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THE BINDING CHARACTERISTICS OF [^{3}H]-DIHYDROERGOCRYPTINE ON INTACT HUMAN PLATELETS

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1 We have characterized the binding of [^{3}H]-dihydroergocryptine to intact human platelets.

2 The values of the association and dissociation rate constants, affinity and capacity of specific [^{3}H]-dihydroergocryptine binding on intact cells closely resemble those previously reported on the human platelet lysate preparation.

3 The affinity of α-adrenoceptor antagonists, determined from inhibition of [^{3}H]-dihydroergocryptine binding, is similar in intact and lysed platelet preparations, but the affinity of agonists is considerably lower in intact cells.

4 The potency of α-adrenoceptor antagonists as inhibitors of noradrenaline-induced platelet aggregation and as inhibitors of [^{3}H]-dihydroergocryptine binding on intact platelets demonstrate a significant correlation ($r = 0.92$, $P < 0.01$).

5 The affinity and capacity of [^{3}H]-dihydroergocryptine binding to platelets from a group of healthy, young, male subjects show a high degree of consistency both between subjects ($K_d = 2.81 \pm 0.27$ nM; $B_{max} = 63 \pm 3$ fmol/10^8 platelet: mean ± s.e.mean, $n = 10$) and between sampling occasions in a single subject ($K_d = 3.28$ nM ± 13%; $B_{max} = 70$ fmol/10^8 platelet ± 16%: mean ± coefficient of variation, $n = 5$).

6 There is no significant difference in the binding capacity of platelets from a group of elderly male subjects (mean age 73) compared to those from young males (mean age 27) or elderly females (mean age 77). The affinity of binding is slightly but significantly ($P < 0.05$) higher in the elderly male group compared to the two other groups.

7 We conclude that [^{3}H]-dihydroergocryptine binds to the α_2-adrenoceptor of intact human platelets which is responsible for noradrenaline-induced platelet aggregation. The high consistency of the binding characteristics of [^{3}H]-dihydroergocryptine indicate that this assay may be useful as a monitor of platelet α-adrenoceptor sensitivity in clinical investigation.

Introduction

The characterization of membrane receptors in intact tissues by radioligand binding techniques has been hampered by the physical inaccessibility of the receptors. In several of those cases investigated, a diffusion element appears to affect significantly the establishment of equilibrium (Ward & Young, 1977; Elliott, Tayler & Young, 1978) and attempts have been made to quantify this 'biophase' barrier (Rang, 1966; Thron & Waud, 1968). The majority of receptor studies have therefore been performed on subcellular tissue preparations, either in the form of a crude homogenate or as a purified receptor fraction. Comparisons between receptor binding characteristics in intact tissue and in homogenates are few but have revealed some significant differences between the two tissue preparations (Ward & Young, 1977; Gilbert, Hanley & Iversen, 1979). Platelets, being unicellular, are particularly useful cells in which to make such a comparison since the diffusional problem is minimized. Furthermore, a highly purified platelet fraction can be prepared from blood, avoiding contamination by other cell types, unlike the situation in tissues such as brain, where the preparation inevitably contains receptors from blood vessels, glia and blood cells, in addition to those from neurones.

We have therefore investigated the binding characteristics of [^{3}H]-dihydroergocryptine on intact human platelets and compared these results with similar studies carried out on human platelet lysates (Newman, Williams, Bishopric & Lefkowitz, 1978; Alexander, Cooper & Handin, 1978). [^{3}H]-dihydroergocryptine is an established radioligand which binds with high affinity to both α_1 and α_2-adrenoceptors in a number of tissues (Williams, Mullikin & Lefkowitz, 1976; Miach, Dausse & Meyer, 1978; Hoffman, Dukes & Lefkowitz, 1981) as well as to dopamine receptors in brain (Titeler, Weinreich & Seeman, 1977).

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We have also evaluated the possible clinical application of the binding assay of [^{3}H]-dihydroergocryptine on intact human platelets. In this respect it is essential that the observed binding characteristics for control subjects are consistent both between individuals and for a single individual when tested on different occasions. Some of these results have already been published in preliminary form (Boullin & Elliott, 1977; Elliott & Grahame-Smith, 1980).

Methods

Tissue preparation

Blood was taken from the antecubital vein with a 19-gauge needle and anticoagulated by addition of nine volumes of blood to either one volume of disodium edetate (EDTA) (1% w/v) in saline for the binding assay or one volume of trisodium citrate (3.8% w/v) for platelet aggregation studies. Platelet-rich plasma (PRP) was prepared by centrifugation of the blood at 180 *g* for 12 min at 20°C. For the binding assay the platelets were separated from the plasma by further centrifugation at 1700 *g* for 7 min at 10°C then resuspended in the incubation medium comprising 0.1% w/v EDTA, 150 mM NaCl, pH 7.5 by repeated vortex mixing. This produced a homogeneous unicellular platelet suspension. Contamination by erythrocytes and leukocytes did not exceed 0.5% of the cell number on examination by low-power microscopy. Platelet cell density was routinely estimated by means of a Coulter Counter Industrial-D model and for the binding assay was adjusted to between 0.6 – 1.0×10^8 cell/ml.

