binding. *J. Biol. Chem.* **251**: 6915–6923.
8. Greenberg, D. A., D. C. U'Prichard, and S. H. Snyder. 1976. Alpha-noradrenergic receptor binding in mammalian brain: differential labeling of agonist and antagonist states. *Life Sci.* **19**: 69–76.
9. Ruffalo, R. R., J. W. Fowble, D. D. Miller, and P. Patil. 1976. Binding of [^{3}H]dihydroazapetine to alpha-adrenoreceptor-related proteins from rat vas deferns. *Proc. Natl. Acad. Sci. U. S. A.* **73**: 2730–2734.
10. Strittmatter, W. J., J. N. Davis, and R. J. Lefkowitz. 1977. α-Adrenergic receptors in rat parotid cells. I. Correlation of [^{3}H]dihyroergocryptine binding and catecholamine-stimulated potassium efflux. *J. Biol. Chem.* **252**: 5472–5477.
11. Salomon, Y., C. Londos, and M. Rodbell. 1974. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**: 541–548.
12. Cooper, B., and R. I. Gregerman. 1976. Hormone-sensitive fat cell adenylase cyclase in the rat: influences of growth, cell size, and aging. *J. Clin. Invest.* **57**: 161–168.
13. Buchanan, G., V. Martin, P. Levine, K. Scoon, and R. I. Handin. 1977. The effects of "anti-platelet" drugs on bleeding time and platelet aggregation in normal human subjects. *Am. J. Clin. Pathol.* **68**: 355–359.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.
15. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**: 660–672.
16. Cheng, Y. and W. Prusoff. 1973. Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**: 3099–3108.
17. Nickerson, M., and N. K. Hollenberg. 1967. Blockade of α-adrenergic receptors. *In* Physiological Pharmacology. Vol. 4. The Nervous System Part D: Autonomic Nervous System Drugs. W. S. Root and Z. G. Hoffman, editors. Academic Press, Inc., New York. 243–305.
18. Davis, J. N., W. J. Strittmatter, E. Hoyer, and R. J. Lefkowitz. 1977. [^{3}H]dihydroergocryptine binding in rat brain. *Brain Res.* **132**: 327–336.
19. Berry, D. G., and J. Miller. 1974. The effects of sympathomimetic agents on ADP aggregation of rat platelets in vitro. *Eur. J. Pharmacol.* **28**: 164–171.
20. Drummond, A. 1976. Interactions of blood platelets with biogenic amines: uptake, stimulation and receptor binding. *In* Research Monographs in Cell and Tissue Physiology. I. Platelets in Biology and Pathology. J. Gordon, editor. American Elsevier Publishing Co. Inc., New York. 203–239.
21. Robison, G. A., R. W. Butcher, and E. W. Sutherland. 1967. Adenyl cyclase as an adrenergic receptor. *Ann. N. Y. Acad. Sci.* **139**: 703–723.
22. Cherrington, A. D., F. D. Assimacopoulos, S. Harper, J. Borbin, C. Park, and J. Exton. 1976. Studies on the α-adrenergic activation of hepatic glucose output. II. Investigation of the roles of adenosine 3′:5′-monophosphate and adenosine 3′:5′-monophosphate dependent protein kinase in the actions of phenylpherine in isolated hepatocytes. *J. Biol. Chem.* **251**: 5209–5218.
23. Mills, D. C. B., and G. C. K. Roberts. 1967. Effects of adrenaline on human blood platelet. *J. Physiol. (Lond.).* **143**: 443–453.
24. Niewiarowski, S., and D. P. Thomas. 1966. Platelet aggregation by ADP and thrombin. *Nature (Lond.).* **212**: 1544–1547.
25. Thomas, D. P. 1968. The role of catecholamines in the aggregation of platelets by collagen and thrombin. *Exp. Biol. Med.* **3**: 129–134.
26. Levine, P. H. 1973. An acute effect of cigarette smoking on platelet function. *Circulation.* **48**: 619–623.
27. Haft, J. I., and R. Levites. 1974. Exercise-induced stress and platelet aggregation. *Circulation.* **50**(Suppl. III): 288A. (Abstr.)
28. Haft, J. I., and Y. S. Arkel. 1975. Effect of emotional stress on platelet aggregation in humans. *Clin. Res.* **28**: 186A. (Abstr.)
29. Shattil, S. J., R. Anaya-Galindo, J. Bennett, R. W. Colman, and R. A. Cooper. 1975. Platelet hypersensitivity induced by cholesterol. *J. Clin. Invest.* **55**: 636–643.
30. Carvalho, A. C. A., R. W. Colman, and R. S. Lees. 1974. Platelet function in hyperlipoproteinemia. *N. Engl. J. Med.* **290**: 434–438.