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Serotonin Receptor-Mediated Activation of Adenylate Cyclase in the Neuroblastoma NCB.20: A Novel 5-Hydroxytryptamine Receptor

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SUMMARY

Serotonin (5-hydroxytryptamine; 5-HT) and its analogs activate adenylate cyclase in membrane particles from neuroblastoma NCB.20 cells. Low concentrations of GTP (EC_{50} = 60 nM) were required for activation by serotonin. Guanosine 5′-*O*-(2-thiodiphosphate) inhibited serotonin-activated cyclase in these cells. The nonhydrolyzable GTP analogs guanosine 5′-*O*-(3-thiotriphosphate) (EC_{50} = 3 nM) and guanylyl-imidodiphosphate (EC_{50} = 100 nM) substituted for GTP in potentiating serotonin activation. Pretreatment of the cells with cholera toxin potentiated enzyme activation by serotonin, whereas pertussis toxin was found to have little effect, indicating the involvement of the α subunit of a stimulatory GTP-binding protein in enzyme activation. Homologous desensitization of the serotonin-stimulated adenylate cyclase was demonstrated in membranes prepared from intact cells pretreated with serotonin. Cell membrane particles that were desensitized to serotonin were still responsive to β-adrenergic agonists and to prostaglandin E_1. Evidence is presented indicating that serotonin stimulation of adenylate cyclase is mediated by receptors that are distinct from other positively coupled receptors (β-adrenergic, histamine, and prostacyclin). Equilibrium binding analysis with [^{3}H]serotonin, [^{3}H]lysergic acid diethylamide, and [^{3}H]dihydroergotamine suggested that the site density was below the level of detection of binding of these radioligands. The pharmacological characteristics of the serotonin-activated cyclase were analyzed in order to compare these serotonin receptors with the family of different receptor subtypes. Correlation analysis between the potencies of different agonists and antagonists at the cyclase in these cells and their reported relative potencies for different serotonin receptor subtypes showed no correlation with the 5-HT_{1A}, 5HT_{1B}, 5HT_{1D}, 5-HT_2, and 5-HT_3 receptors. On the other hand, the analysis showed that the NCB.20 serotonin receptors are similar but not identical to the rat and pig brain 5-HT_{1C} receptors and to the serotonin receptors coupled to adenylate cyclase in the trematodes *Schistosoma mansoni* and *Fasciola hepatica*. The results point to a novel serotonin receptor which has a low density in these cells.

The ability of serotonin (5-HT) to activate adenylate cyclase was first demonstrated in a trematode, the liver fluke *Fasciola hepatica*, as early as 1960 (1). Since that time, there have been several attempts to find a mammalian model to study serotonin receptor-activated cyclase. Because of the role of serotonin in brain function, these studies have focused primarily on the effect of serotonin on adenylate cyclase in different parts of the central nervous system. Low serotonin-activated adenylate cyclase activity was demonstrated in newborn and was almost absent in adult animals (2, 3). Fillion *et al.* (4) demonstrated serotonin-sensitive adenylate cyclase in horse brain synaptosomal membranes. With the availability of more selective ligands that affect specific serotonin receptors, attempts were made to characterize some of these effects. De Vivo and Maayani (5), using guinea pig and rat hippocampal membranes, reported inhibition of forskolin-stimulated adenylate cyclase by serotonin through 5-HT_{1A} receptors. On the other hand, Shenker *et al.* (6) reported activation of adenylate cyclase through receptors that are functional correlates of the 5-HT_{1A} site. MacDermot *et al.* (7) reported an activation of similar magnitude in NCB.20 neuroblastoma-brain hybrid cells and demonstrated minor dependency of serotonin action on GTP (8).

In this report, we have studied the nature of serotonin activation of adenylate cyclase and some aspects of its regula-

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine, serotonin; LSD, *d*-lysergic acid diethylamide; BOL, 2-bromo-lysergic acid diethylamide; Gpp(NH)p, guanylyl-imidodiphosphate; GTPβS, guanosine-5′-*O*-(3-thiotriphosphate); GDPβS, guanosine-5′-*O*-(2-thiodiphosphate); 8-OH-DPAT, 8-hydroxy-*n*-(dipropylamino)tetralin; EC_{50}, concentration that produces 50% of maximal effect; IC_{50}, concentration that inhibits stimulation of adenylate cyclase or radioligand binding by 50%; G protein, GTP-binding protein; G_s, GTP-binding protein that is involved in activation of adenylate cyclase.

