Biochemical Pharmacology, Vol. 33, No. 17, pp. 2789–2797, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

NOVEL SEROTONIN RECEPTORS IN *FASCIOLA*

CHARACTERIZATION BY STUDIES ON ADENYLATE CYCLASE ACTIVATION AND [^{3}H]LSD BINDING*

STEVEN J. MCNALL and TAG E. MANSOUR†
Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305, U.S.A.

(*Received* 1 *October* 1983; *accepted* 15 *February* 1984)

Abstract—Serotonin (5-HT) receptors coupled to adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] in the liver fluke *Fasciola hepatica* have been characterized by adenylate cyclase activation studies and by direct binding studies using [^{3}H]-*d*-lysergic acid diethylamide ([^{3}H]LSD) as a radioligand. Inhibition of 5-HT stimulation of adenylate cyclase by a series of 5-HT antagonists revealed a potency order of LSD = 2-bromo-LSD > methiothepin > metergoline = cyproheptadine > methysergide > spiroperidol. [^{3}H]LSD binding to a cell-free fluke particle preparation was rapid, stereospecific, and proportional to protein concentration. Scatchard analysis indicated multiple binding sites which, when resolved into two components, gave for the high affinity site an apparent dissociation constant of 25 nM and a receptor concentration of 160 fmoles/mg protein. The ability of a series of compounds to compete for [^{3}H]LSD binding sites correlated closely with their ability to inhibit 5-HT stimulation of adenylate cyclase. [^{3}H]LSD binding sites were most concentrated in the anterior region of the fluke which was consistent with the higher levels of 5-HT activated adenylate cyclase found in this region. GTP and 5′-guanylyl imidophosphate, a poorly hydrolyzable GTP analog, decreased the affinity of the agonist 5-HT for the binding sites but had little effect on the affinity of the antagonist 2-bromo-LSD. Calcium at concentrations above 300 μM significantly reduced both [^{3}H]LSD binding and 5-HT activation of adenylate cyclase. The results indicate that [^{3}H]LSD can be used to label the 5-HT receptors coupled to adenylate cyclase activity. The pharmacological specificity and other characteristics of the fluke receptors appear to differ from the properties of reported mammalian 5-HT receptors. As a result, serotonin receptors in the flukes represent sites that may be amenable to selective manipulation by new chemotherapeutic agents useful in the treatment of these parasite infections.

Previous studies in our laboratory described a serotonin (5-HT‡) sensitive adenylate cyclase in the liver fluke, *Fasciola hepatica* [1, 2]. This system appears to have a physiological role in the regulation of carbohydrate metabolism and motility in trematodes [3–5]. Activation of adenylate cyclase by 5-HT occurs in both intact flukes and cell-free particles. Further study of this system indicated that there is a single class of adenylate cyclase in the fluke, which is regulated by only one type of 5-HT receptor [6]. Thus, the fluke offers an excellent model system for the study of 5-HT receptors coupled to adenylate cyclase activity.

Activation of adenylate cyclase by 5-HT has been shown to occur in the mammalian brain [7, 8]. In addition, 5-HT receptors in the mammalian brain have been identified by direct radioligand binding studies [9–12]. These receptors, however, were not shown to be coupled to adenylate cyclase activity, and their physiological function remains unclear. The different pharmacological profiles of these receptors suggest the existence of several types of 5-HT receptors. Characterization of a 5-HT receptor coupled to adenylate cyclase may provide information which will be useful in differentiating between these multiple receptor types. Drummond *et al.* [13] reported on the properties of a 5-HT receptor coupled to adenylate cyclase activity in the snail *Helix pomatia*. It would be of interest to compare this receptor with other adenylate cyclase coupled 5-HT receptors to determine what similarities exist between them.

Northup and Mansour [6] reported that *d*-lysergic acid diethylamide (LSD) had a high affinity for the fluke 5-HT receptors that are coupled to adenylate cyclase (K_d = 40 nM). This suggested that [^{3}H]LSD might be useful in the characterization of these receptors. In this report we examine the abilities of several serotonin antagonists to inhibit serotonin activation of adenylate cyclase. In addition, we show that [^{3}H]LSD specifically binds to fluke particles and appears to label the adenylate cyclase coupled 5-HT receptors. Our results indicate that the

* This investigation was supported by U.S. Public Health Service Grants MH 23464 and AI 16501. These studies constitute a part of the work submitted to the Graduate School, Stanford University, by S. J. M. in partial fulfillment of the requirement for the degree of Doctor of Philosophy. S. J. M. was a recipient of a predoctoral trainee fellowship, U.S. Public Health Service, Training Grant GM 07149.

† To whom reprint requests should be sent.

‡ Abbreviations: 5-HT: serotonin, 5-hydroxytryptamine; LSD: *d*-lysergic acid diethylamide; Gpp(NH)p: 5′-guanylyl imidophosphate; K_d: dissociation constant; IC_{50}: concentration which inhibits 5-HT stimulation of adenylate cyclase or [^{3}H]LSD binding by 50%; PPO: 2,5-diphenylopazole; POPOP: 1,4-bis-[2-(4-methyl-5-phenyl-oxazolyl)]benzene; and EGTA, ethyleneglycolbis(amino-ethylether) tetra acetate.

fluke receptors are pharmacologically distinct from the mammalian 5-HT receptors that have been characterized. Finally, studies of the possible regulation of the receptor site by nucleotides and ions are presented. A preliminary note of some of this investigation has been reported [14].