For characterization of the human platelet α-adrenoceptor, blood was obtained from a group of 12 healthy male laboratory personnel (mean age 27, range 19–40). To investigate the effects of ageing, blood was taken with informed consent from elderly male (mean age 73, range 56–89) and female (mean age 77, range 53–89) patients who had recovered from the acute phase of various medical illnesses. Several of these patients were receiving no drug therapy and those drugs used (including digoxin, frusemide and ampicillin) had no effect on the binding of [^{3}H]-dihydroergocryptine to platelets *in vitro* at concentrations up to 0.1 mM.

Radioligand binding assay

Before each assay an aliquot of [^{3}H]-dihydroergocryptine was diluted from the stock solution into incubation medium containing 10% v/v ethanol and 5 mM HCl. The acidified ethanolic medium was found to be essential to maintain the [^{3}H]-dihydroergocryptine in solution. The presence of equivalent amounts of ethanol and HCl (final concentrations 1% and 0.5 mM respectively) has been shown to have no effect on binding of [^{3}H]-dihydroergocryptine in other tissues (Williams *et al.*, 1976) and did not affect the aggregation response of human PRP to noradrenaline. EDTA was chosen as the anticoagulant for binding studies because of its strong Ca-chelating effect which abolished the possibility of the platelets aggregating during the incubation step. Incubations comprising 800 μl platelet suspension, 100 μl diluted [^{3}H]-dihydroergocryptine and 100 μl incubation medium with or without phentolamine (50 μM) were maintained at 37°C for 20 min (unless otherwise specified) and terminated by centrifugation at room temperature for 1 min at 6500 *g*. To determine the free concentration of radioligand, samples of the supernatant were counted. Each platelet pellet was then sonicated in 500 μl distilled water and a 400 μl aliquot counted by liquid scintillation in 5 ml PCS scintillation fluid (Amersham-Searle, USA) at an efficiency of 35%.

During competitive inhibition studies, the competing ligand was added to both the total and non-specific incubates in order to determine accurately the degree of inhibition of specific binding. Between four and eight concentrations of each ligand were studied in order to bracket the IC_{50} value. Within each assay both total and non-specific samples were replicated four fold. Specific binding of [^{3}H]-dihydroergocryptine represented 20–30% of total radioactivity bound to the cells. Total binding itself did not exceed 4% of the total amount of radiolabel present in any incubation.

Platelet aggregation study

The platelet aggregation response was monitored using a Payton dual-channel 300 BD-S aggregometer by the standard method of measuring the change in optical density of stirred PRP following addition of noradrenaline. Immediately after preparation, the PRP was diluted to a density of approximately 2.5×10^8 cells/ml with autologous plasma, then gassed with 95% O_2:5% CO_2 for 5 min and stored at room temperature. The limits of platelet aggregation were defined for each subject using platelet-rich plasma as the baseline and platelet-free plasma to indicate 100% aggregation. Platelet aggregation studies were begun 45 min after taking the blood and were completed within the following 90 min, during which time the aggregation response to noradrenaline remained unchanged. All aggregation studies were performed at 37°C following precisely 2 min preincubation of the PRP at this temperature. The α-adrenoceptor antagonist or saline was added in a volume of 10 μl to each 0.5 ml sample of PRP at the

start of the 2 min preincubation. Ethanol was used to dissolve the hydrophobic compounds but the final concentration of ethanol in PRP did not exceed 1% v/v which itself did not affect aggregation. Noradrenaline was added in volume of 5 μl to a final concentration of 5 μM and the rate of the primary aggregation response was monitored. Each antagonist was tested at 4–6 different concentrations in order to bracket the IC_{50} value.

Analysis of data

The equilibrium binding characteristics of [^{3}H]-dihydroergocryptine were calculated from Scatchard analysis of the specific binding data according to the method of least squares linear regression. Comparisons between the binding characteristics of [^{3}H]-dihydroergocryptine in different groups were made on the basis of Student's unpaired t test. Probit analysis was used to linearize the competitive inhibition curves and obtain an estimate of the IC_{50}. The binding affinity constant (K_i) for unlabelled ligands was then calculated according to the expression (Cheng & Prusoff, 1973):

$$K_i = \frac{IC_{50}}{1 + \frac{[D]}{K_d}}$$

where IC_{50} = concentration of ligand producing 50% inhibition of specific binding of [^{3}H]-dihydroergocryptine, [D] = free concentration of [^{3}H]-dihydroergocryptine, and K_d = dissociation equilibrium binding constant for [^{3}H]-dihydroergocryptine on intact human platelets.