tion in the NCB.20 cell line. We have also undertaken a pharmacological analysis of serotonin activation of cyclase in these cells, with the intent of comparing these receptors with other mammalian and nonmammalian serotonin receptor subtypes. Our results indicate that in these cells a single set of serotonin receptors signals to adenylate cyclase through a G protein that is similar to the stimulatory α subunit of G_s of the β-adrenergic receptors. The serotonin receptors are capable of undergoing homologous desensitization. On the basis of our studies on the effect of different serotonin agonists and antagonists, we have concluded that, although these receptors are similar to serotonin 5-HT_{1C} receptors and those in the trematodes *F. hepatica* and *Schistosoma mansoni*, they appear to be distinct from the well characterized serotonin receptor subtypes in mammals.

Materials and Methods

Cell culture. NCB.20 cells (9) (generously provided by Dr. Marshall Nirenberg) NIH, Bethesda, MD, were grown in high glucose Dulbecco's modified Eagle's medium, 10% newborn calf serum, HAT supplement (100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine). Cells were harvested, when confluent, in phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, adjusted to pH 7.5) supplemented with 2 mM EDTA.

Membrane preparation. Harvested intact NCB.20 cells were washed twice in phosphate-buffered saline and resuspended in 0.29 M sucrose, 1 mM EDTA, 50 mM Tris, pH 7.5 (sucrose buffer). The resuspended cells were frozen rapidly on dry ice and stored at −80° for up to 3 months with no loss of enzyme activity. All subsequent steps were performed at 4°. Cells were thawed and homogenized in sucrose buffer with 30 strokes of a motor-driven Teflon pestle and glass tube. The nuclear fraction was removed with a 20-min centrifugation at 800 × *g*. The supernate was centrifuged at 30,000 × *g* for 20 min. The pellet was resuspended in sucrose buffer to a protein concentration of 2 to 10 mg/ml and placed on ice for subsequent use in the adenylate cyclase assay. Frozen and fresh preparations produced similar results.

Adenylate cyclase assay. Adenylate cyclase assays were performed by the method of Salomon (10), with some modifications. The final assay volume was 200 μl, consisting of 100 mM sucrose, 50 mM Tris buffer (pH 7.5), 5 mM phosphocreatine, 1 mM EDTA, 0.5 mM isobutylmethylxanthine, 10 mM $MgCl_2$, 0.1 mM ATP, 10 μM pargyline,[2] 5 μM GTP, 6.25 μCi/ml [α-^{32}P]ATP, and 20 units/ml creatine phosphokinase (prepared in 1% bovine serum albumin). The reaction was initiated by the addition of the particle preparation (100–500 μg of protein) and incubated for 20 min at 30°. Under these conditions, the reaction was linear with respect to time, for 30 min, and protein concentration, within the indicated range. The reaction was terminated by the addition of 250 μl of a stop solution consisting of 2% sodium dodecyl sulfate, 1 mM cAMP, 10 mM Na_2ATP, and 10 mM EDTA, adjusted to pH 7.5. After the reaction was terminated, a [^{3}H]cAMP recovery standard (~10,000 cpm) was added to each tube. The volume was brought up to 1 ml with distilled water and the samples were boiled for 5 min to solubilize the protein to prevent column clogging. Isolation of labeled cAMP and calculations of enzyme activity were performed as described by Salomon (10). Each condition was repeated in triplicate and expressed as the mean ± standard deviation. The standard deviation was usually ≤5% of the mean.

Assay components were found to provide antioxidant capacity to prevent oxidation of tryptamine derivatives. Using thin layer chromatography, we have found no significant oxidation or enzymatic degradation of serotonin in the standard cyclase assay mixture. Furthermore, dilution of serotonin and 5,6-dihydroxytryptamine in a solution containing ascorbic acid did not result in changes in EC_{50} or V_{max} values, when compared with results from experiments without ascorbic acid.

ADP-ribosylation with bacterial toxins. ADP-ribosylation with cholera toxin was performed using the protocol described by Ribeiro-Neto *et al.* (11), with some modifications. Toxin was activated by incubation in 50 mM dithiothreitol for 30 min at 37°. Activated toxin was incubated with NCB.20 cell membranes (prepared in sucrose buffer) in 50 mM Tris (pH 7.5), 1 mM ATP, 0.5 mM GTP, 2 mM NAD, 20 mM dithiothreitol, 2 mM EDTA, at 30° for varying periods of time. The incubation mixtures were centrifuged in a Beckman microfuge for 5 min at 4°. The pellet was resuspended in sucrose buffer and recentrifuged. The remaining pellet was resuspended and placed on ice for use in adenylate cyclase assay.

