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DIHYDROERGOTAMINE BINDING TO RAT BRAIN MEMBRANES

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SUMMARY

^{3}H-Dihydroergotamine, which is used clinically to treat orthostatic hypotension and migraine, binds saturably, reversibly and with high affinity (K_D = 0.2 nM) to rat brain membranes. The binding is time, temperature and pH dependent and is highest in the hippocampus and the corpus striatum. Serotonin was the only neurotransmitter tested capable of inhibiting ^{3}H-DHE binding.

Several groups have recently described stereospecific high-affinity binding of d-LSD (lysergic acid diethylamide) (Fig. 1) and serotonin (5-HT) to rat brain membranes and its possible relationship to postsynaptic serotonin receptors (1-5). Williams and Lefkowitz (6) identified a specific binding site for ^{3}H-dihydroergokryptine (Fig. 1) on rabbit uterine membranes as α-adrenergic receptor.

We report here the identification and characterization of a stereospecific,high affinity binding site for dihydroergotamine *) (DHE, Fig. 1) on rat brain membranes. DHE and the before-mentioned dihydroergokryptine are both derivatives of genuine ergot peptide alkaloids (7,8). DHE is clinically used for the treatment of orthostatic hypotension and migraine (9). Its venoconstrictor effect, which has been shown pharmacologically both in vitro (10) and in vivo (11), is mediated partially by α-adrenergic receptors (10). In the canine or bovine basilar arteries DHE antagonizes responses to 5-HT in a non-competitive way (E.Müller-Schweinitzer, unpublished results).

*) dihydroergotamine mesylate = DHE 45, Dihydergot®

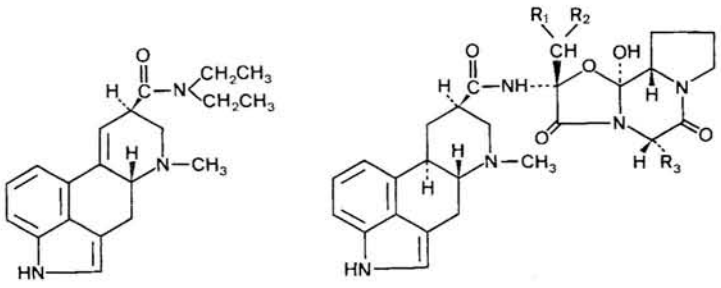

FIG. 1

d-LSD

	R_1	R_2	R_3
DH-ergotamine	H	H	$-CH_2-C_6H_5$
DH-α-ergokryptine	CH_3	CH_3	$-CH_2-CH(CH_3)_2$
DH-ergonine	H	CH_3	$-CH(CH_3)_2$
DH-β-ergosine	H	H	$-CH(CH_3)-CH_2CH_3$

METHODS

Male rats (OFA, 120-200 g) were decapitated and their brains were rapidly removed. Several brains together were homogenized in 40 volumes (weight for volume) of ice-cold 0.05 M TRIS-HCl buffer (pH 7.7 at 20°) for 30 sec with a Polytron PT-20 ST at a setting of 7 (full scale 10). After centrifugation at 48,000 x g for 10 min, the supernatant was discarded and the pellet was resuspended (Polytron, 30 sec) in the original buffer and recentrifuged. The second pellet was usually resuspended (Polytron, 30 sec) in 60 volumes of the same buffer.

In some experiments the dilution of the homogenate was different and is therefore indicated in the text.

DHE and other ergot alkaloids in dilute aqueous solution adhere to glass and plastics. In a 10 µM aqueous solution of ^{3}H-DHE 10 % of the alkaloid binds to the glass tube in a time dependent manner, and in a 100 pM solution as much as 50 % was bound to the glass. The binding studies were therefore carried out in the presence of 2 % ethanol. Preliminary experiments had shown that this alcohol concentration inhibits the binding of the alkaloid to glass surfaces and does not affect the binding to brain membranes.

[13-^{3}H]-Dihydroergotamine (17,8 Ci/mmole) was prepared by Drs.E.Schreier and R.Voges, SANDOZ Ltd., Basle. It was stored in ethanol solution at 4° and diluted with water or water-ethanol.