The initial rate of noradrenaline-induced platelet aggregation was calculated as % aggregation/min. As for binding, probit analysis was used to identify the concentration of α-adrenoceptor antagonist which reduced the initial rate of aggregation by 50%. The affinity constant (K_i) for inhibition of noradrenaline-induced platelet aggregation was also calculated by the method of Cheng & Prusoff (1973) as:

$$K_i = \frac{IC_{50}}{1 + \frac{[NA]}{K_{NA}}}$$

where IC_{50} = concentration of ligand producing 50% inhibition of the initial rate of noradrenaline-induced aggregation, [NA] = concentration of noradrenaline used to stimulate aggregation (5 μM) and K_{NA} = affinity constant for noradrenaline-induced aggregation.

The value of K_{NA} was determined immediately before and after each inhibition study by measuring the initial rate of aggregation induced by noradrenaline (range 1–50 μM) and analysing these data according to the Michaelis-Menton plot. During this study $K_{NA} = 7.91 \pm 1.25$ μM (mean ± s.e.mean, $n = 10$).

Drugs

Drugs were dissolved on the day of use in incubation medium for the binding assay or in saline for platelet aggregation studies. Yohimbine, prazosin, dihydroergocryptine, phenoxybenzamine and haloperidol were insoluble in aqueous solution and were dissolved initially in ethanol then diluted in the appropriate aqueous medium.

[^{3}H]-dihydroergocryptine (specific activity 25.7–38.8 Ci/mmol) was obtained from New England Nuclear Ltd.

The following drugs were donated by the firms named in parentheses phentolamine hydrochloride, naphazoline nitrate, tolazoline hydrochloride, imipramine hydrochloride (Ciba-Geigy); prazosin hydrochloride (Pfizer); thymoxamine hydrochloride (Warner); oxymetazoline hydrochloride (Merck); clonidine hydrochloride (Boehringer-Ingelheim); methoxamine hydrochloride (Burroughs Wellcome); phenoxybenzamine hydrochloride (Smith, Kline & French); dihydroergocryptine mesilate (Sandoz); (+)-adrenaline bitartrate, (+)-noradrenaline bitartrate (Sterling-Winthrop); (−)-propranolol hydrochloride (ICI); chlopromazine hydrochloride (May & Baker); haloperidol (Janssen). All other drugs were purchased from Sigma London Chemical Co.

Results

Definition of specific binding

The total binding of [^{3}H]-dihydroergocryptine to intact human platelets was progressively inhibited by phentolamine in the range 10^{-8}–10^{-6}M as shown in Figure 1. Between 10^{-6}–2×10^{-5}M phentolamine, the binding of [^{3}H]-dihydroergocryptine remained almost constant, then as the concentration of phentolamine was further increased, the level of binding again began to fall. A concentration of 5 μM phentolamine was therefore chosen to define the specific binding of [^{3}H]-dihydroergocryptine. As outlined below, this fraction of the binding displayed the anticipated characteristics of a functional receptor site. Substitution of 200 μM for 5 μM phentolamine abolished the saturability of [^{3}H]-dihydroergocryptine binding and considerably reduced the difference in affinity between the stereoisomers of adrenaline and of noradrenaline.

Kinetics of [^{3}H]-dihydroergocryptine binding

Specific binding of [^{3}H]-dihydroergocryptine to in-

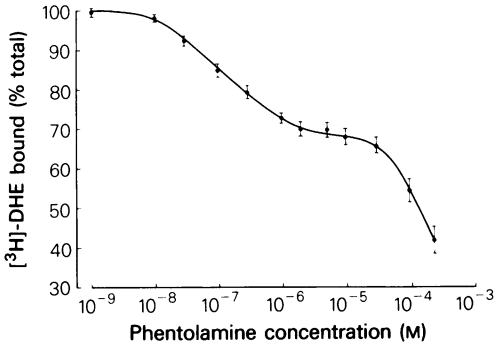

Figure 1 Total binding of [^{3}H]-dihydroergocryptine to intact human platelets in the presence of phentolamine. Intact human platelets were incubated with [^{3}H]-dihydroergocryptine ([^{3}H]-DHE; 3 nM) for 20 min at 37°C with varying concentrations of phentolamine. Each point represents the mean from 4 individual assays; vertical lines show s.e.mean. The 100% binding was defined as that observed in the absence of phentolamine.

tact human platelets increased exponentially with an association half-time of approximately 7.5 min at a free concentration of 2.4 nM, as shown in Figure 2. At this concentration, binding reached equilibrium within 20 min, therefore this was chosen as the routine incubation time for further studies. The apparent association rate constant based on the assumption of first-order kinetics and calculated from the slope of the inset to Figure 2 was $k_{ob} = 0.090$/min. The second-order association rate constant (k_1) was then calculated from the expression (Williams & Lefkowitz, 1978):

$$k_1 = \frac{k_{ob} - k_2}{[D]} = 1.93 \times 10^7\ M^{-1}\ min^{-1}$$

where k_2 is the dissociation equilibrium rate constant and [D] the free concentration of [^{3}H]-dihydroergocryptine.