Experimental design of pharmacological analysis. Compounds were dissolved in distilled water or dimethyl sulfoxide, depending on solubility. Antagonist potency was determined in the presence of 5 μM serotonin. Agonist and antagonist dose-response curves were plotted as a function of the logarithm of the concentration of the compound. EC_{50} values were determined directly from these figures. IC_{50} values for full antagonists were calculated as the concentration of the compound required to reduce the incremental serotonin response by 50%. It was necessary to use a concentration of serotonin (5 μM) that was near the E_{max} in order to get reliable activity. With full antagonists, the incremental response was calculated after subtraction of basal enzyme activity. In the case of partial agonists, the incremental response was calculated after subtraction of the elevated enzyme activity observed with saturating concentrations of the partial agonists. The IC_{50} values were modified by the Cheng-Prusoff (12) relationship, to provide estimates of apparent K_i values. Unless otherwise specified, at least six drug concentrations were used for each concentration-response curve.

Equilibrium binding assay. Intact cells were harvested and frozen, and membrane particles were prepared as described above, except that the pellet was resuspended in 20 mM Tris (pH 7.5), centrifuged a second time for 20 min at 30,000 × *g*, and then resuspended in 50 mM Tris (pH 7.5) for use in the binding assay. Binding assays were performed using the protocol of Peroutka and Snyder (13), with some minor modifications. Incubations were carried out at 37°, in a total volume of 0.15 ml, in 10 μM pargyline and 50 mM Tris (pH 7.5).

Experimental design and data analysis of desensitization studies. Pretreatment with serotonin was performed with intact cells as monolayers in culture. Routinely, each treated and control membrane preparation was assayed for both basal adenylate cyclase activity and maximal serotonin response (0 and 10 μM serotonin in the presence of 5 μM GTP). Untreated controls were analyzed for each time point. Values were standardized to the controls to allow averaging and comparison within experiments and between separate experiments.

Determination of protein concentration. Sample protein concentrations were determined by the method of Bradford (14), using reagents purchased from Bio-Rad.

Materials. The following chemicals were purchased from Sigma Chemical Company: 5-HT (creatine sulfate salt), creatine phosphokinase, creatine phosphate, neutral alumina (type WN-3), HAT medium supplement, cAMP, GTP, prostaglandin E_1, bufotenine, 1-(*m*-chlorophenyl)piperazine, dopamine, isoproterenol, epinephrine, norepinephrine, cyproheptadine, haloperidol, promethazine, imipramine, clonidine, amitriptyline, propranolol, and pindolol. 5,6-Dihydroxytryptamine was purchased from Regis Chemical Company. The following compounds were obtained from Calbiochem: 5-methoxytryptamine, tryptamine, 5-hydroxytryptophan, 5-hydroxyindoleacetic acid, and histamine. *N*,ω-Methyltryptamine,*N*,*N*-dimethyltryptamine,*N*,*N*,-dimethylserotonin,*N*,*N*,-dimethyl-5-methoxytryptamine, 5-benzyloxytryptamine, and 5-hydroxy-*N*,ω-methyltryptamine were purchased from Aldrich. Dowex 1-X8 and 50W-X4 were obtained from Bio-Rad. [^{3}H]cAMP and [α^{32}P]ATP were purchased from Amersham. ATP, GTPγS, GDPβS,, and Gpp(NH)p were purchased from Boehringer Mannheim. Dulbecco's modified Eagle's medium and newborn calf serum were obtained from Irvine Scientific. Cholera toxin and pertussis toxin were

[2] Optimal concentration to inhibit oxidation by monoamine oxidase in these membrane assay mixtures.

purchased from List Laboratories. LSD and BOL were obtained through the National Institutes of Mental Health. [^{3}H]5-HT and [^{3}H] LSD were obtained from New England Nuclear. Forskolin was a generous gift from Dr. Daniel Ellis of Hoechst. MDL 72222 was a gift from Dr. J. Fozard, University of Paris, France. Other compounds were generous gifts from Dr. Stephen Peroutka, Dr. Roland Ciaranello, and Dr. Howard Schulman of Stanford University.