Solutions of unlabeled DHE were prepared as follows: 7.25 mg DHE (base) were dissolved in 0.5 ml of acetic acid-water (1:3), diluted with 50 ml of water, and the pH was adjusted with NaOH to 6.5. Filling up with water to 250 ml volume yielded a $5 \cdot 10^{-5}$ M

stock solution. The latter was further diluted with 0.05 M TRIS-HCl buffer to the desired concentration.

For routine measurements of binding each glass test tube (16 x 100 mm) contained 750 µl of membrane suspension, 250 µl of various concentrations of non-radioactive drugs and 250 µl of 5 nM ^{3}H-DHE. It proved to be important to fill the incubation tubes with the respective solutions in the cold. 100 µM sodium metabisulfite (12), or 10 µM l-ascorbic and µM pargyline (4) had no influence on IC_{50} values in experiments containing catecholamines or 5-HT, and were usually omitted. The final concentration of protein was approximately 0.6 mg/ml, as determined by the method of Lowry et al. (13). The tubes, prepared in triplicate, were incubated at 37°C for 30 min and the incubation was terminated by cooling to 0°C. Samples of 200 µl each in triplicate were taken from the incubation tubes and centrifuged in 400 µl plastic tubes for 10 min at 48,000 x g and 0°. The supernatant was aspirated by the method of Rodbell et al. (14), and the pellet was washed once with 200 µl of ice-cold 0.05 M TRIS-HCl buffer. In an alternative procedure the supernatant was spun off by placing the micro tubes into cavities of a rubber belt, which had been attached to the shaft of a stirrer. The stirrer was immersed in a glass baker in order to collect the supernatant fluid. This latter method made washing of the pellet unnecessary, and gave better reproducibility. With either procedure the tips of the micro tubes were cut off with a razor blade and transferred to plastic vials containing 10 ml of Insta-Gel (Packard Instrument Company). The vials were vortexed and left at room temperature overnight. The vials were then vortexed again and counted in a liquid scintillation spectrometer (Packard, Model 3385) at 45 % efficiency. All values are the means of at least two separate determinations.

In some experiments intended for Scatchard analysis (15) higher dilutions of tissue were used (16). In these cases the incubations were performed in 1.5 ml plastic tubes, which were subsequently centrifuged in a Sorval HS-4 rotor for 30 min at 8,000 x g.

Due to high blank values, the filtration technique (17) was unsatisfactory for the ergot alkaloids.

Saturable or specific ^{3}H-DHE binding, which was measured as the difference between the binding of ^{3}H-DHE in the presence and absence of 10 µM DHE, represented approximately 55 % of the total binding. In Scatchard analyses total binding was used instead of specific binding.

Subcellular fractionation studies were performed with minor modifications of the procedure of Gray and Whittaker (18): All subsequent operations were performed at 0°C. The brains of four rats were minced with a razor blade and then homogenized in 20 volumes of 0.32 M sucrose by using three strokes of a loose fitting Dounce homogenizer. The homogenate was centrifuged at 1000 x g for 10 min, yielding a crude "nuclear" pellet (P 1). The supernatant was centrifuged at 17,500 x g for 60 min, yielding a crude "mitochondrial" pellet P 2. The supernatant was centrifuged

at 100,000 x g for 60 min, leading to a crude "microsomal" pellet. P 2 was subfractionated by resuspension in 15 volumes of 0.32 M sucrose, layering on a discontinuous gradient of 0.8, 1.2 and 1.4 M sucrose, and centrifugation in a swing-out rotor at 64,200 x g for 90 min. The following bands were collected: A at the interface of 0.32 and 0.8 M sucrose, B between 0.8 and 1.2 M sucrose, and C between 1.2 and 1.4 M sucrose.

The fractions were diluted with equal volumes of water and spun down at 100,000 x g for 60 min. The pellets were resuspended in 20 volumes of distilled water and homogenized, using a Polytron PT-20 ST (setting 7) for 30 sec. The homogenates were diluted (P 1: 100 fold; P 2: 250 fold; P 3, A, B, C: 500 fold) with the same buffer and assayed as described before. "Wet weight" was determined by centrifuging the final homogenates at 100,000 x g for 60 min. and weighing the resulting pellet.