Addition of phentolamine (final concentration 5 μM) to platelets which had been incubated with [^{3}H]-dihydroergocryptine for 20 min caused reversal of 95% of specific binding within a further 50 min. Dissociation of [^{3}H]-dihydroergocryptine was exponential with a half-time of approximately 16 min and a first-order dissociation rate constant $k_2 = 0.044$/min, as calculated from the slope of the logarithmic dissociation curve.

The kinetically derived value for the equilibrium dissociation constant (K_d) for [^{3}H]-dihydroergocryptine on intact human platelets was therefore

$$K_d = \frac{k_2}{k_1} = 2.30 \times 10^{-9}\ M$$

Binding characteristics of [^{3}H]-dihydroergocryptine on intact human platelets

The specific binding of [^{3}H]-dihydroergocryptine to intact human platelets was saturable in the range 1.5–15 nM free concentration, as shown in Figure 3. Linear regression analysis of the resulting Scatchard plot indicated a correlation coefficient $r = 0.98$, consistent with the presence of a single binding site. Analysis by the Hill plot was not possible since this method requires an estimate of the binding at saturation. Attempts to determine this figure were unsuccessful since at free concentrations greater than 20 nM the specific binding became such a small proportion of the total binding (less than 5%) that frequently it was not significantly different from zero. Typical figures observed in a single assay from four fold replicates of total and non-specific binding were 2325 ± 117 ct/min and 1784 ± 112 ct/min respective-

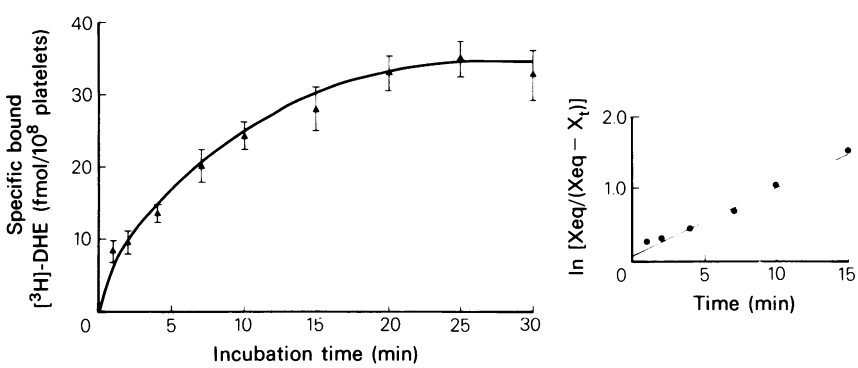

Figure 2 Association time-course of specific binding of [^{3}H]-dihydroergocryptine to intact human platelets. Intact human platelets were incubated with [^{3}H]-dihydroergocryptine ([^{3}H]-DHE; 2.4 nM) at 37°C for 1–30 min. Each point represents the mean result for specific binding in 5 individual assays; vertical lines show s.e.mean. Inset shows the same data plotted logarithmically as a pseudo-first order rate curve.

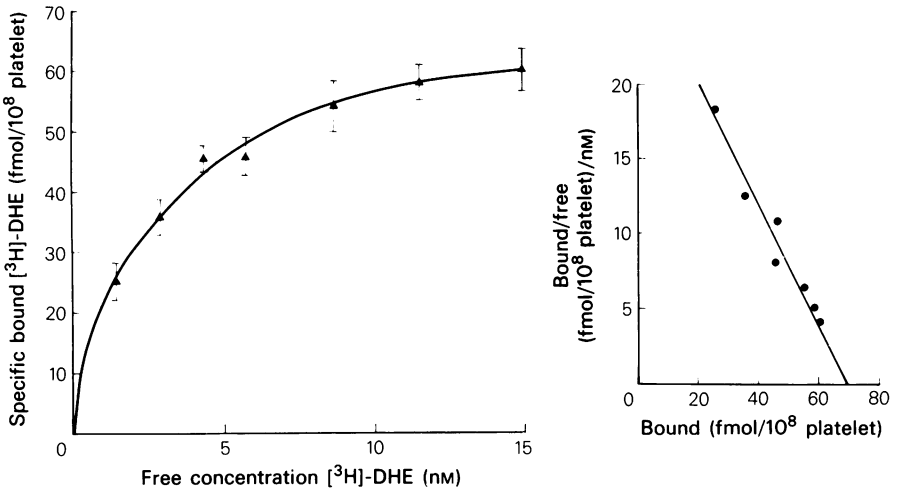

Figure 3 Specific binding of [^{3}H]-dihydroergocryptine to intact human platelets. Intact human platelets were incubated with [^{3}H]-dihydroergocryptine ([^{3}H]-DHE, range 1.5–15 nM) for 20 min at 37°C. Each point represents the mean result from 10 separate assays, each using blood from a different subject; vertical lines show s.e.mean. Inset shows a Scatchard plot of the same data.

ly at 3 nM concentration of label and 10097 ± 427 ct/min and 9257 ± 221 ct/min at 15 nM concentration, all figures expressed as mean ± standard deviation. Individual assessments of 10 healthy young male subjects indicated a mean binding affinity $K_d = 2.81 \pm 0.27$ nM (mean ± s.e.mean) and capacity $B_{max} = 63 \pm 3$ fmol/10^8 platelet, equivalent to approximately 400 receptor sites per cell.