Results

Kinetics of activation of adenylate cyclase by serotonin. A representative concentration-response curve for activation of adenylate cyclase by serotonin in NCB.20 cell particle preparations in the presence of GTP is shown in Fig. 1. The concentration of serotonin that produces a half-maximal response (EC_{50}) was 260 nM in this experiment. A Hill plot of the dose-response data indicates that the activation of adenylate cyclase occurs with first-order kinetics, which is consistent with a single population of these receptors. The maximum serotonin-stimulated response varied from approximately 2 to 10 pmol of cAMP/min/mg of protein. Cells with an early passage number (3 to 18 passages) showed higher sensitivity to serotonin and were used for this investigation. The magnitude of the response declined as the cell passage number increased, eventually to levels that were not detectable. This may be attributed to the instability of the hybrid cell genome.

Effects of GTP and its nonhydrolyzable analogs on serotonin activation of adenylate cyclase. Serotonin activation of adenylate cyclase in cell homogenates or unwashed membrane fractions showed little requirement for GTP. However, in washed membrane preparations there is a marked dependence of the serotonin response on the presence of GTP. The effect of GTP on basal activity and the activity observed in the presence of a saturating serotonin concentration (10 μM) are illustrated in Fig. 2, *inset*. GTP causes minor stimulation

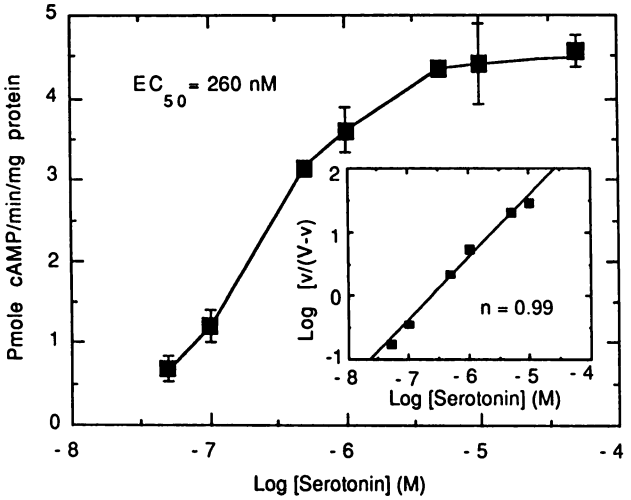

Fig. 1. Serotonin activation of adenylate cyclase activity in particle preparations. Particle preparations were incubated with the indicated concentrations of serotonin in the presence of 5 μM GTP. Basal enzyme activity (8.0 ± 0.1 pmol of cAMP min^{-1} mg of protein^{-1}) was subtraced from each value. *Points*, mean ± standard deviation of triplicate determinations. *Inset*, Hill plot of the concentration-response curve. The *abscissa* is plotted as log ($v/V - v$), where v equals the velocity at any given serotonin concentration with basal activity subtracted and V equals the maximum velocity, which was approximated from the concentration-response curve. *Line*, the nonweighted linear regression line with slope equal to 0.99.

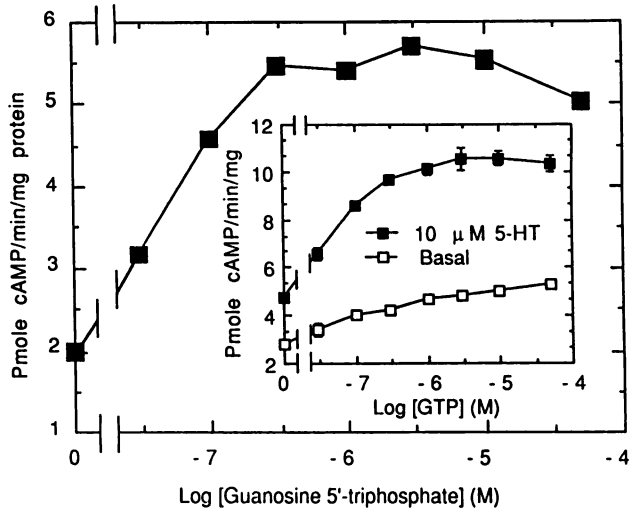

Fig. 2. Effects of GTP on basal and serotonin-stimulated adenylate cyclase activity. Particle preparations were washed four times, as described in Materials and Methods, and then incubated with varying concentrations of GTP with or without 10 μM serotonin, as shown in the *inset*. *Points*, mean ± standard deviation of triplicate determinations. *Main panel*, effect of GTP on the incremental serotonin response. The data were plotted after subtraction of the basal enzyme activity from the serotonin-stimulated activity for each GTP concentration (shown in *inset*).

of basal adenylate cyclase activity and potentiates activation by serotonin. The results in Fig. 2 show that the GTP potentiation of the serotonin response saturates at 1 μM and the EC_{50} is 0.06 μM.