The sources of drugs were as follows: Fluka A.G.: serotonin, 5-methoxy-tryptamin, dopamine, l-norepinephrine, GABA, glycine, adenosine, atropin, d-tubocurarin, tyramine; ICN Pharmaceuticals: guanylyl imidodiphosphate; Sigma: l-isoproterenol; Winthrop: d-norepinephrine, d-isoproterenol; Roche: methiothepin; Organon: mianserine; Ayerst: butaclamol; Squibb: fluphenazine; Merck, Darmstadt: oxymetazolin; CIBA: xylometazolin, naphazolin, phentolamine; MSD: cyproheptadine; ICI: propranolol; Goedecke: thymoxamine; Boehringer Ingelheim: clonidine, synephrin; Burroughs Wellcome: methoxamin; Janssen: cinnarizin; UCB: piracetam. All other drugs were from SANDOZ.

RESULTS

Kinetic analysis of ^{3}H-DHE binding

At 37°C and various concentrations of ^{3}H-DHE binding reaches equilibrium by 30 min and remains constant up to 60 min (Fig. 2). At 22°C equilibrium is attained within 60 min. Binding in the presence of 10 µM DHE (unspecific binding) at 0°, 22° and 37°C is complete within a few seconds and is unchanged after 60 min.

Plotting the data of Fig. 2 according to the equation (19,20):

$$\frac{\ln 2}{t_{1/2}} = k_{on} \cdot [H] + k_{off}$$

yields a straight line (Fig.2,inset) with $k_{on} = 1.2 \times 10^6\ M^{-1}s^{-1}$ and $k_{off} = 0.001\ s^{-1}$ at 37°C. The kinetically derived K_D is 0.83×10^{-9} M. The rate of dissociation of bound ^{3}H-DHE, determined independently by displacing ^{3}H-DHE with a large excess (10 µM) of non-radioactive DHE, is shown in Fig. 3. When plotted semilogarithmically (Fig. 3, inset), the curve reveals the existence of more than one component. The half-life of the slow component is approximately 20 min and hence $k_{off} = 0.0058\ s^{-1}$, which is in reasonable agreement with the value obtained from the forward reaction.

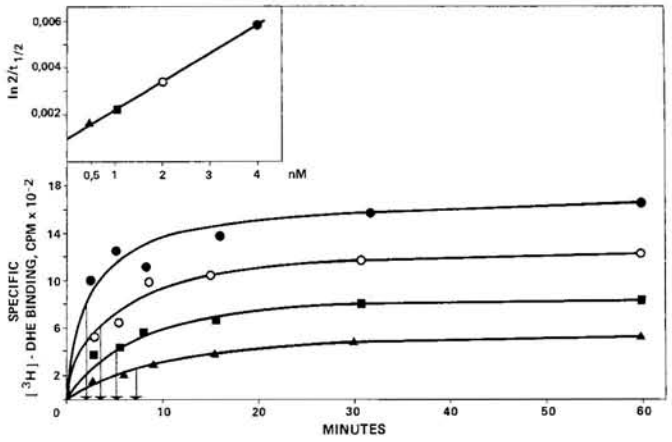

FIG. 2

Time course of association of ^{3}H-DHE binding at 37°. ^{3}H-DHE (▲—▲ 0.5 nM; ■—■ nM; o—o 2 nM; ●—● 4 nM) was incubated with rat brain membranes in the presence and absence of 10^{-5} M DHE as described in METHODS. Aliquots were cooled to 0° and centrifuged for 10 min at 48,000 x g. Inset: Plot of $\ln 2/t_{1/2}$ versus ^{3}H-DHE concentration. $t_{1/2}$ is the time at which specifically bound ^{3}H-DHE reaches one-half of equilibrium value.

Saturation of specific ^{3}H-DHE-binding

Specific ^{3}H-DHE binding is linear with tissue over the range of 0.1 to 1.6 mg/ml of protein. All experiments were conducted routinely within this range.

Saturability of specific ^{3}H-DHE binding at various tissue concentrations was determined by plotting specifically bound ^{3}H-DHE versus free ^{3}H-DHE (21). Using an incubation mixture, in which the pellet is diluted 1:500 as described under METHODS, half-maximal saturation of specific ^{3}H-DHE binding occurs at approximately 0.2 nM (Fig. 4, inset). Scatchard analysis of the same data reveals a curve with upward concavity (Fig. 4), which can be interpreted as negative cooperativity of a single class or the existence of multiple classes of binding sites (22). The dissociation constant K_D of the high affinity binding site is 0.23 nM and the binding capacity 127 fmol/mg protein. Using the usual tissue dilution of 1:60, which was applied in the displacement experiments, the K_D is shifted to 1.3 nM, a phenomenon, which has recently been discussed in detail (16).

