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CHARACTERISATION OF [^{3}H]LYSERGIC ACID DIETHYLAMIDE BINDING TO A 5-HYDROXYTRYPTAMINE RECEPTOR ON HUMAN PLATELET MEMBRANES

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Specific binding of [^{3}H]lysergic acid diethylamide (LSD) to human platelet membranes, as defined by 300 nM spiperone, was saturable over the concentration range of 0.25-2.5 nM [^{3}H]LSD. At 0.5 nM [^{3}H]LSD the half-time for association at 37°C was 56 min, half-time for dissociation was 173 min, and the kinetically derived affinity was 0.24 nM. In 19 control subjects equilibrium binding studies gave an affinity of 0.53 ± 0.02 nM (mean ± S.E.M.) and capacity of 57.1 ± 5.6 fmol/mg protein (mean ± S.E.M.). The inhibition profile was consistent with that of a 5-hydroxytryptamine (5-HT) receptor. There was a significant correlation between the inhibition of [^{3}H]LSD binding and the inhibition of 5-HT-induced shape change, but not inhibition of active platelet uptake of 5-HT. There was also a significant correlation between the inhibition of [^{3}H]LSD binding to human platelet membranes and human frontal cortex. Platelet [^{3}H]LSD binding may therefore be a useful model for study of peripheral and central 5-HT receptors in man.

Human platelets 5-Hydroxytryptamine [^{3}H]Lysergic acid diethylamide Receptor binding

1. Introduction

5-Hydroxytryptamine (5-HT) interacts with human platelets in two separate ways. Firstly, it induces shape change and aggregation and secondly, it is actively taken up by an energy-dependent transport system. Inhibition studies with structural analogues of 5-HT suggest that shape change and aggregation are mediated through the same receptor, while 5-HT uptake occurs at a different site (Born et al., 1972). Radioligand binding studies using [^{3}H]5-HT have demonstrated the presence of two separate 5-HT binding sites on intact human platelets, the high affinity site being associated with 5-HT-induced aggregation and the low affinity site with 5-HT uptake (Peters and Grahame-Smith, 1980). The analysis of binding of a single ligand to two independent sites is complex and therefore [^{3}H]5-HT is not an ideal ligand for the investigation of these two sites. In addition, 5-HT is taken up into the platelets, and any endogenous 5-HT released from intact platelets could dilute the [^{3}H]5-HT.

D-lysergic acid diethylamide (LSD) is a potent inhibitor of 5-HT-induced shape change and aggregation but is virtually inactive against 5-HT uptake in resuspended human platelets (Laubscher and Pletscher, 1979). [^{3}H]LSD has been used to label 5-HT receptors in rat brain (Bennett and Snyder, 1975; Peroutka and Snyder, 1979), human brain (Cross, 1982) and rabbit platelets (Laubscher et al., 1981), and classical inhibitors of 5-HT uptake are weak inhibitors of binding in these systems. We have therefore investigated the binding of [^{3}H]LSD to human platelet membranes in order to characterise the specific receptor responsible for 5-HT-induced shape change and aggregation.

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2. Materials and methods

2.1. Materials

[^{3}H]LSD (D-isomer, specific activity 40.8-48.5 Ci/mmol was obtained from New England Nuclear Ltd. Drugs were obtained from the following sources: (+)- and (−)-butaclamol (Ayerst, Hampshire; metoclopramide (Beecham, Middlesex); clonidine (Boehringer-Ingelheim, Berkshire); phentolamine (Ciba, West Sussex); metergoline (Farmitalia, Hertfordshire); acetylsalicylic acid (Fisons, Leicestershire); imipramine (Geigy, Cheshire); (+)- and (−)-propranolol (ICI, Cheshire); ketanserin, pimozide, pirenperone and spiperone (Janssen, Beerse); fluoxetine (Lilly, Surrey); α- and β-flupenthixol (Lundbeck, Copenhagen); chlorpromazine and mepyramine (May and Baker, Essex); cyproheptadine (Merck, Sharpe & Dohme, Hertfordshire); quipazine (Miles Laboratories, Buckinghamshire); mianserin (Organon, Surrey); prazosin (Pfizer, Kent); amitriptyline, diazepam and methiothepin (Roche, Basel); bromo-LSD, D-LSD, ergotamine, methysergide and pizotifen (Sandoz, Basel); haloperidol (Searle, Buckinghamshire); ADP, atropine and noradrenaline (Sigma, Dorset); cimetidine (Smith, Kline & French, Hertfordshire); fluphenazine (Squibb, Middlesex); naloxone (Winthrop, Surrey).