Both GTPγS and Gpp(NH)p caused a significant stimulation of adenylate cyclase activity in the absence of serotonin. Furthermore, both GTP analogs potentiated stimulation by serotonin.

Effects of GDP and the nonhydrolyzable analog GDPβS. The observation that serotonin activated adenylate cyclase to some extent in the absence of added guanine nucleotides in washed particle preparations raised the possibility that some of the serotonin response was guanine nucleotide independent. This possibility was addressed using GDP and GDPβS, under the assumption that these compounds would antagonize GTP-dependent responses. GDP inhibited both serotonin-stimulated activity and basal activity in the presence of 1 μM GTP. The inhibition of the serotonin response is illustrated in Fig. 3. However, the potency of GDP was quite low, and it was difficult to determine whether the effects were specific. The GDP analog GDPβS was a potent inhibitor of the serotonin response. GDPβS decreased basal and serotonin-stimulated activity in the presence of GTP. In Fig. 3, the effects of varying concentrations of GDPβS on serotonin-stimulated activity in the presence of 0.3 or 5 μM GTP are illustrated. In the presence of a low GTP concentration (0.3 μM), nearly all of the serotonin response is inhibited (94% with 1 mM GDPβS), indicating that the activity previously observed that was "GTP independent" was probably the result of endogenous or contaminating GTP. In addition, the shift of the GDPβS dose-response curve to the right upon addition of 5 μM GTP suggests a competitive interaction of GDPβS with GTP, presumably at the guanine nucleotide binding site.

Effects of cholera toxin on adenylate cyclase activity. The effects of cholera toxin on adenylate cyclase activity in particle preparations were investigated to determine whether

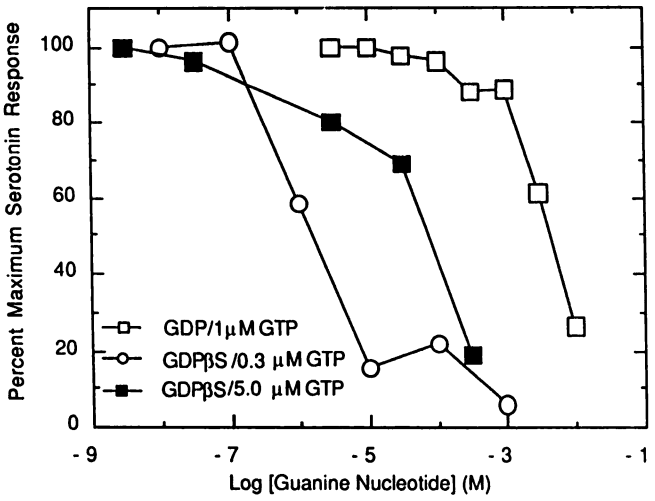

Fig. 3. Effects of GDP and GDPβS on serotonin-stimulated adenylate cyclase activity. Particle preparations were incubated with varying concentrations of GDP or GDPβS, with or without 10 μM serotonin. Enzyme activity observed in the absence of serotonin was subtracted from that observed in the presence of serotonin, and the resultant values were standardized to 100% to allow comparison of separate experiments. Each curve was produced in the presence of the indicated concentration of GTP. IC_{50} values were determined graphically. *Points*, mean of triplicate determinations.

TABLE 1

Effects of cholera toxin on adenylate cyclase activity in particle preparations

Particle preparations were incubated with or without cholera toxin (10 μg/ml) for 5 min and subsequently washed, as described in Materials and Methods, before adenylate cyclase activity was assayed. Values are the mean ± standard deviation of triplicate determinations.

Additions to assay	Adenylate cyclase activity	
	Control	+Cholera toxin
	pmol of cAMP min^{-1} mg of $protein^{-1}$	
None (basal)	17.5 ± 0.37	101 ± 1.9
GTP (50 μM)	16.9 ± 0.51	116 ± 6.6
GTPγS (10 μM)	149 ± 7.5	202 ± 5.5
5-HT (20 μM), GTP (50 μM)	27.9 ± 0.70	161 ± 6.5
Forskolin (2 mM)	95.8 ± 3.11	231 ± 8.9