Dependence of ^{3}H-DHE binding on pH and ions

Specific ^{3}H-DHE binding is maximal between pH 7.2 and 8.0 and

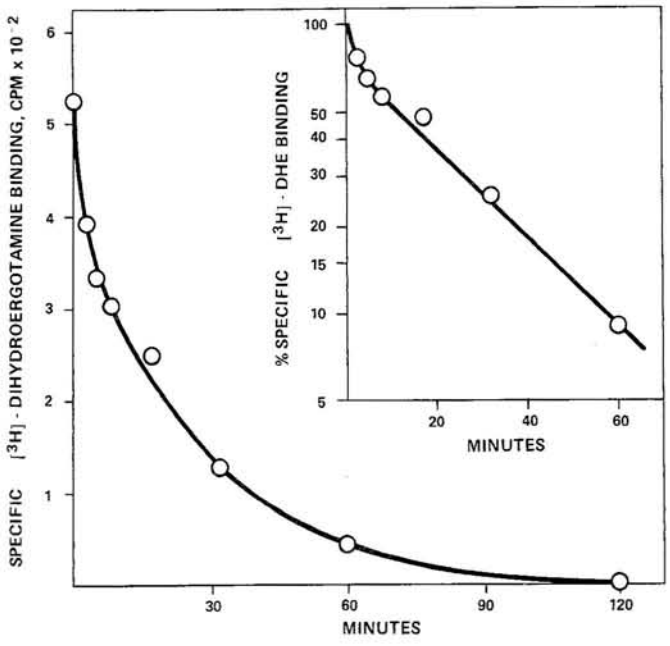

FIG. 3

Time course of dissociation of bound ^{3}H-DHE. Rat brain membranes were incubated with ^{3}H-DHE (nM) for 30 min at 37°. Non-radioactive DHE (20 µM final concentration) was added and ^{3}H-DHE binding was assayed at various times as described in METHODS. Inset: Semilogarithmic plot of same data.

declines steeply at more acidic or alkaline pH values. The thermal stability of the binding site was examined by prior incubation of the tissue at various temperatures and then conducting the binding assay at 37°. Specific binding was not influenced by prior incubation at 0 to 40°, but was drastically reduced at higher temperature. Specific binding was also affected by the addition of divalent cations. At 0.002 M $ZnCl_2$ specific binding was completely inhibited, at 0.005 M $CoCl_2$ it was 45% of the control. With Ca^{++} and Mg^{++} ions, this effect was less pronounced and monovalent cations as Na^{+}, K^{+} or Li^{+} influenced binding only at higher concentrations.

Identification of bound ^{3}H-DHE by thin-layer chromatography

^{3}H-DHE bound to rat brain membranes is unaltered as determined by thin-layer chromatography. 10 nM ^{3}H-DHE was incubated with rat brain membranes for 30 min at 37°C. The membranes were isola-