To determine the intra-subject variability, the binding assay was repeated five times at weekly intervals on one young healthy male. Affinity and capacity were similar to other young male subjects and showed little variation; $K_d = 3.28$ nM ± 13%, $B_{max} = 70$ fmol/10^8 platelet ± 16%, expressed as mean ± coefficient of variation. This investigation was repeated one year later in the same subject with almost identical results; $K_d = 2.98$ nM ± 16%, $B_{max} = 69$ fmol/10^8 platelet ± 14%

Specific binding of [^{3}H]-dihydroergocryptine increased linearly with platelet cell density between $0.32-2.0 \times 10^8$ cell/ml but was lower than anticipated at cell densities exceeding 2×10^8 cell/ml. This was due to the high degree of adsorption of [^{3}H]-dihydroergocryptine by the platelets effectively reducing the free concentration of the radioligand, as confirmed by samples taken from the incubation supernatant following centrifugation.

To investigate the possibility that resuspension of the platelets in the incubation medium may introduce artefactual changes in the binding characteristics, the binding assay was repeated using either platelet-rich plasma or platelets resuspended by the normal method in autologous plasma. As shown in Table 1, the platelet binding capacity was unaffected by presence of the plasma but the affinity of [^{3}H]-dihydroergocryptine was reduced both in platelet-rich plasma and in platelets resuspended in autologous plasma. Filtration of the plasma through Amicon CF50A filters, which retain molecules exceeding 50000 daltons, abolished this inhibitory effect. Since dihydroergocryptine is a lipophilic compound, it seemed likely that the high molecular weight plasma inhibitory factor may be albumin, adsorbing the [^{3}H]-dihydroergocryptine and thereby reducing its effective free concentration. When added to platelets resuspended in the normal incubation medium, egg albumin (3 g/100 ml) reduced the binding affinity of [^{3}H]-dihydroergocryptine ($K_d = 5.01$ nM) without altering the binding capacity.

Table 1 Effect of plasma on the binding characteristics of [^{3}H]-dihydroergocryptine on intact human platelets

	Affinity (K_d) (nM)	*Capacity* (B_{max}) (fmol/10^8 platelet)
Rususpended platelets	2.85 ± 0.37	71 ± 4
Resuspended platelets + autologous plasma	5.82 ± 0.53*	74 ± 7
Platelet-rich plasma	6.03 ± 0.73*	75 ± 5

Each value represents the mean ± s.e.mean of 3 separate estimates. Significance values (*$P < 0.05$) refer to comparison with respective values for resuspended platelets.

Table 2 Inhibition of specific binding of [^{3}H]-dihydroergocryptine to intact human platelets

Antagonists	K_I (μM)	*Agonists*	K_i (μM)
Dihydroergocryptine	0.006	(−)-Adrenaline	4.1
Phentolamine	0.055	(−)-Noradrenaline	14.9
Phenoxybenzamine	0.14	Dopamine	83
Pizotifen	4.7	(+)-Adrenaline	135
Haloperidol	5.9	5-HT	153
Imipramine	8.2	(+)-Noradrenaline	412
Chlorpromazine	9.4	Isoprenaline	730
(−)-Propranlol	29.0	Histamine	> 500
Yohimbine	0.005	Oxymetazoline	0.039
Tolazoline	1.2	Clonidine	0.23
Prazosin	2.3	(−)-Phenylephrine	9.3
Thymoxamine	10.9	Methoxamine	153.0

Intact human platelets were incubated with [^{3}H]-dihydroergocryptine (3 nM) ± phentolamine (5 μM)+ inhibitor at 4–8 concentrations per assay. The affinity of each compound is represented by the inhibitory equilibrium constant (K_i) calculated from the IC_{50} value as described in the text. Each value is the mean of 3–9 individual estimates.

Specificity of the [^{3}H]-dihydroergocryptine binding site

The specific binding of [^{3}H]-dihydroergocryptine to intact human platelets was more potently inhibited by α-adrenoceptor ligands, both agonists and antagonists, than by those for 5-hydroxytryptamine, dopamine and histamine receptors or β-adrenoceptors as shown in Table 2. Examination of the inhibition curves in the form of the Hill plot indicated a slope of approximately 1.0 in the case of all compounds, both agonists and antagonists. Inhibition by adrenaline and noradrenaline showed a high degree of stereospecificity, the biologically active (−)-isomers being over 20 fold more potent than the (+)-isomers. The adrenergically inert compounds catechol, phenol and indole were inactive as inhibitors of [^{3}H]-dihydroergocryptine with affinity constants greater than 500 μM. The affinity of unlabelled dihydroergocryptine ($K_i = 5.58 \pm 1.78$ nM, mean ± s.e.mean, $n = 6$) was not significantly different from that of [^{3}H]-dihydroergocryptine identified from Scatchard analysis of the binding curves ($K_d = 2.81 \pm 0.27$ nM, mean ± s.e.mean, $n = 10$).