2.2. Membrane preparation

Blood was taken from the antecubital vein with a 19-gauge needle and anticoagulated by the addition of nine volumes of blood to one of EDTA (1% w/v in saline). Platelet-rich plasma (PRP) was obtained by centrifugation of the blood at $180 \times g$ for 15 min at 20°C. This was then centrifuged at $1200 \times g$ for 7.5 min at 10°C to produce a platelet pellet which was resuspended in hypotonic Tris-EDTA (5 mm Tris, 0.1% EDTA, pH 7.5). This suspension was homogenised by 20 strokes of a motor-driven pestle (Citenco Ltd., Hertfordshire) and then centrifuged at $30000 \times g$ for 15 min at 4°C. The membrane pellet was then homogenised and centrifuged again in hypotonic Tris-EDTA, then once in the incubation buffer (50 mM Tris, 120 mM sodium chloride, 5 mM potassium chloride, 2 mM magnesium chloride and 0.05% ascorbic acid, pH 7.3). The platelet pellet was then resuspended in the same incubation buffer to form the final membrane suspension.

2.3. [^{3}H]LSD binding assay

Incubations were performed at 37°C for 4 h and contained 400 μl platelet membrane suspension, 50 μl [^{3}H]LSD (0.25-2.5 nM), and 50 μl of incubation buffer with or without spiperone (final concentration 300 nM). Spiperone was chosen as the displacing agent because like LSD it is a potent inhibitor of 5-HT-induced shape change and aggregation and is a weak inhibitor of 5-HT uptake (Laubscher and Pletscher, 1979), but it is structurally dissimilar to LSD. The incubations were stopped by addition of 5 ml ice-cold Tris buffer (50 mM Tris, 0.01% bovine serum albumin, pH 7.7) and then filtered under reduced pressure through Whatman GF/F filters. The filters were washed twice more with the Tris buffer, dried in air at 25°C and counted in 10 ml Instagel (Packard Corporation) scintillation fluid at an efficiency of approximately 40%. The total bound [^{3}H]LSD did not exceed 1% of the total radioactivity. Aliquots of the membrane suspension were frozen at −20°C and protein content later assayed by the method of Lowry (Lowry et al., 1951).

During inhibition studies, the competing ligand was added to incubates without spiperone to determine the inhibition of total binding, and also to incubates with 300 nM spiperone to determine any inhibition of non-specific binding which may have occurred. By subtracting any inhibition of non-specific binding from the inhibition of total binding, the degree of inhibition of specific binding could be accurately determined for each inhibitor concentration. Between four and twelve concentrations of each ligand were studied in order to determine the IC_{50} value. Within each assay, total and non-specific samples were replicated in triplicate.

2.4. Analysis of data

The equilibrium binding characteristics of [^{3}H]LSD were calculated from Scatchard analysis

of the specific binding data according to the method of least squares linear regression. Log-probit analysis was used to estimate the IC_{50} from the inhibition curves.

3. Results

3.1. Definition of specific binding

The total binding of [^{3}H]LSD was inhibited by spiperone over approximately 2 orders of magnitude to reach a plateau at 300 nM spiperone (fig. 1). We have therefore used this concentration of spiperone to define the specific binding of [^{3}H]LSD. A plateau occurs at the same level for inhibition of binding by D-LSD between 10^{-8} and 10^{-7} M (fig. 2). 5-HT has a shallower displacement curve than either D-LSD or spiperone.