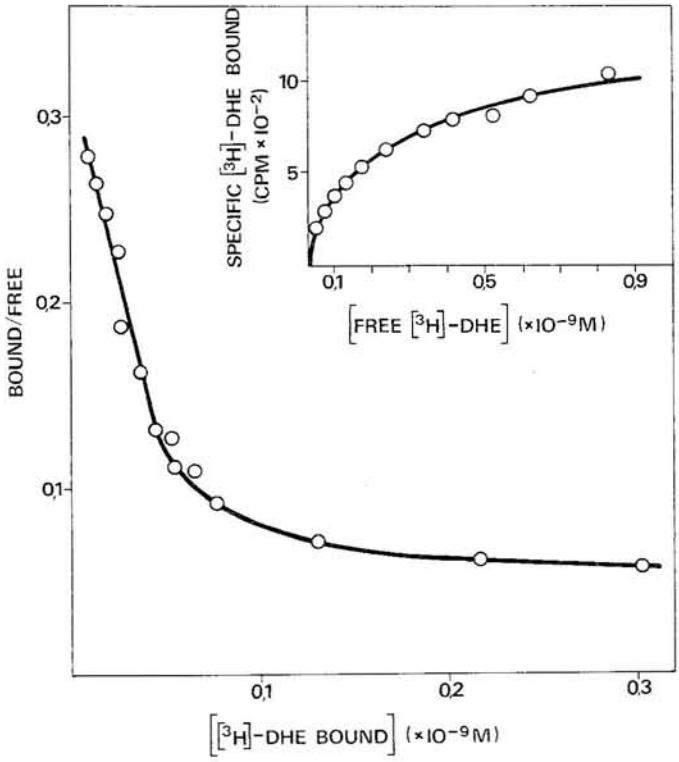

FIG. 4

Binding of ^{3}H-DHE to rat brain membranes as a function of ^{3}H-DHE concentration. Increasing concentrations of ^{3}H-DHE were incubated with rat brain membranes in the presence (non-specific) and absence (total) of 10 µM non-radioactive DHE and assayed as described in METHODS. Values of total ^{3}H-DHE binding are plotted according to Scatchard (15).
Inset: Saturation of specific ^{3}H-DHE binding.

ted by centrifugation and reincubated in the presence of 10 µM non-radioactive DHE. After centrifugation the supernatant was extracted twice with methylene chloride. The organic layer was washed with water and saturated sodium chloride solution. After evaporation of the solvent, the extract was analyzed on silica and alox sheets using the solvent systems chloroform-methanol (19:1 and 9:1) and benzene-methanol (8:2). In all three systems the eluted ^{3}H-DHE was indistinguishable from the original ^{3}H-DHE. In a separate experiment under identical conditions of incubation, the membranes obtained by centrifugation were reincubated in 0.05 M TRIS-HCl buffer, and centrifuged. The supernatant, whose concentration of ^{3}H-DHE was 3.8 nM as calculated from its radioactivity, was used for a displacement experiment under the usual conditions. The resulting curve was identical with that obtained

with fresh material. Both experiments suggest, that at least 99% of the ^{3}H-DHE reversibly bound to the membranes is unaltered.

Regional localization of specific ^{3}H-DHE binding

The regional distribution of specific ^{3}H-DHE binding (Tab.1) shows some similarity with the localization of ^{3}H-serotonin binding in rat brain (4). In both cases binding is highest in the hippocampus and the corpus striatum and lowest in the cerebellum.

TABLE 1

Regional Localization of Specific ^{3}H-DHE Binding in Rat Brain

Region	Specific binding	
	fmoles/mg wet weight (± s.e.m.)	fmoles/mg protein (± s.e.m.)
Cerebellum	15 ± 1.3	287 ± 28
Pons/Medulla	16 ± 1.4	407 ± 13
Midbrain	28 ± 2.3	612 ± 23
Thalamus/Hypothalamus	30 ± 1.3	603 ± 23
Striatum	32 ± 2.0	679 ± 16
Cortex	34 ± 2.5	606 ± 33
Hippocampus	34 ± 2.3	767 ± 43

Specific binding of ^{3}H-DHE (nM) to rat brain membranes from different regions was assayed as described in METHODS. Values are the means from five separate determinations s.e.m.

Electron microscopy of the various fractions showed that fraction A contained almost exclusively myelin, the "synpatosomal" fraction B included also mitochondria and other structures. Some of the latter might correspond to gliasomes as described by Henn et al. (23). Fraction C was enriched in mitochondria, P 3 in microsomes and ribosomes. P 1 contained a considerable amount of incompletely homogenized material. The ratio of specific versus unspecific binding, as indicated in Table 2, reflects the concentration of specific ^{3}H-DHE binding sites and might be related to the protein-lipid composition of the fraction.

Displacement of bound ^{3}H-DHE

(-)-Dihydroergotamine (DHE) inhibits ^{3}H-DHE binding at 4 nM, while the pharmacologically (24) inactive (+)-dihydroergotamine (antipode of DHE) is more than 2000 times less potent in displacing specifically bound ^{3}H-DHE (Table 3). Ergotamine, 1-methylergotamine, dihydroergotoxine and the other dihydroergot derivatives have comparable affinities for the DHE binding sites.