METHODS AND MATERIALS

Liver fluke particle and membrane preparation. Fresh liver flukes were obtained from bovine liver at a local slaughterhouse and kept overnight in fluke saline media containing antibiotics and dextrose as previously described [2]. In some experiments, fluke "heads" were prepared by cutting the oral end of the animal just below the posterior sucker. The remainder of the fluke was taken as the "tail". Whole flukes or fluke parts were then washed in medium, blotted dry on filter paper, and their wet weight determined. Three volumes (w/v) of homogenization buffer (0.25 M sucrose, 5 mM dithiothreitol, and 1 mM EDTA) were added, and the flukes were homogenized either in a motor-driven Teflon pestle tissue grinder or by brief exposure to a "Tekmar Tissumizer". The homogenized suspension was spun at 10,000 *g* for 15 min, and the pellet was resuspended in homogenization buffer. The suspension was incubated at 37° for 10 min and then respun at 10,000 *g* for 15 min. The final pellet was suspended in homogenization buffer at a concentration of 5–15 mg/ml and used directly or stored at −70° until just prior to use.

"Enriched" membrane fractions were prepared by a modification of the procedure described by Neville [15]. The final fluke particle pellet was suspended in a small volume of buffer contain 0.25 M sucrose, 25 mM glycylglycine, pH. 7.5, 5 mM dithiothreitol, 5 mM $MgCl_2$, and 2 mM EDTA. The suspension was rehomogenized by several passes in a Teflon pestle tissue grinder, and the homogenate was brought up to 53% sucrose by the addition of a solution of 69% sucrose and 5 mM dithiothreitol. Eighteen milliliters of this solution was placed in tubes to fit the Beckman SW 27 rotor, and 20 ml of a solution of 42.3% sucrose and 5 mM dithiothreitol was carefully layered on top. The rotor was spun at 25,000 rpm for 2 hr in a Beckman L5–50 ultracentrifuge. Material from the interface (enriched membrane fraction) was collected, diluted 3-fold with homogenization buffer, and spun at 10,000 *g* for 15 min. The final pellet was suspended in homogenization buffer at a final concentration of 5–15 mg protein/ml and used directly or stored in liquid nitrogen until just prior to use.

Adenylate cyclase assay. Adenylate cyclase activity was measured by the addition of fluke particles or "enriched" membranes to a reaction mixture containing (final concentrations) 0.1 M sucrose, 20 μM EGTA, 50 mM glycylglycine, pH 7.5, 5 mM phosphocreatine, 2 mM $MgCl_2$, 0.1 mM sodium-ATP with 0.75 to 1.5 μCi [α-^{32}P]ATP per 0.25 ml assay, 5 units creatine phosphokinase, 0.5 mM 3-isobutyl-l-methylxanthine, and other substances as indicated in a final volume of 0.25 ml. The samples were incubated for 10 min at 37° unless otherwise indicated and the reaction was terminated by the addition of a stop solution containing (final concentrations) 2% sodium dodecyl sulfate, pH 7.4, 10 mM EDTA, 10 mM sodium-ATP, and 1 mM cAMP. Ten microliters of a [^{3}H]cAMP standard (about 5000 cpm) was then added to each sample. The cyclic nucleotide content of each sample was determined by column chromatography as described by Salomon *et al.* [16]. The samples were first passed through a Dowex 50-Wx4 column which had been regenerated using 1.0 M HCl. This was followed by the passage of the samples through a neutralized alumina column equilibrated with 0.1 M imidazole, pH 7.4. Insta-gel (Packard) was added to the samples and ^{3}H and ^{32}P activities were determined in a Tricarb scintillation spectrometer. Under these conditions, adenylate cyclase activity was linear with time for up to 10 min, and [^{3}H]cAMP recovery was between 50 and 60%. All values given are the average of duplicate samples from which a zero assay time blank value was subtracted.

[^{3}*H*]*LSD binding assay.* Fresh or just thawed fluke particles or "enriched" membrane fractions were diluted 5-fold in binding buffer containing (final concentrations) 0.5 M sucrose, 0.1 M glycyglycine, pH 7.5, 10 mM dithiothreitol, 5 mM $MgCl_2$ and 2 mM EDTA. This suspension was spun at 10,000 *g* for 15 min and the pellet was resuspended to a desired volume in the same buffer. The final incubation mixture unless otherwise stated consisted of fluke particles (1–3 mg protein/ml), 0.25 M sucrose, 50 mM glycylglycine, pH 7.5, 5 mM dithiothreitol, 2.5 mM $MgCl_2$, 1 mM EDTA, 10 nM [^{3}H]LSD (15–60 Ci/mmole), and other agents as indicated in a final volume of 150 μl. LSD was found to be light sensitive [17], so to avoid decomposition assay tubes were kept in the dark as much as possible throughout the entire procedure. Samples were incubated in a shaking water bath for 5 min at 37° unless otherwise indicated. After incubation, the tubes were removed and 4 ml of cold wash buffer (10 mM glycylglycine, pH 7.5) was added. The mixture was quickly passed through a glass fiber filter (Whatman GF/C) under vacuum. Each tube and filter was then washed three times with 4 ml of the same wash buffer. The filter was placed in a scintillation vial to dry, and 10 ml of scintillation fluid (toluene, PPO, POPOP) was added. ^{3}H-Activity was determined on a Tricarb liquid scintillation spectrometer. In some experiments, the final incubation volume was 0.5 ml. After incubation of these samples, three 140–150 μl aliquots were removed and added to 4 ml of cold wash buffer. The remainder of the procedure was as described above. Specific [^{3}H]LSD binding was determined by subtracting from the total [^{3}H]LSD binding of the test sample the amount of binding from an identical sample containing an excess of unlabeled LSD (100 μM) or 5-HT (1 mM) as indicated. Values given are the average of triplicate samples.

Thin-layer chromatography of LSD and [^{3}H]LSD was done on silica gel with a fluorescent indicator (Eastman Chromogram) in a solvent system of chloroform–methanol–acetic acid (40:30:30). Identification of the compounds was determined by u.v. irradiation. ^{3}H-Activity of the gel was determined by liquid scintillation counting of the gel slices.

Protein concentrations were determined by the

method of Bradford [18] or Lowry *et al.* [19], using bovine serum albumin as a standard.