Within the group of α-adrenoceptor ligands, those with a preferential affinity for α_2-adrenoceptors, such as yohimbine and clonidine, were more potent inhibitors of [^{3}H]-dihydroergocryptine binding on intact human platelets than the α_1-selective agents, such as prazosin and methoxamine. Similarly, the α_2-adrenoceptor antagonists, yohimbine and tolazoline, proved to be more potent inhibitors of noradrenaline-induced platelet aggregation than the α_1-antagonists, prazosin and thymoxamine. As shown in Figure 4, comparison of the affinity of these antagonists as determined from inhibition of noradrenaline-induced platelet aggregation, with that determined from inhibition of [^{3}H]-dihydroergocryptine binding to intact human platelets demonstrated a significant correlation ($r = 0.92$, $P < 0.01$).

The pattern of inhibition of binding therefore suggests that [^{3}H]-dihydroergocryptine binds to α_2-adrenoceptors on intact human platelets. The affinity of antagonists for this site is similar to that at other α_2-adrenoceptors, whereas the affinity of agonists, particularly (−)-adrenaline and (−)-noradrenaline, on the intact platelet appears much lower than that at other α_2-adrenoceptors or on the platelet lysate preparation (Newman *et al.*, 1978; Alexander *et al.*, 1978). We therefore repeated the inhibition study for (−)-adrenaline first in the presence of 2.5 mM ascorbic acid and 1 μM pargyline to prevent the oxidation and/or metabolism of the amine then in the presence of 1 μM imipramine to prevent any active uptake into the platelet but on both occasions the affinity was unchanged. In tests on nine different individuals the affinity constant for (−)-adrenaline always fell in the range 2–7 μM.

Binding of [^{3}H]-dihydroergocryptine to intact human platelets from elderly subjects

The specific binding of [^{3}H]-dihydroergocryptine was tested at five different concentrations in the

range 1.5–15 nM on intact platelets from each of eight elderly male subjects (mean age 73, range 56–89, and eight elderly female subjects (mean age 77, range 53–89). The binding capacity of the elderly males $B_{max} = 63 \pm 4$ fmol/10^8 platelet (mean ± s.e.mean) was not significantly different from that of the elderly females ($B_{max} = 65 \pm 6$ fmol/10^8 platelet) or the younger males described above. The affinity of [^{3}H]-dihydroergocryptine was significantly higher ($P < 0.05$) in the elderly male group ($K_d = 2.01 \pm 0.15$ nM) than either the elderly female group ($K_d = 2.83 \pm 0.31$ nM) or the younger male group ($K_d = 2.81 \pm 0.27$ nM). However, no significant difference was observed between any of the three groups in terms of the affinity or maximum rate of noradrenaline-induced platelet aggregation.

Discussion

The specific binding of [^{3}H]-dihydroergocryptine to intact human platelets fulfils the essential criteria for binding to a functional α-adrenoceptor inasmuch as that binding is saturable, of high affinity, kinetically follows the law of mass action for a simple bimolecular interaction and demonstrates the specificity and stereospecificity of a typical α-adrenoceptor. Functionally, this binding site appears to correspond with the α-adrenoceptor responsible for noradrenaline-induced platelet aggregation since characteristic α-adrenoceptor antagonists show a good correlation between their potency to inhibit [^{3}H]-dihydroergocryptine binding and their potency to inhibit noradrenaline-induced aggregation.

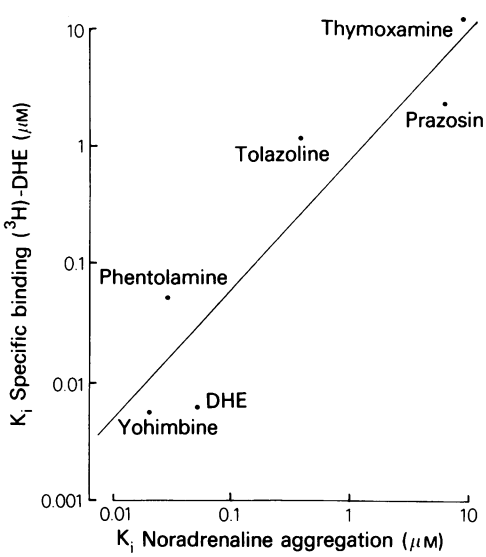

Figure 4 Correlation of affinity of α-adrenoceptor antagonists on binding and aggregation response of intact human platelets. The affinity of α-adrenoceptor antagonists for competitive inhibition of specific binding of [^{3}H]-dihydroergocryptine ([^{3}H]-DHE) and for inhibition of noradrenaline-induced platelet aggregation were calculated as described in the text. Linear regression according to the method of least squares indicated a significant correlation ($r = 0.92$; $P < 0.01$: slope = 1.09).