3.2. Kinetics of [^{3}H]LSD binding

Specific binding of [^{3}H]LSD reached equilibrium by 4 h and remained constant at 10 h at 37°C (fig. 3). Thus, 4 h was chosen as the routine incubation time. On addition of 300 nM spiperone dissociation of the specific binding occurred. At a free concentration of [^{3}H]LSD of 0.5 nM the half-time for association was 56 min with an association rate constant of 0.017 ± 0.004 nM^{-1} min^{-1} (mean $k_1 \pm$ S.E.M., $n = 7$). The half-time for dissociation was 173 min with a dissociation rate constant of 0.0040 ± 0.0003 min^{-1} (mean $k_2 \pm$ S.E.M., $n = 4$). The kinetically derived value for the equilibrium dissociation constant was therefore $K_D = k_2/k_1 = 0.0040/0.017 = 0.24$ nM.

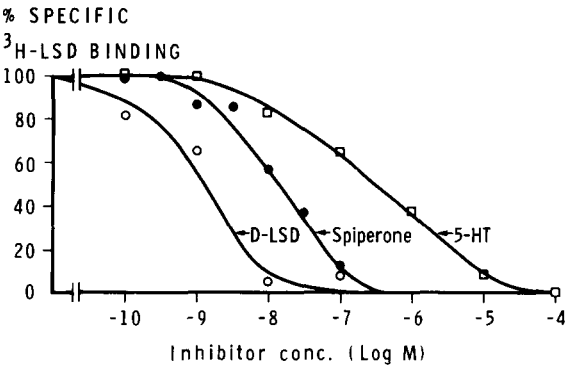

Fig. 2. Specific binding of [^{3}H]LSD to human platelet membranes in the presence of D-LSD, spiperone or 5-HT ([^{3}H]LSD 0.5 nM). Each point represents the mean of 3 separate experiments in different subjects.

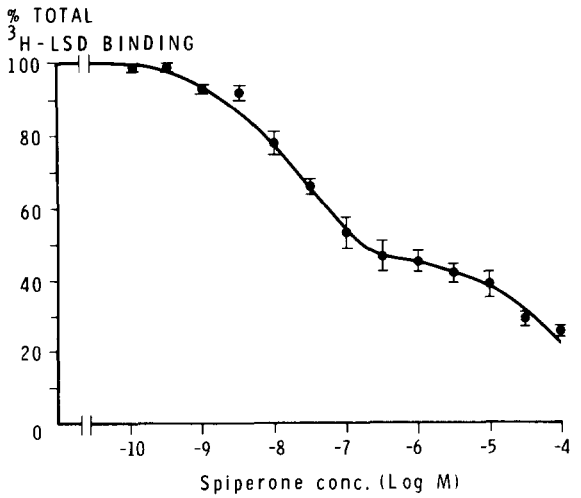

Fig. 1. Total binding of [^{3}H]LSD to human platelet membranes in the presence of spiperone ([^{3}H]LSD 0.5 nM). Each point represents the mean, and vertical line the S.E.M. of 3 separate experiments in different subjects.

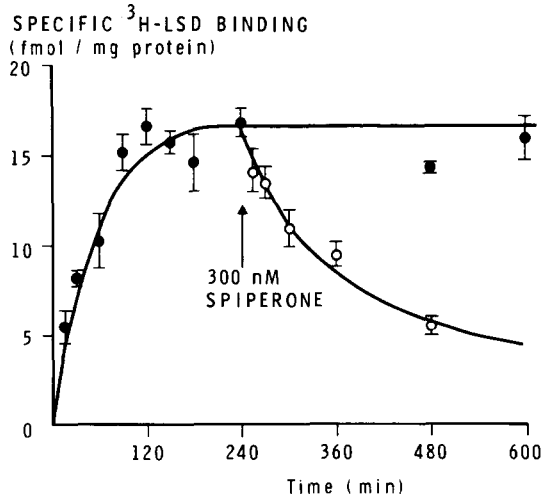

Fig. 3. Combined association and dissociation time course of specific [^{3}H]LSD binding. Each point represents the mean, and vertical line the S.E.M. of triplicate assays ([^{3}H]LSD 0.5 nM).