the G protein involved in the serotonin activation of adenylate cyclase had characteristics of the cholera toxin-sensitive $G_{s\alpha}$ (Table 1). Toxin treatment increased basal enzyme activity 5- to 6-fold. Maximum activity observed after activation of the receptor (with serotonin), the G protein (with GTPγS or GTP), or adenylate cyclase itself (with forskolin) was also increased by pretreatment with cholera toxin. The incremental serotonin response was increased 4-fold (from 11 to 45 pmol of cAMP/min/mg of protein) after toxin treatment (Table 1). Pertussis toxin moderately reduced enzyme activity in particle preparations under all of the activating conditions listed in Table 1 but had no effect on the response to serotonin (data not shown). In parallel experiments, the ADP-ribosylation of the membrane particles by cholera toxin and pertussis toxin was confirmed using [^{32}P]NAD and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

The possibility that serotonin receptors might mediate inhibition of adenylate cyclase in NCB.20 cell membranes was tested. Serotonin did not inhibit basal, forskolin-activated, or prostacyclin receptor-activated adenylate cyclase activity. In fact, serotonin potentiated forskolin activation of adenylate cyclase (data not shown).

Specificity of the serotonin response. NCB.20 cells have several receptors that activate or inhibit adenylate cyclase. In addition to serotonin, isoproterenol (and other β-adrenergic agents), histamine, and prostaglandin were found to stimulate adenylate cyclase to varying degrees. Several experiments indicated that serotonin stimulation of adenylate cyclase was mediated by a receptor distinct from other positively coupled receptors. The serotonin response was not mediated by a β-adrenergic receptor, because the β-adrenergic antagonists propranolol and pindolol did not antagonize the serotonin response. In addition, pretreatment of intact cells with serotonin resulted in the desensitization of the serotonin response (see below) with no reduction of the β-adrenergic response. The histamine response was small and variable. In membrane preparations where histamine activation was nonexistent, the serotonin response remained normal, indicating that the two effects are distinct. In addition, the histamine receptor antagonist mepyramine had no effect on the serotonin response. Prostaglandin E_1 causes a 5- to 10-fold increase in adenylate cyclase activity. Several lines of evidence indicate that the serotonin and prostaglandin E_1 responses do not share a common receptor: 1) serotonin did not act as a partial agonist or antagonist of the prostaglandin E_1 response; 2) pretreatment of intact cells with serotonergic compounds resulted in a desensitization of the serotonin response with no attenuation of the prostaglandin E_1 response; and 3) the serotonin antagonists cyproheptadine and BOL did not antagonize the prostaglandin E_1 response.

Effects of serotonergic agonists. A variety of compounds were tested for their ability to activate adenylate cyclase in NCB.20 cell membranes. Representative concentration-response curves for these agonists are shown in Fig. 4A. Hill plots for 10 of the agonists examined, which include those shown in Fig. 4A, gave slopes that varied from 0.9 to 1.2. The potencies and relative efficacies of these compounds are presented in Table 2. All of the tryptamine derivatives tested displayed agonist activity, with EC_{50} values that ranged from 0.13 to 3.8 μM. This is a small range of potencies (50-fold), when compared with other serotonin receptor subtypes. All these agonists were capable of stimulating adenylate cyclase activity to a level 70–100% of that observed with serotonin. None of the tryptamine derivatives were significantly more potent than serotonin. Hydroxyl or methoxy substitutions at the 5-position of the indole ring increased potency relative to the parent compound (e.g., compare the EC_{50} values of 5-methoxytryptamine and serotonin with that of tryptamine or *N,N*-dimethyl-5-methoxytryptamine and *N,N*-dimethyl-5-hydroxytryptamine with *N,N*-dimethyltryptamine). More complex substitutions at the 5-position had varied effects on potency (compare the potency of 5-carboxamidotryptamine with that of 5-benzyloxytryptamine). Hydroxyl substitutions at the 6-position greatly reduced potency, relative to serotonin. In general, indole ring modifications appear to modulate agonist affinity, whereas *N*-methyl additions moderately reduced efficacy. Monomethylated compounds were typically more potent that their dimethylated analogs. Neither 5-hydroxytryptophan, the precursor of serotonin, nor 5-hydroxyindoleacetic acid, its main metabolic product, caused any significant stimulation of adenylate cyclase.