The binding of [^{3}H]-dihydroergocryptine to intact human platelets occurs rapidly with an association rate constant ($k_1 = 1.93 \times 10^7$ M^{-1} min^{-1}) very similar to that observed using the platelet lysate preparation ($k_1 \times 1.8 = 10^7$ M^{-1} min^{-1}; Newman *et al.*, 1978). This implies that the α-adrenoceptors of intact platelets are readily accessible to the medium containing the cells and argues against the existence of any diffusion barrier such as that which appears to restrict the access of ligands to the muscarinic cholinoceptor of longitudinal smooth muscle strips from the guinea-pig ileum (Elliott *et al.*, 1978). The binding of [^{3}H]-dihydroergocryptine to intact human platelets is reversible with a dissociation rate which also resembles that of the lysate preparation.

The affinity of [^{3}H]-dihydroergocryptine as independently determined from the two rate constants ($K_d = k_2/k_1 = 2.30$ nM), Scatchard analysis of the equilibrium binding curves ($K_d = 2.81 \pm 0.27$ nM) and inhibition of binding by unlabelled dihydroergocryptine ($K_i = 5.58 \pm 1.78$ nM) show general agreement, suggesting a simple bimolecular interaction between ligand and receptor which obeys the law of mass action. A similar affinity has been reported for the lysate preparation (Newman *et al.*, 1978; Alexander *et al.*, 1978) though Kafka, Tallman, Smith & Costa (1977) identified a binding site with ten fold lower affinity. We were unable to investigate the possibility of a second lower-affinity binding site on intact platelets due to the high level of non-specific binding and consequent inaccuracy of specific binding determination at free concentrations of [^{3}H]-dihydroergocryptine exceeding 20 nM. However, Scatchard analysis of binding in the region 1.5–15 nM gave no indication of such a site, in fact the linear correlation in the young male group was highly significant ($r = 0.98$, $P < 0.001$). Furthermore, the inhibition of [^{3}H]-dihydroergocryptine binding by each ligand resulted in a Hill coefficient close to unity, supporting the proposal of a single binding site for [^{3}H]-dihydroergocryptine on intact human platelets.

The capacity of that binding site on intact platelets (400 sites/cell) is slightly greater than that estimated from lysate studies (220 sites/cell, Newman *et al.*, 100 sites/cell, Alexander *et al.*) but this may simply represent a loss of tissue during preparation of the washed lysate. Under similar incubation conditions

to our own, Kaywin, McDonough, Insel & Shattil (1978) observed 460 sites/cell on normal intact platelets. A somewhat lower capacity of 220 sites/cell for intact platelets was reported by Newman *et al.* (1978), possibly due to their use of adrenaline to define specific binding rather than phentolamine. In contrast, Alexander *et al.* (1978) were unable to identify any saturable binding of [^{3}H]-dihydroergocryptine to intact cells when using phentolamine 100 μM to define the level of specific binding. Reference to Figure 1 of this paper demonstrates that at such a high concentration, phentolamine displaces not only the specific binding of [^{3}H]-dihydroergocryptine to intact platelets but also a considerable amount which is non-specifically bound. Since the non-specific binding increases linearly with the free concentration of radioligand, then specific binding defined in this way would indeed be unsaturable and using 200 μM phentolamine we observed a similar phenomenon. The high level of non-specific binding associated with intact tissue makes displacement by the competing ligand more likely than in subcellular preparations, therefore as illustrated by this case greater care is needed in defining the specific binding component of intact tissues.

The inhibition of [^{3}H]-dihydroergocryptine binding follows the typical pattern of an α-adrenoceptor, yohimbine and phentolamine being the most potent antagonists, and inhibition by agonists following the order (−)-adrenaline > (−)-noradrenaline > 5-HT > dopamine > isoprenaline. The possibility that [^{3}H]-dihydroergocryptine may bind to dopamine receptors on the platelet, as in calf brain (Titeler *et al.*, 1977), is countermanded by the low affinity of both dopamine and haloperidol. These findings, together with a previous report that [^{3}H]-haloperidol does not bind to specific sites on human platelets (Boullin, Molyneux & Roach, 1978), argues against the presence of dopamine receptors on human platelets. Receptors for 5-HT on human platelets have been identified (Peters & Grahame-Smith, 1980; Kim, Steiner & Baldini, 1980) but the low affinity of 5-HT and pizotifen indicate that these sites are not labelled by [^{3}H]-dihydroergocryptine.