3.3. Binding characteristics of [^{3}H]LSD

The specific binding of [^{3}H]LSD to human platelet membranes was saturable in the range 0.25-2.5 nM (fig. 4). No further specific binding sites were observed at concentrations up to 15 nM. Scatchard analysis of the binding curve revealed a straight line. Specific binding represented 30-60% of the total radioactivity bound to the platelet membranes. In 19 control subjects (10 males, 9 females, age 17-60 years) the binding affinity (K_D) was 0.53 ± 0.02 nM (mean $\pm$ S.E.M.) and the capacity (B_{max}) was 57.1 ± 5.6 fmol/mg protein. Binding studies in a single male subject repeated on 4 separate occasions during a 10-day period showed a coefficient of variation of 16% for the K_D and 11% for the B_{max}.

Specific binding of [^{3}H]LSD was linearly related to protein concentration in the range 0.05-0.50 mg/ml (data not shown).

TABLE 1

Inhibition of specific binding of [^{3}H]LSD to human platelet membranes. Human platelet membranes were incubated with [^{3}H]LSD (0.5 nM) (±)-spiperone (300 nM)+inhibitor at 4-12 concentrations per assay. The inhibition of specific binding was determined by subtracting any inhibition of non-specific binding (incubates containing 300 nM spiperone) from the inhibition of total binding (incubates without spiperone) at each inhibitor concentration. The IC_{50} was estimated from log-probit analysis of the inhibition curves. The Hill coefficient (n_H) is given for 5-HT antagonists and agonists (each value is the mean of experiments on 2-4 different subjects).

IC_{50}	(nM)	n_H	IC_{50}	(nM)	n_H
5-HT antagonists			*Endogenous amines*		
D-LSD	1.5	0.99	5-HT	185	0.60
Methiothepin	2.1	0.77	Dopamine	>100000	
Bromo-LSD	2.7	0.99	Histamine	>100000	
Metergoline	2.8	0.85	Isoprenaline	>100000	
Pirenperone	7.3	0.95	Noradrenaline	>100000	
Spiperone	13.2	0.98			
Ketanserin	13.5	0.97			
Pizotifen	15.3	0.76	*5-HT agonists*		
Ergotamine	18.3	1.08	Quipazine	80.1	0.60
Cyproheptadine	23.7	0.72	5-MDMT	116	0.48
(+)-Butaclamol	26.3	1.11	Mescaline	5929	0.54
Methysergide	30.7	0.93			
Mianserin	38.4	0.90			
α-Flupenthixol	38.8	0.81	*5-HT uptake blockers*		
β-Flupenthixol	4748	1.28	Amitriptyline	321	
(−)-Butaclamol	>10000		Fluoxetine	1256	
			Imipramine	1324	
Dopamine antagonists			*Histamine antagonists*		
Fluphenazine	71.5		Mepyramine	2927	
Chlorpromazine	128		Cimetidine	>10000	
Pimozide	158				
Haloperidol	1128		*Others*		
			Acetylsalicylic acid	>10000	
			ADP	>10000	
Adrenergic antagonists			Atropine	>10000	
(−)-Propranolol	3358		Clonidine	>10000	
(+)-Propanolol	>10000		Diazepam	>10000	
Phentolamine	>10000		Metoclopramide	>10000	
Prazosin	>10000		Naloxone	>10000	

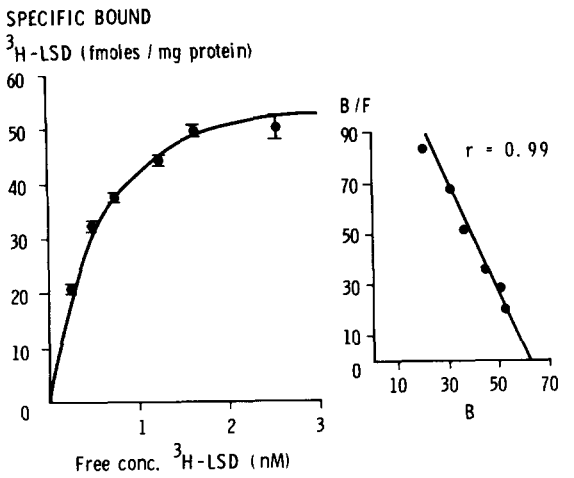

Fig. 4. Specific binding of [^{3}H]LSD to human platelet membranes. Each point represents the mean, and vertical line the S.E.M. of triplicate assays in one experiment. Scatchard analysis of the same data is shown on the right.