Subcellular distribution of ^{3}H-DHE binding

The highest concentration of specific ^{3}H-DHE binding sites (per mg wet weight) was found in fraction B (Table 2), in which usually membrane fragments and synaptosomes are enriched (18).

TABLE 2

Subcellular Distribution of Specific ^{3}H-DHE Binding in Rat Brain

Subcellular fractions	Specific binding		Spec.binding / Unspec.binding
	fmoles/mg wet weight (± s.e.m.)	fmoles/mg protein (± s.e.m.)	
Whole homogenate	28 ± 1.6	439 ± 59	1.15
P 1: crude "nuclear"	25 ± 3.1	480 ± 34	1.22
P 2: crude "mitochondrial"	28 ± 2.0	529 ± 42	1.15
A: "myelin"	13 ± 1.1	344 ± 62	0.79
B: "synaptosomal"	42 ± 4.3	586 ± 68	1.22
C: "mitochondrial"	28 ± 3.6	282 ± 52	0.73
P 3: crude "microsomal"	23 ± 4.0	447 ± 71	1.41

Subcellular fractions were prepared and assayed with ^{3}H-DHE (nM) as described in METHODS. Values are the means from four separate determinations ± s.e.m. Spec. binding/unspec. binding means the ratio of reversibly versus irreversibly bound ^{3}H-DHE.

2-Bromo-α-ergokryptine (CB 154), a prolactin inhibitor and dopaminergic drug (9), lysergolacetate and d-LSD are 1 to 3 % as potent as DHE. Methergin and the classical 5-HT antagonists methysergide (27), pizotifen (25) and cyproheptadine (26) are weak inhibitors of specific ^{3}H-DHE binding. Methiothepin, which has been characterized as a central 5-HT antagonist (27), and the imidazoline derivatives oxymetazolin and xylometazolin, known as sympathomimetics (28,29), are 25 to 60 times less potent than DHE. The neuroleptic drugs clozapine, thioridazine, butaclamol, fluphenazine and chlorpromazine are almost 1000 times less effective at the DHE binding sites. Of all the neurotransmitter candidates tested only serotonin displays significant affinity for the DHE binding sites with 50 % inhibition at about 8 µM. The displacement curve of serotonin (Fig. 5) reveals two components, half-maximal inhibition of the high-affinity component occurring at about 30 nM. The various adrenergic agonists and antagonists tested had hardly any potency in displacing ^{3}H-DHE. Vincamin, cinnarizin, and piracetam were equally ineffective.

TABLE 3

Inhibition of Specific ^{3}H-DHE Binding to Rat Brain Membranes

Drug	IC_{50} (nM)	Drug	IC_{50} (nM)
(-)-DHE	4	Clozapine	2,000
(+)-DHE	>10,000	Thioridazine	2,000
Ergotamine	5	(+)-Butaclamol	2,000
13-Brom-DHE	6	Fluphenazine	3,000
Dihydroergonine	9	Mianserine	4,000
Dihydro-β-ergosine	10	Chlorpromazine	4,000
Dihydroergotoxine	12	Phentolamine	4,200
1-Methyl-ergotamine	12	Apomorphine	4,500
2-Bromo-α-ergokryptine	135	Naphazolin	5,000
Lysergolacetate	350	Pizotifen	6,000
d-LSD	460	Cyproheptadine	7,000
Methylergometrine	1,000	Serotonin	8,000
Methysergide	5,000	Pindolol	>10,000
		Propranolol	>10,000
Oxymetazolin	100	5-Methoxy-tryptamine	>10,000
Xylometazolin	160	Thymoxamine	>10,000
Methiothepin	280	Clonidine	>10,000

Dopamine, epinephrine, l-norepinephrine, d-norepinephrine, synephrin, methoxamin, l-isoproterenol, d-isoproterenol, GABA, glycine, guanylyl imidodiphosphate, tyramine, adenosine, atropine, d-tubocurarine, indometacin, vincamin, cinnarizin, piracetam: less than 50 % displacement at 10^{-4} M.

IC_{50} values shown are the concentration of each drug required to inhibit specific binding of nM ^{3}H-DHE to rat brain membranes by 50%. Non-specific binding occurring in the presence of 10 μM unlabeled DHE was substracted from each of the respective determinations. IC_{50} values were determined by log probit analysis, utilizing at least five concentrations of each drug. Values are the means of at least three determinations.