Of the α-adrenoceptor ligands, the α_2-selective compounds, such as yohimbine and clonidine, are more potent inhibitors of [^{3}H]-dihydroergocryptine binding than their α_1-selective counterparts, prazosin and methoxamine. The absence of biphasic inhibition curves by selective α-adrenoceptor ligands and the close approximation of the Hill coefficient to unity in the case of each inhibitor suggests that [^{3}H]-dihydroergocryptine binds to a single class of α_2-adrenoceptors. Ligand binding studies in platelet lysate preparations concluded likewise (Wood, Arnett, Clarke, Tsai & Lefkowitz, 1979; Hoffman, De Lean, Wood, Schoken & Lefkowitz, 1979) but studies of platelet aggregation suggested the presence of α_1 and α_2-adrenoceptors on human platelets, both of which have a high affinity for dihydroergocryptine (Grant & Scrutton, 1979; Scrutton & Grant, 1979). If the proportion of α_1 to α_2-adrenoceptors was small, less than 10%, then their detection may be beyond the limits of resolution of the present assay. Investigation by selective α_1 and α_2-adrenoceptor radioligands would then be the most reliable method by which to resolve the postulated existence of the platelet α_1-adrenoceptors.

The low affinity of α-adrenoceptor agonists observed with intact platelets compared to the values found with platelet lysate preparations (Newman *et al.*, 1978; Alexander *et al.*, 1978) have been mentioned above. However, attempts to prevent possible metabolism and active uptake by the platelet did not alter the particularly low affinity of (−)-adrenaline. In the platelet lysate preparation the affinity of agonists has been shown to be sensitive to the presence of specific cations and purine nucleotides (Tsai & Lefkowitz, 1979; Michel, Hoffman & Lefkowitz, 1980). Studies in our laboratory suggest that similar factors may account for the low affinity of agonists on intact platelets (Elliott & Grahame-Smith, 1981).

It appears, therefore, that the binding characteristics of [^{3}H]-dihydroergocryptine on intact human platelets closely resemble those found with the platelet lysate preparation. Both the specificity and stereospecificity of binding of the lysate preparation are retained in the intact cells but the absolute affinity of agonists is substantially less. This suggests that although the receptor binding site is not significantly altered by the process of cell rupture and homogenization, certain essential components which normally modulate receptor activity within the cell are lost during the extraction procedure. The human platelet α-adrenoceptor may therefore be an ideal case in which to compare the properties of an isolated receptor to those of a functional receptor within an intact membrane system.

As discussed above, there is good evidence that [^{3}H]-dihydroergocryptine binds to the platelet α-adrenoceptor responsible for noradrenaline-induced aggregation, thus fulfilling the primary requirement for use of this assay as a monitor of peripheral adrenergic sensitivity in clinical investigation. The small degree of variability in binding characteristics both between different individuals and between different sampling occasions for a single subject even as long as a year apart, suggest that the platelet α-adrenoceptor population from healthy male subjects is normally highly consistent. Any deviation from the norm should therefore be detectable using this assay.

Ageing has been associated with a decrease in the capacity of several receptors in both rat and human

brain (Maggi, Schmidt, Ghetti & Enna, 1979; Shih & Young, 1978). Similar changes have been observed in some peripheral receptor systems in man, notably the lymphocyte β-adrenoceptor (Schocken & Roth, 1977) and the platelet binding site for [^{3}H]-imipramine (Langer, Briley, Raisman, Henry & Morselli, 1980). Our studies revealed no such change in the case of the platelet α-adrenoceptor when comparing old and young men. A small but significant increase in receptor affinity was observed in the older group but this was not paralleled by any difference in aggregation response to noradrenaline.

Similarly no significant difference in binding capacity was observed between platelets from elderly male and female subjects. In contrast, the binding capacity of platelets from young, healthy females during the normal menstrual cycle (B_{max} 81–84 fmol/10^8 platelets; Peters, Elliott & Grahame-Smith, 1979) is considerably higher than that of the young male group. Previous work has demonstrated that the platelet α-adrenoceptor capacity of both rabbits (Elliott, Peters & Grahame-Smith, 1980; Roberts, Goldfien, Tsuchiya, Goldfien & Insel, 1979) and women (Peters *et al.*, 1979) is sensitive to the circulating level of oestrogen/progestogen. It is probable, therefore, that the higher binding capacity of young women compared to young men is related to their higher levels of circulating oestrogen/progestogen. Such a hypothesis is supported by the observation that the binding capacity of the elderly women, all of whom were postmenopausal, is substantially lower than that of the young women. It also suggests that age-related changes in other tissues and concerning other receptors may, at least in part, result from indirect changes other than the ageing process itself.

In conclusion, [^{3}H]-dihydroergocryptine binds to a single class of sites on intact human platelets which correspond to the α-adrenoceptor responsible for noradrenaline-induced platelet aggregation. The binding characteristics of [^{3}H]-dihydroergocryptine itself are similar to those previously described using the platelet lysate preparation and are sufficiently reproduceable that this assay should prove useful as a monitor of platelet α-adrenoceptor sensitivity in clinical investigation.

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