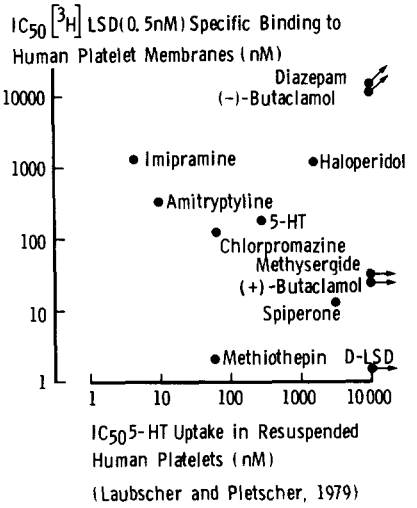

Fig. 5. Correlation of affinity of inhibitors of [^{3}H]LSD binding to human platelet membranes and of 5-HT uptake in resuspended human platelets (from Laubscher and Pletscher, 1979).

3.4. Inhibition studies

5-HT antagonists were all found to be potent inhibitors of [^{3}H]LSD binding to human platelet membranes (table 1). Haloperidol, which like spiperone is a butyrophenone, but with predominantly dopamine antagonist properties, was a weaker inhibitor of binding than spiperone by approximately 2 orders of magnitude. The 5-HT uptake inhibitors were also weak inhibitors of binding. Histamine, α- and β-adrenergic antagonists were all weak inhibitors, and the isomers of butaclamol displayed a marked stereospecificity with the (+)-isomer, the active form being over 2 orders of magnitude more potent than the (−)-isomer. Similar stereospecificity was displayed by the α- and β-isomers of flupenthixol. Of the biogenic amines tested, 5-HT was by far the most potent inhibitor, but much less potent than the 5-HT antagonists. 5-HT agonists quipazine and 5-methoxydimethyltryptamine (5-MDMT) had a similar affinity to 5-HT.

Hill plots of the inhibition of specific [^{3}H]LSD binding revealed slopes close to unity for LSD, spiperone and nearly all the other 5-HT antagonists tested, but 5-HT and the other 5-HT agonists were all considerably lower. Inhibition by 5-HT was not altered by the presence of 10 μM guanosine triphosphate (GTP), or 100 μM 5'-guanylylimidodiphosphate (Gpp(NH)p).

There was no correlation between the inhibition of [^{3}H]LSD binding to human platelet membranes

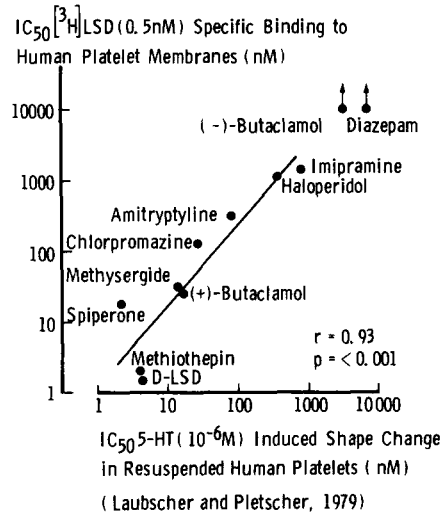

Fig. 6. Correlation of affinity of inhibitors of [^{3}H]LSD binding to human platelet membranes and of 5-HT-induced shape change in resuspended human platelets (from Laubscher and Pletscher, 1979). Linear regression analysis by the method of least squares.

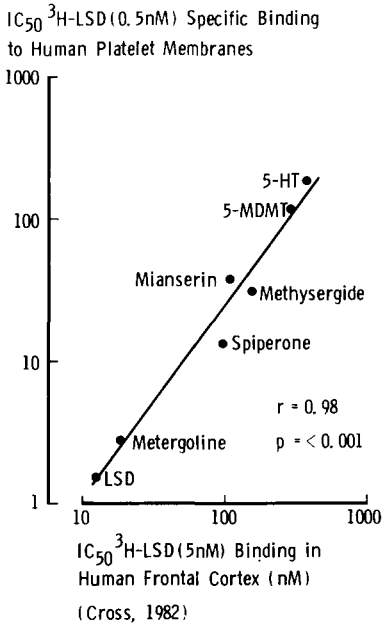

Fig. 7. Correlation of affinity of inhibitors of [^{3}H]LSD binding to human platelet membranes and [^{3}H]LSD binding in human frontal cortex (Cross, 1982). Linear regression analysis by the method of least squares.