Materials. *d*-Lysergic acid diethylamide, *l*-lysergic acid diethylamide, and 2-bromo-D-lysergic acid diethylamide were products of Sandoz, obtained through the National Institute of Mental Health. [^{3}H]Adenosine 3′,5′-cyclic monophosphate (37 Ci/mmole), and [α-^{32}P]ATP (400 Ci/mmole) were purchased from Amersham. [^{3}H]LSD (15–60 Ci/mmole) was a product of Amersham or New England Nuclear. ATP was from Boehringer-Mannheim. 5′-Guanylyl imidophosphate was from ICN. Adenosine 3′,5′-cyclic monophosphate, rabbit skeletal muscle creatine phosphokinase, and 5-hydroxytryptamine were obtained from Sigma. Methiothepin, metergoline, mianserin, cinanserin, cyproheptadine, methysergide, haloperidol, ketanserin, and spiroperidol were provided by Dr. Roland Ciaranello, Stanford. All other materials were of reagent grade and were obtained from various sources.

RESULTS

Adenylate cyclase studies. As we previously reported [6], 5-HT markedly stimulated adenylate cyclase activity in the fluke particle preparation. In a typical experiment a saturating concentration of 5-HT (100 μM) activated adenylate cyclase over 100 times above the basal level, to a final value of about 1500 pmoles cAMP per min per mg protein. The ability of compounds to compete for these 5-HT receptor sites was determined by measuring their ability to inhibit 5-HT activation of adenylate cyclase. The inhibition curves for several of these compounds are shown in Fig. 1. The concentrations needed to inhibit 5-HT activation by 50% (IC_{50}) for all the compounds tested are summarized in Table 1. LSD, methysergide and metergoline at concentrations up to 100 μM were capable of partially activating adenylate cyclase. Respectively, they activated adenylate cyclase to the extent of 15, 10 and 2% of the level obtained with 100 μM 5-HT. Only LSD and 2-bromo-LSD had very high affinity for the receptor site. The other LSD derivatives

Table 1. IC_{50} Values for inhibition of 5-HT-stimulated adenylate cyclase by different compounds*

Compound	IC_{50} (μM)
d-Lysergic acid diethylamide	0.08
l-Lysergic acid diethylamide	>10
2-Bromo-lysergic acid diethylamide	0.05†
Methysergide	9.0
Metergoline	3.7
Methiothepin	1.1
Cinanserin	3.0
Cyproheptadine	5.2
Mianserin	12
Haloperidol	>100
Spiroperidol	>100
Ketanserin	45

* Inhibition of 5-HT stimulation of adenylate cyclase was determined by assaying for adenylate cyclase activity in fluke particles as described in Methods and Materials for 10 min at 37° in the presence of 1 μM 5-HT and different concentrations (minimum of six) of the indicated compounds. IC_{50} Values were determined graphically on semilog paper. The values given are the mean of two or more experiments, each done in duplicate.

† IC_{50} Values were determined previously in liver fluke particles [6].

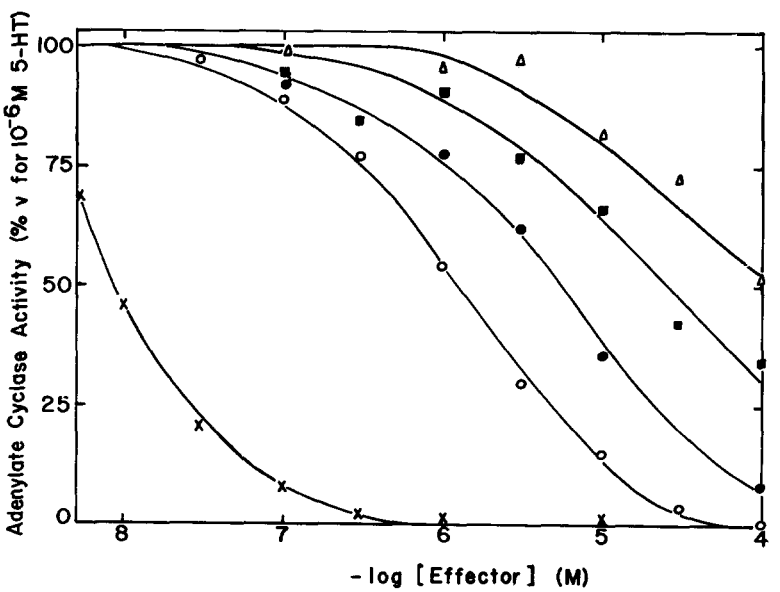

Fig. 1. Inhibition of 5-HT stimulation of adenylate cyclase by different effectors. Adenylate cyclase activity was determined in liver fluke particles as described in Methods and Materials after incubation for 10 min at 37° in the presence of 1 μM 5-HT and the indicated concentrations of 2-bromo-LSD (×), methiothepin (○), cyproheptadine (●), ketanserin (■), or spiroperidol (△). The data shown represent mean values of duplicate samples from a single representative experiment for each compound.

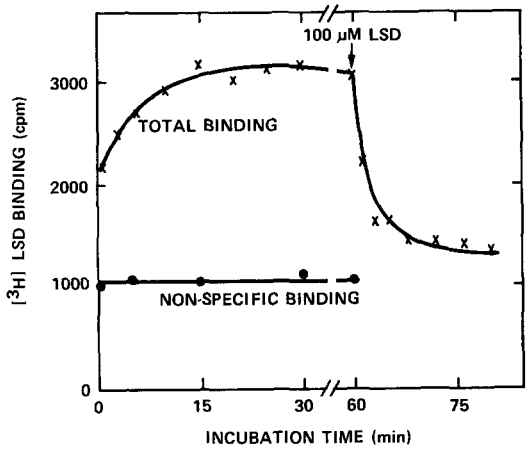

Fig. 2. Time course of [^{3}H]LSD binding to liver fluke particles at 0°. Fluke particles were incubated with 10 nM [^{3}H]LSD at 0° with no additions (×) or with 100 μM unlabeled LSD (●). At the times indicated, [^{3}H]LSD binding was determined as described in Methods and Materials. After 60 min (arrow) 100 μM unlabeled LSD was added to the sample, and binding was determined at the times indicated. The data shown are the means of triplicate assays in a single representative experiment.

metergoline and methysergide had significantly lower affinity. Classical 5-HT antagonists like cyproheptadine, cinanserin, and mianserin also had relatively low affinity for the fluke 5-HT receptor site as did spiroperidol and ketanserin. The order of potency of these compounds for the fluke receptor strongly suggests that these receptors may differ significantly from the 5-HT receptors previously described.