Several compounds unrelated to tryptamine were tested be-

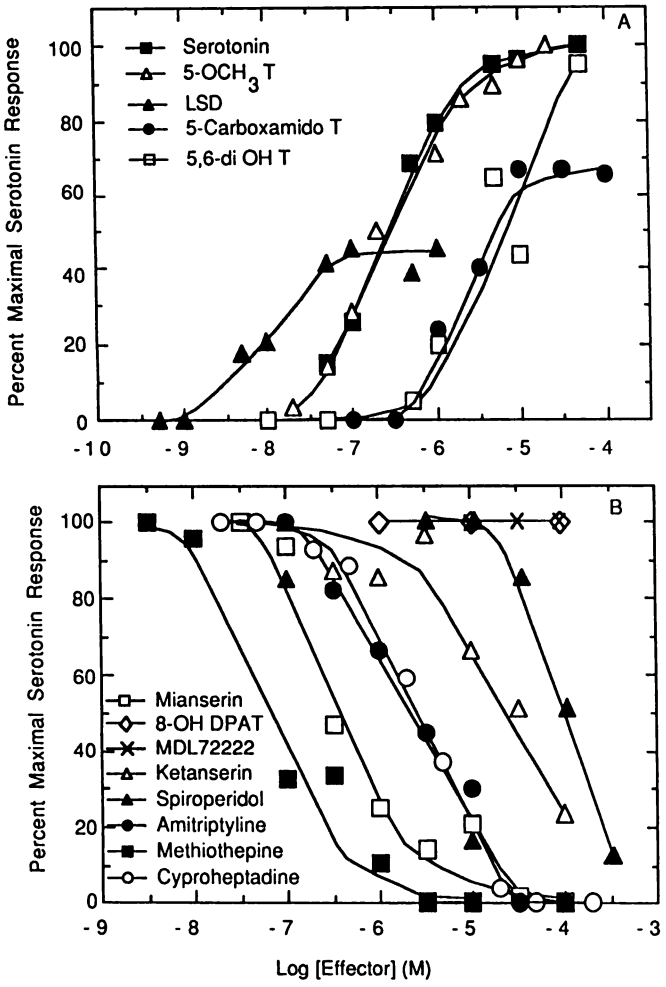

Fig. 4. Concentration-response curves. A, Representative agonist concentration-response curves. *5-OCH$_3$T*, 5-methoxytryptamine; *5-CarboxamidoT*, 5-carboxamidotryptamine; *5,6-di OHT*, 5,6-dihydroxytryptamine. B, Representative antagonist concentration-response curves.

cause of their previously described ability to interact with serotonin receptors. Only the erogline derivatives (LSD, dihydroergotamine, BOL, and methysergide) increased adenylate cyclase activity (Table 2). The ergoline derivatives were partial agonists, capable of increasing adenylate cyclase activity to less than 50% of that seen with serotonin. As a group, the ergoline derivatives were considerably more potent than the tryptamine derivatives. Neither the tetralin derivative, 8-OH-DPAT, which displays potent agonist activity at some serotonin receptors coupled to adenylate cyclase (6, 15), or the central serotonergic agonist 1-(*m*-chlorophenyl)piperazine (16) displayed detectable stimulatory effects in NCB.20 particle preparations.

Several compounds that interact with nonserotonergic biogenic amine receptors were tested for their effect on adenylate cyclase activity. The catecholamine receptor agonists dopamine, norepinephrine, and isoproterenol did not behave pharmacologically like serotonin receptor agonists (see above). In addition, neither histamine (histamine receptor agonist) or carbachol (muscarinic receptor agonist) displayed serotonergic agonist activity.

Effects of serotonergic antagonists. A variety of compounds were tested for their ability to inhibit the serotonin-stimulated adenylate cyclase activity in membrane particle preparations. The relative potencies of these compounds are presented in Table 3. Representative inhibition curves for some of these compounds are shown in Fig. 4B. The ergoline derivatives as a group displayed the highest affinity for the NCB.20 cell serotonin receptor, except for mesulergine, which had no antagonist activity (Fig. 4B; Table 3). The nature of antagonism was investigated in the case of cyproheptadine. The serotonin concentration-response curve was found to shift in a parallel manner to the right (higher concentration levels) in the pres-

TABLE 2

Agonist potency and efficacy

Six or more concentrations of each compound were tested. EC_{50} values were determined directly from semilogarithmic concentration-response curves. Values are the average of duplicate, triplicate, or quadruplicate determinations ± the range of standard deviation. Relative efficacy was defined as the ratio of the adenylate cyclase velocity observed in the presence of a saturating concentration of each test compound to that observed with saturating 5-HT (10 μM).