and the inhibition of the active uptake of 5-HT by human platelets (fig. 5). However, there was a highly significant correlation between the inhibition of [^{3}H]LSD binding and the inhibition of 5-HT-induced shape change in resuspended human platelets (fig. 6). In addition, there was a close correlation between inhibition of [^{3}H]LSD binding to human platelet membranes and that to human frontal cortex defined by 1 μM LSD (fig. 7).

4. Discussion

The specific binding of [^{3}H]LSD fulfils the criteria for binding to a receptor in that it is saturable and of high affinity, kinetically follows the law of mass action for a bimolecular interaction and it demonstrates the specificity and stereospecificity of a 5-HT receptor. Functionally this binding site correlates with the 5-HT receptor responsible for shape change and aggregation, but not that associated with the active uptake of 5-HT.

The binding of [^{3}H]LSD to human platelet membranes occurs slowly, reaching equilibrium by 4 h. The dissociation is also slow and hence the affinity calculated by kinetic analysis is high (K_D 0.24 nM). This is in reasonable agreement with the affinity obtained by analysis of equilibrium binding (K_D 0.53 nM). The slow rate of association in platelet membranes contrasts with the relatively rapid association of [^{3}H]LSD to rat cerebral cortical membranes where equilibrium is reached by 10 min at 37°C (Bennett and Snyder, 1975). However, the concentration of [^{3}H]LSD we have used for kinetic studies is considerably lower than that used by Bennett and Snyder (1975) and the rate of association is dependent upon the concentration of radioligand. The assay-receptor concentration used (0.0114 nM) is considerably lower than that used by Bennett and Snyder (1975). Also, LSD is a mixed agonist-antagonist of the shape change mediated by the 5-HT receptor. In rabbit platelets the shape change induced by LSD has a slower, more gradual onset than that due to 5-HT (Laubscher et al., 1981), though there may be species differences between rabbit and human platelets.

The platelet has previously been proposed as a model for the central 5-HT neurone (Pletscher, 1968; Sneddon, 1973; Graf and Pletscher, 1979; Laubscher and Pletscher, 1979) and the correlation between [^{3}H]LSD binding to human platelet membranes and that to human frontal cortex supports this analogy. It has been suggested that there are two populations of central 5-HT receptors in addition to central 5-HT uptake sites. These have been termed 5-HT_1 and 5-HT_2 receptors (Peroutka and Snyder, 1979). Agonists possess the higher affinity for 5-HT_1 sites and antagonists the higher affinity for 5-HT_2 sites. The inhibitory profile of the platelet 5-HT receptor for shape change and aggregation which we have described is intermediate between that of the proposed central 5-HT_1 and 5-HT_2 sites. It more closely resembles the 5-HT_2 site in that 5-HT antagonists are much more potent inhibitors of platelet [^{3}H]LSD binding than agonists. However, the 5-HT agonists are relatively more potent inhibitors of binding to platelets than of binding to central 5-HT_2 sites. Accordingly, there is a close correlation between [^{3}H]LSD binding to human platelet membranes and [^{3}H]LSD

binding to human frontal cortex defined by 1 μM LSD only. This raises the possibility that [^{3}H]LSD binding to platelet membranes is to two separate sites. This seems unlikely as the Hill coefficients for inhibition of specific binding of [^{3}H]LSD by 5-HT antagonists, including spiperone, were almost unity, suggesting binding to a single platelet site. However, it is not yet clear why the Hill coefficients for inhibition by 5-HT and 5-HT agonists are considerably less than unity.

[^{3}H]LSD binding to human platelet membranes may prove useful in studying pathological and drug-induced changes in 5-HT receptors in man, both as a direct assessment of the platelet 5-HT receptor for shape change and aggregation, and as a correlate for central 5-HT receptors.

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