[^{3}H]LSD binding studies. To further characterize the fluke 5-HT receptor, a radioligand binding assay was developed. [^{3}H]LSD was used as a radioligand in these studies because of its previously reported high affinity for the receptor. The concentration of [^{3}H]LSD used was 10 nM. This low concentration was used to ensure that the binding would be primarily to high affinity sites. The amount of [^{3}H]LSD that specifically bound was linearly proportional to the protein concentration in the range of 0 to 2.5 mg protein. All binding experiments were done within this protein range. [^{3}H]LSD binding measured against time at 0° gave the result shown in Fig. 2. Equilibrium was reached within 20 min and the binding was rapidly dissociated upon the addition of 100 μM unlabeled LSD. Displacement of [^{3}H]LSD binding by unlabeled LSD is shown in Fig. 3. Scatchard analysis of the data indicated that multiple binding sites were present. Resolution of the data into a two-site model gave, for the high affinity site, an apparent dissociation constant (K_d) value of 25 nM and a receptor concentration of 160 fmoles/mg protein.

To directly compare the properties of the 5-HT receptor determined from the adenylate cyclase studies with the binding results required that the binding studies be done at 37°. It was found that temperature had a marked effect on [^{3}H]LSD binding. Figure 4 shows that, unlike at 0° where binding required 20 min to reach a maximum, [^{3}H]LSD binding at 37° was very rapid, reached a maximum within 2 min, and then steadily decreased for up to 30 additional min. We believe that this time-dependent decrease in binding is a receptor-specific phenomena representing a desensitization process which is further characterized and discussed in the accompanying paper [20]. Of importance here is that the maximum numbers of binding sites measured at 0° and at 37° were similar. Furthermore, Fig. 5 shows that when [^{3}H]LSD binding was measured after 5 min of incubation at 37°, at which time binding was near its maximum level, the resulting displacement curve and Scatchard analysis determined in the presence of unlabeled LSD were similar to those obtained at 0° under equilibrium conditions. The ability of several different compounds to compete for [^{3}H]LSD bind-

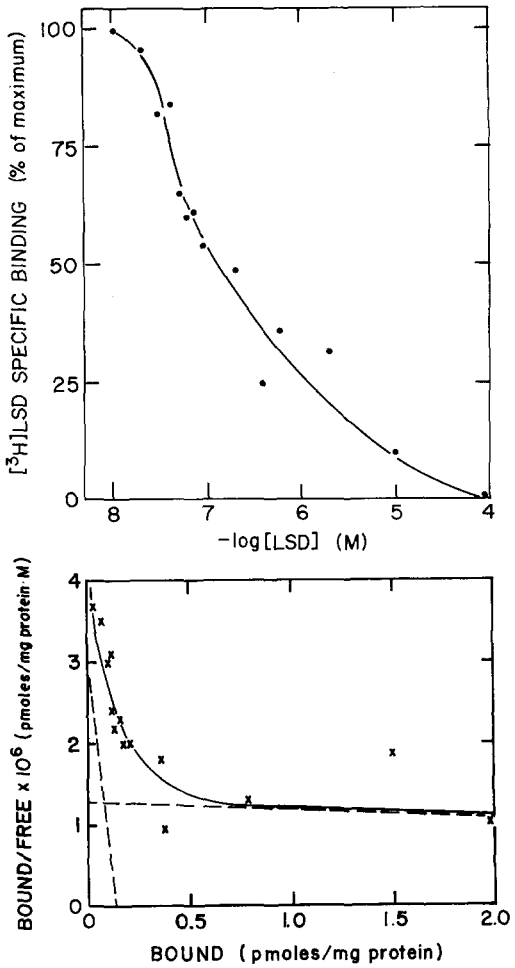

Fig. 3. [^{3}H]LSD binding to liver fluke particles at 0°. Liver fluke particles were incubated with 10 nM [^{3}H]LSD and the indicated concentrations of unlabeled LSD for 20 min at 0°. Specific [^{3}H]LSD binding was then determined as described in Methods and Materials. The data shown are the means of triplicate assays in a single representative experiment. Top panel: Displacement curve of [^{3}H]LSD binding by unlabeled LSD. Bottom panel: Scatchard plot of the data. Computer fit curves for a two-site model (dashed lines) gave for the high affinity site: dissociation constant, K_d = 25 nM; receptor concentration = 160 fmoles/mg protein.

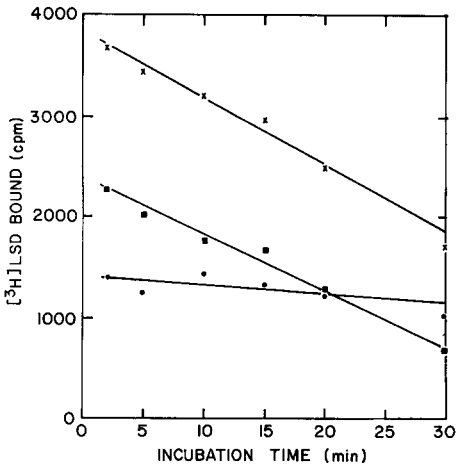

Fig. 4. Time course of [^{3}H]LSD binding to liver fluke particles at 37°. Fluke particles were incubated with 10 nM [^{3}H]LSD at 37° with no additions (×) or with 100 μM unlabeled LSD (●). At the times indicated, [^{3}H]LSD binding was determined as described in Methods and Materials. Specific binding (■) was determined by taking the difference in the amount of [^{3}H]LSD bound in the samples incubated with and without unlabeled LSD. The data shown are the means of triplicate assays in a single representative experiment.