DISCUSSION

Our results demonstrate that ^{3}H-DHE binds saturably, reversibly and with high affinity (K_D = 0.23 nM) to rat brain membranes. ^{3}H-DHE binding is stereo- and ligand-specific. The unnatural antipode of DHE is more than 2000 times less potent than DHE in inhibiting specific ^{3}H-DHE binding. The rates of association and dissociation are temperature dependent. The regional and subcellular distribution of ^{3}H-DHE binding resembles the distribution of ^{3}H-serotonin binding reported by Bennett and Synder (4). Highest concentrations of binding sites are found in the hippocampus and the corpus striatum. On the cellular level specific ^{3}H-DHE binding is enriched in the "synaptosomal" fraction. Hence the ^{3}H-DHE binding site could be related to a synaptic receptor. Of all the neurotransmitters tested as inhibitors of speci-

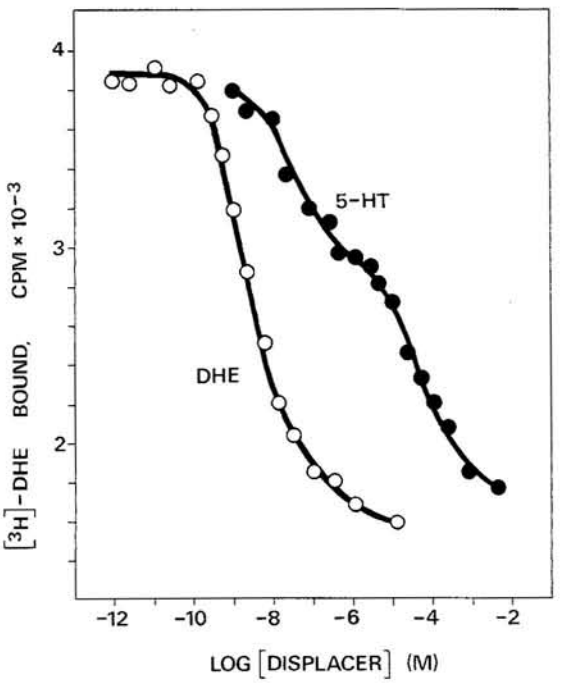

FIG. 5

Displacement of ^{3}H-DHE by unlabeled DHE and 5-HT. ^{3}H-DHE (nM) was incubated with rat brain membranes (about 0.2 mg/ml protein) in the presence of increasing concentrations of unlabeled DHE and 5-HT and assayed as described in METHODS. The data represent total ^{3}H-DHE binding. The experiment was replicated five times.

fic ^{3}H-DHE binding, only serotonin was able to displace ^{3}H-DHE. Furthermore, DHE is a potent inhibitor of ^{3}H-serotonin binding to rat brain membranes (A.Closse and D.Hauser, unpublished). Not only epinephrine, norepinephrine and dopamine, but a variety of adrenergic and dopaminergic agonists and antagonists are ineffective or only weak inhibitors at ^{3}H-DHE binding sites. The same holds for methysergide, pizotifen and cyproheptadine, which are potent serotonin antagonists at smooth muscles. This is in contrast to ^{3}H-LSD binding (4), which at 3 nM is fairly well inhibited by methysergide and cyproheptadine. Methiothepin, a central serotonin antagonist (27), is both a potent inhibitor of ^{3}H-LSD (4) and ^{3}H-DHE binding.

It is tempting to assume that ^{3}H-DHE is interacting at least in part with sites related to the putative (4) postsynaptic serotonin receptor. But the ligand specificity is not so clear as to exclude other sites being labeled by ^{3}H-DHE.

The findings of our study differ from the results with ^{3}H-dihydroergokryptine recently published by Williams and Lefkowitz (6). In their rabbit uterine membrane preparation the equilibrium of ^{3}H-dihydroergokryptine binding is reached within 12 minutes at 25°C, whereas with rat brain membranes ^{3}H-dihydroergotamine binding equilibrates after approximately 20 minutes at 37°C. Furthermore, the binding sites of ^{3}H-dihydroergokryptine in peripheral tissue were characterized as α-adrenergic, whereas the binding sites of ^{3}H-dihydroergotamine in the brain have no or only weak affinity for adrenergic compounds.

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