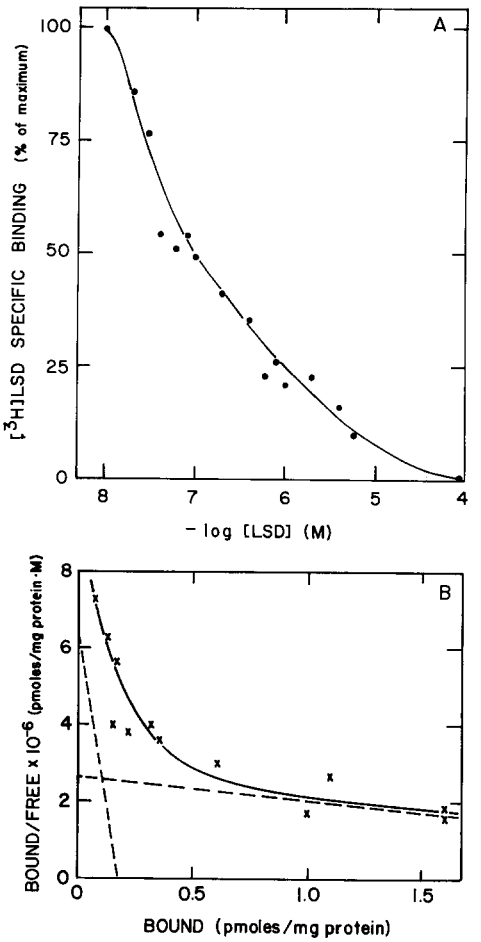

Fig. 5. [^{3}H]LSD binding to liver fluke particles at 37°. Liver fluke particles were incubated with 10 nM [^{3}H]LSD and the indicated concentrations of unlabeled LSD for 5 min at 37°. Specific [^{3}H]LSD binding was then determined as described in Methods and Materials. The data shown are the means of triplicate assays in a single representative experiment. (A) Displacement curve of [^{3}H]LSD binding by unlabeled LSD. (B) Scatchard plot of the data. Computer fit curves for a two-site model (dashed lines) gave for the high affinity site: dissociation constant, K_d = 51 nM; receptor concentration = 140 fmoles/mg protein

ing sites at 37° is shown in Fig. 6, and the results from all the compounds tested are summarized in Table 2. Comparison of Fig. 1 and 6 and Tables 1 and 2 shows a strong correlation between the ability of the compounds tested to inhibit 5-HT activation of adenylate cyclase and their ability to compete for [^{3}H]LSD binding sites. In both cases, a potency order of LSD = 2-bromo-LSD > methiothepin > classical smooth muscle 5-HT antagonists > 5-HT$_2$ antagonists spiroperidol and ketanserin. From these studies we conclude that the binding at 37° by [^{3}H]-LSD is representative of the properties of the 5-HT receptor coupled to adenylate cyclase. In addition, the correlation of the binding potency order with the adenylate cyclase studies emphasizes the significant differences between the affinity of these compounds for the fluke receptors and their affinity for other reported 5-HT receptors.

Distribution of [^{3}H]LSD binding sites and adenylate cyclase activity in the liver fluke. Previous studies [2] showed that adenylate cyclase from the anterior region ("head") of the liver fluke was activated to a greater extent in response to 5-HT stimulation than the cyclase from the posterior end ("tail"). Experiments were designed to determine if this increased response was due in part to a higher concentration of 5-HT receptors being present in the head than in the tail of the fluke. Cell-free particles from liver fluke heads and tails were prepared, and both [^{3}H]LSD binding and adenylate cyclase activity were measured from the same samples. Table 3 shows the results of three experiments using three different particle preparations. In each case it was verified that 5-HT activation of adenylate cyclase was higher in the particles prepared from fluke heads. The number of [^{3}H]LSD binding sites was also greater in the fluke heads than in the tails. However, the relative differences between receptor concentration and adenylate cyclase activation varied in the three experiments, which made it difficult to relate the higher concentration of [^{3}H]LSD binding sites directly to increased adenylate cyclase activity. Sodium fluoride activation of adenylate cyclase was also greater in the heads than in the tails. Thus, in addition to having a higher receptor concentration, fluke heads also had more enzyme capable of being activated.

Effect of guanine nucleotides on [^{3}H]LSD binding to fluke particles. Our previous observation that GTP was required for 5-HT activation of adenylate cyclase [21], along with several reports [22–24] indicating that GTP can modify agonist binding to adenylate cyclase receptors, prompted us to examine the effects

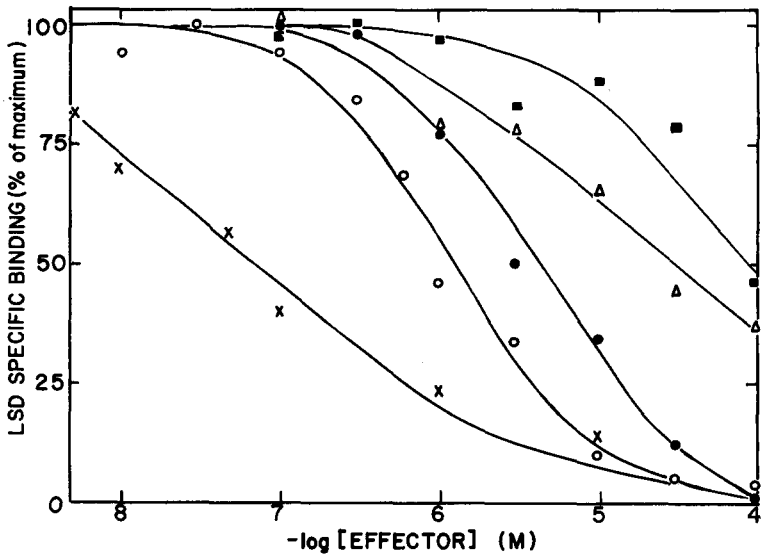

Fig. 6. Inhibition of [^{3}H]LSD binding to fluke particles at 37° by different effectors. Liver fluke particles were incubated with 10 nM [^{3}H]LSD and the indicated concentrations of 2-bromo-LSD (×), methiothepin (○), cyproheptadine (●), spiroperidol (△), or ketanserin (■) for 5 min at 37°. Specific [^{3}H]LSD binding was then determined as described in Methods and Materials. The data shown represent mean values of triplicate samples from a single representative experiment for each compound.

of guanine nucleotides on [^{3}H]LSD binding in the fluke. GTP, at concentrations up to 100 μM, had little effect on the displacement of [^{3}H]LSD by unlabeled LSD. It also had no effect on the displacement of [^{3}H]LSD binding by the antagonist 2-bromo-LSD. However, as shown in Fig. 7, GTP markedly inhibited the displacement of [^{3}H]LSD binding by the agonist 5-HT. Similar effects were seen when Gpp(NH)p was substituted for GTP. The addition of 100 μM Gpp(NH)p resulted in about a ten-fold reduction in the apparent affinity of 5-HT for the [^{3}H]LSD binding sites. The affinity of LSD for the binding sites was also reduced in the presence of Gpp(NH)p although not to the extent seen with

Table 2. IC_{50} Values for inhibition of [^{3}H]LSD binding to fluke particles by different compounds*

Compound	IC_{50} (μM)
d-Lysergic acid diethylamide	0.09
l-Lysergic acid diethylamide	12
2-Bromo-lysergic acid diethylamide	0.09
Methysergide	9.3
Metergoline	2.1
Methiothepin	1.3
Cinanserin	1.5
Cyproheptadine	3.8
Serotonin (5-HT)	4.0
Mianserin	5.0
Levorphanol	6.0
Haloperidol	12
Spiroperidol	26
Ketanserin	>100
Epinephrine	>500
Dopamine	>500

* To measure inhibition of [^{3}H]LSD binding, fluke particles were incubated with 10 nM [^{3}H]LSD and different concentrations (minimum of five) of the indicated compounds for 5 min at 37°. Following incubation, specific [^{3}H]-LSD binding was determined as described in Methods and Materials. IC_{50} Values were determined by graphical analysis on semilog paper. The values given are the mean of at least two experiments, each done in triplicate.

Table 3. [^{3}H]LSD binding and adenylate cyclase activity in particles from different sites in the liver fluke*

	[^{3}H]LSD binding (fmoles LSD bound/mg protein)	Adenylate cyclase activity (pmoles cAMP/min · mg protein)		
		Basal	5-HT + GTP	NaF
Experiment I:				
Fluke heads	59	8	726	2870
Fluke tails	18	2	210	1575
Experiment II:				
Fluke heads	121	7	715	2857
Fluke tails	79	1	165	1438
Experiment III:				
Fluke heads	139	11	1146	2508
Fluke tails	39	2	364	1583

* Specific [^{3}H]LSD binding using 10^{-8} M [^{3}H]LSD was determined as described under Methods and Materials after incubation for 2, 5, and 10 min at 30°. The values given are the maximum number of specific binding sites determined and are the average of triplicate samples. The same particle preparations were used to measure adenylate cyclase activity. Samples were assayed for 5 or 10 min at 30°, and adenylate cyclase activity was determined as described in Methods and Materials. The concentrations of the effectors tested were 100 μM for 5-HT and GTP and 10 mM for NaF. Values given are the average of duplicate samples.

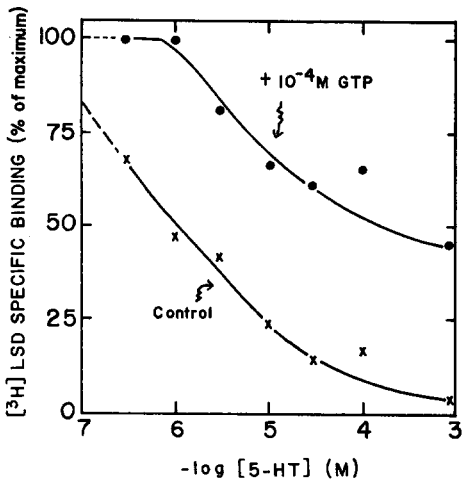

Fig. 7. Effect of GTP on 5-HT displacement of LSD binding in fluke particles. Liver fluke particles were incubated with 10 nM [^{3}H]LSD and the indicated concentrations of 5-HT with no additions (×), or with 100 μM GTP (●) for 5 min at 37°. Specific [^{3}H]LSD binding was then determined as described in Methods and Materials. The results shown are the mean values of three separate experiments, each done in triplicate.

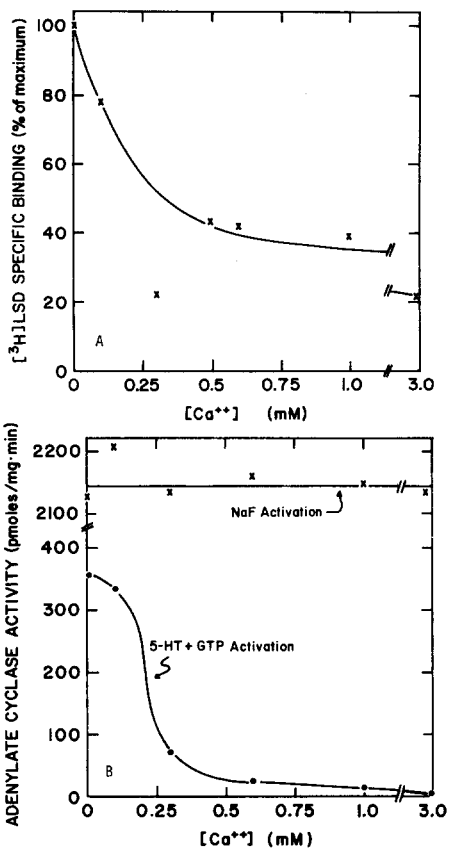

Fig. 8. Effect of Ca^{2+} on [^{3}H]LSD binding and adenylate cyclase activity in fluke particles. (A) Effect on [^{3}H]LSD binding. Fluke particles were incubated with 10 nM [^{3}H]-LSD and the indicated concentrations of $CaCl_2$ for 5 min at 37°. Specific [^{3}H]LSD binding was then determined as described in Methods and Materials. (B) Effect on adenylate cyclase activity. The same particles used above were assayed for adenylate cyclase activity at 37° as described in Methods and Materials with the indicated concentration of $CaCl_2$ and 10 mM sodium fluoride (×) or 100 μM 5-HT and GTP (●). EGTA was not included in the reaction mixture for this experiment.

5-HT. Gpp(NH)p had no effect on the affinity of the antagonist 2-bromo-LSD. Thus, these results were consistent with the binding properties of other receptors coupled to adenylate cyclase in that agonist binding was inhibited by GTP to a greater extent than was antagonist binding.

Effect of Ca^{2+} on LSD binding and activation of adenylate cyclase. Ca^{2+} has been reported to increase the amount of specific binding to 5-HT receptors in the mammalian brain [9]. In addition, millimolar concentrations of Ca^{2+} have been routinely used by others in their studies of mammalian 5-HT receptors [12,25]. When tested in the fluke, however, Ca^{2+} at concentrations above 300 μM markedly reduced [^{3}H]LSD binding (Fig. 8A). 5-HT activation of adenylate cyclase was reduced by Ca^{2+} at similar concentrations (Fig. 8B). Sodium fluoride activation of adenylate cyclase was not affected by Ca^{2+} at these concentrations, which strengthened the evidence that Ca^{2+} was inhibiting interactions at the receptor site. Mg^{2+} inhibited [^{3}H]LSD binding, but only when present at 10-fold higher concentrations (5 mM and above) than that needed for Ca^{2+} inhibition. The monovalent ions Na^+ and K^+ also reduced [^{3}H]LSD binding but only at very high concentrations (10 mM and above).

DISCUSSION

This investigation describes some novel properties of the 5-HT receptors coupled to adenylate cyclase in the liver fluke *F. hepatica*. The receptor properties were examined by both the adenylate cyclase assay and by a radioligand binding assay. [^{3}H]LSD rather than [^{3}H]5-HT was used as the radioligand in this study because of its higher affinity for the receptor sites. The high affinity of LSD is consistent with what we reported before on the high potency of LSD when determined by activation of adenylate cyclase [6] or by stimulation of rhythmical movement [2] in the fluke. We have designated these receptors as 5-HT receptors because 5-HT agonists and antagonists influence the binding properties as well as the physiological and biochemical effects of these receptors. Other neurotransmitters such as dopamine, epinephrine, and norepinephrine do not affect the binding of LSD to these receptors and do not mimic any of the physiological effects of the indoleamines. [^{3}H]-LSD has also been used to study 5-HT receptors in the mammalian brain [9–12]. These studies have been complicated by reports that LSD, in addition to binding to 5-HT receptors, can also bind to dopamine [26] and α-adrenergic receptors [27]. This was not a problem in the present studies for, as indicated above, neither of these receptor types appears to be functional in the flukes.

The kinetics of LSD binding to the fluke particles indicated that the binding sites were heterogeneous.

There is a population of high affinity sites and a population of low affinity sites. Studies on the displacement of [^{3}H]LSD binding by 5-HT and related compounds showed that they competed with [^{3}H]-LSD for the high affinity sites. The affinity of LSD and 2-bromo-LSD for the fluke receptors was greater than that of 5-HT. This corresponds with what we have reported on the relative effect of these agents on the fluke adenylate cyclase. The concentration of 5-HT required for half-maximal activation of adenylate cyclase was 2.1 μM while for LSD it was 46 nM [6]. The relative binding data for LSD and 5-HT also reflect the potency of these agents to stimulate carbohydrate metabolism and motility. Stimulation of carbohydrate metabolism in intact flukes required micromolar concentrations of LSD, while with 5-HT a 100-fold higher concentration was necessary to show a significant effect [3]. In the case of motility, the minimum effective concentration that caused stimulation of rhythmical movement in intact flukes was 100 nM for LSD and 50 μM for 5-HT [2].

There are not enough data on the physiological effects on the flukes of all the ligands tested above to make final conclusions about the relationship between the binding and the pharmacological effects of these agents. However, our data showed a good correlation in the ranking order of these compounds in regard to their ability to displace [^{3}H]LSD binding and their ability to inhibit 5-HT activation of adenylate cyclase. While differences in the buffer compositions for the two sets of measurements may only allow for qualitative comparison, the results indicated that [^{3}H]LSD was binding to the 5-HT receptor coupled to adenylate cyclase. This was supported by the following data: (1) Regional distribution studies of [^{3}H]LSD binding showed the highest concentration of binding sites to be in the "head" of the fluke. This correlated with the greater 5-HT activation of adenylate cyclase found in this region. (2) [^{3}H]LSD binding was very rapid at 37° reaching a maximum within 3 min. This agreed with earlier studies which found no detectable time lag for activation of adenylate cyclase by 5-HT, LSD, or other effectors. (3) GTP and Gpp(NH)p reduced the affinity of the agonist 5-HT for [^{3}H]LSD binding sites while having little effect on the partial agonist LSD and the antagonist 2-bromo-LSD. This effect of GTP and Gpp(NH)p on agonist binding has been reported for several other receptors coupled to adenylate cyclase [22, 28]. (4) Ca^{2+} inhibited [^{3}H]LSD binding and 5-HT activation of adenylate cyclase in a similar manner. Furthermore, as described in the accompanying paper [20], both [^{3}H]LSD binding and 5-HT activation of adenylate cyclase decreased in a similar time-dependent manner.

The pharmacological profile of the fluke receptor appears to differ from those of other reported 5-HT receptors. Peroutka and Snyder [12] have characterized two 5-HT receptors, designated 5-HT$_1$ and 5-HT$_2$, in the rat brain. Both receptors differ greatly from the fluke receptor with respect to affinity for various compounds. For example, 5-HT was reported to have very high affinity for 5-HT$_1$ receptors (K_d in the nanomolar range), while for the fluke receptors 5-HT had much lower affinity (K_d in the micromolar range). Similarly, spiroperidol had very high affinity for 5-HT$_2$ receptors but very low affinity for the fluke receptors. Other reported 5-HT receptors in the vertebrate central nervous system [9–11] also differ from the fluke receptor. The different effect of Ca^{2+} on the fluke and mammalian 5-HT receptors provides further evidence that these receptors are not alike. It has been suggested that, despite the finding of multiple 5-HT receptors in the CNS, the receptor coupled to adenylate cyclase activity has not yet been identified by direct binding studies [29]. A 5-HT receptor coupled to adenylate cyclase activity has been characterized from the CNS of the snail *H. pomatia* [13]. There are many similarities between this receptor and the fluke receptor. However, these two receptors are not pharmacologically identical as indicated by the difference in their relative affinities for methysergide and cinanserin.

Differences between mammalian and fluke 5-HT receptors may provide a rational approach to the selection of new chemotherapeutic agents against trematode infestation. 5-HT regulation of adenylate cyclase appears to have an important role in the carbohydrate metabolism [3–5] and motility [2] of these worms. Studies with *Schistosoma mansoni* [30] have suggested that they have 5-HT receptors similar to those found in the fluke. Furthermore, these receptors may play a role in the development of these trematodes from cercariae to mature adult worms [31]. Disruption of these 5-HT-mediated functions may provide a useful approach towards the chemotherapy of these parasites. Differences in the pharmacological profile of the fluke and mammalian 5-HT receptors have been discussed above. These differences indicate that it may be possible to develop agents which could interact specifically at fluke 5-HT receptors without interfering with mammalian 5-HT functions.

REFERENCES

1. T. E. Mansour, E. W. Sutherland, T. W. Rall and E. Bueding, *J. biol. Chem.* **235**, 466 (1960).
2. S. L. Abrahams, J. K. Northup and T. E. Mansour, *Molec. Pharmac.* **12**, 49 (1976).
3. T. E. Mansour, *J. Pharmac. exp. Ther.* **126**, 212 (1959).
4. T. E. Mansour, *J. Pharmac. exp. Ther.* **135**, 94 (1962).
5. D. B. Stone and T. E. Mansour, *Molec. Pharmac.* **3**, 161 (1967).
6. J. K. Northup and T. E. Mansour, *Molec. Pharmac.* **14**, 804 (1978).
7. A. Enjalbert, M. Hamon, S. Bourgoin and J. Bockaert, *Molec. Pharmac.* **14**, 2 (1978).
8. K. Von Hungen, S. Roberts and D. F. Hill, *Brain Res.* **84**, 257 (1975).
9. J. P. Bennett and S. H. Snyder, *Molec. Pharmac.* **12**, 373 (1976).
10. G. M. B. Fillion, J-C. Rousselle, M-P. Fillion, D. M. Beaudoin, M. R. Goiny, J. M. Deniau and J. J. Jacob, *Molec. Pharmac.* **14**, 50 (1978).
11. P. M. Whitaker and P. Seeman, *Proc natn. Acad. Sci. U.S.A.* **75**, 5783 (1978).
12. S. J. Peroutka and S. H. Snyder, *Molec. Pharmac.* **16**, 687 (1979).
13. A. H. Drummond, F. Bucher and I. B. Levitan, *J. biol. Chem.* **255**, 6679 (1980).
14. S. J. McNall and T. E. Mansour, *Fedn Proc.* **42**, 1876 (1983).

15. D. M. Neville, Jr., *Biochim. biophys. Acta* **154**, 540 (1968).
16. Y. Salomon, C. Londos and M. Rodbell, *Analyt. Biochem.* **58**, 541 (1975).
17. E. B. Walker, S. J. McNall and T. E. Mansour, *Biochem. Pharmac.* **32**, 1251 (1983).
18. M. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
19. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
20. S. J. McNall and T. E. Mansour, *Biochem. Pharmac.* **33**, 2799 (1984).
21. J. K. Northup and T. E. Mansour, *Molec. Pharmac.* **14**, 820 (1978).
22. E. Ross and A. Gilman, *A. Rev. Biochem.* **49**, 533 (1980).
23. R. J. Lefkowitz, D. Mulliken and M. G. Caron, *J. biol. Chem.* **251**, 4686 (1976).
24. N. R. Zahniser and P. B. Molinoff, *Nature, Lond.* **275**, 453 (1978).
25. M. Hamon, M. Mallat, A. Herbet, D. L. Nelson, M. Audinot, L. Pichat and J. Glowinski, *J. Neurochem.* **36**, 613 (1981).
26. D. R. Burt, I. Creese and S. H. Snyder, *Molec. Pharmac.* **12**, 631 (1976).
27. D. C. U'Prichard and S. H. Snyder, *J. biol. Chem.* **253**, 3444 (1978).
28. M. Rodbell, *Nature, Lond.* **284**, 17 (1980).
29. J. Bockaert, D. L. Nelson, A. Herbet, J. Adrien, A. Enjalbert and M. Hamon, *Adv. exp. Med. Biol.* **133**, 327 (1981).
30. M. R. Kasschau and T. E. Mansour, *Molec. biochem. Parasit.* **5**, 107 (1982).
31. M. R. Kasschau and T. E. Mansour, *Nature, Lond.* **296**, 66 (1982).