Compound	EC_{50}	Relative efficacy
	nM	
Tryptamines		
Serotonin	320 ± 120	1.00
5-Hydroxy-*N*,ω-methyltryptamine	130 ± 47	0.71
5-Methoxytryptamine	190 ± 65	0.98
5-Benzyloxytryptamine	190 ± 65	0.94
N,*N*-Dimethyl-5-methoxytryptamine	200 ± 72	0.85
Bufotenine	460 ± 175	0.73
N,ω-Methyltryptamine	530 ± 327	0.72
Tryptamine	1200 ± 550	0.81
N,*N*-Dimethyltryptamine	1600 ± 450	0.56
5-Carboxamidotryptamine	3600 ± 1750	0.76
5,6-Dihydroxytryptamine	3800 ± 750	0.84
5-Hydroxytryptophan	?[a]	0.00
5-Hydroxyindoleacetic acid	?[a]	0.00
Ergolines		
LSD	27 ± 21	0.40
Methysergide	?[a]	0.18
Dihydroergotamine	<50	0.38
BOL	<30	0.45

[a] Potency could not be accurately determined due to low efficacy.

TABLE 3

Antagonist potencies

Six or more concentrations of each compound were incubated with particle preparations in the presence of 5 μM 5-HT. IC_{50} values were determined directly from semilogarithmic concentration-response curves. Apparent K_i values were determined using the Cheng and Prusoff (12) relationship (apparent $K_i = IC_{50}/(1 + [5\text{-HT}]/EC_{50})$. Values are the average of duplicate, triplicate, or quadruplicate determinations ± range or standard deviation. Values routinely varied less than 3-fold.

Compound	Apparent K_i
	nM
Ergolines	
BOL	16 ± 10
LSD	33 ± 20
Methysergide	420 ± 180
Mesulergine	ND[a]
Traditional serotonin antagonists	
Methiothepine	12 ± 4
Mianserin	29 ± 13
Cyproheptadine	183 ± 62
Ketanserin	4600 ± 4390
Cinanserin	ND
Antipsychotics	
Promethazine	620 ± 280
Haloperidol	2300 ± 720
Spiroperidol	4800 ± 1800
Uptake inhibitors	
Amitriptyline	90 ± 40
Imipramine	370 ± 57

[a] ND, no detectable inhibition at 100 μM.

ence of increasing cyproheptadine concentrations, indicating the competitive nature of antagonism.

Serotonin antagonists that bind preferentially to the 5-HT_2 receptor rather than the 5-HT_1 or 5-HT_3 receptor subtypes (17, 18) were examined. The compounds in this class that displayed relatively high potency as antagonists of serotonin-activated cyclase were either tricyclic compounds (cyproheptadine and methiothepine) or tetracyclic compounds (mianserin) with a central seven-membered ring (Fig. 4B; Table 3). Ketanserin, which is structurally related to haloperidol and spiroperidol, had relatively low potency. Cinanserin is structurally distinct and had no detectable effect on the serotonin-stimulated cyclase in NCB.20 cell membranes.

Haloperidol and spiroperidol are *p*-fluorobenzoyl derivatives, whereas the structure of promethazine is related to the tricyclic phenothiazine. These compounds also recognize 5-HT_2 receptors and are fairly useful in distinguishing 5-HT_2 receptors from other serotonin receptor subtypes (13). These compounds had intermediate to poor potencies for the NCB.20 cell serotonin receptor. Imipramine and amitriptyline are tricyclic compounds that are structurally related to mianserin, cyproheptadine, and methiothepine. Our results show that imipramine and amitriptyline are relatively potent antagonists of serotonin receptors in NCB.20 cells (Table 3; Fig. 4B).

Two β-blockers, pindolol and propranolol, were tested because of their ability to differentiate 5-HT_1 receptor subtypes (17). Neither compound displayed detectable antagonism of the serotonin response in membrane particle preparations. The tetralin derivative 8-OH-DPAT and buspirone are known to be potent and specific partial agonists at 5-HT_{1A} receptors (6, 15). These compounds have no agonist or antagonist effects in the serotonin-stimulated adenylate cyclase system. The arylpiperazine derivative 1-*m*-chlorophenyl)piperazine is a member of a family of compounds believed to have central serotonergic agonist activity. The compound showed neither agonist nor antagonist activity in the NCB.20 cell system. The α-adrenergic agents clonidine and phentolamine did not antagonize the serotonin response. MDL 72222, a potent 5-HT_3 receptor antagonist (19) had no effect in the NCB.20 cell system.

Attempts to measure receptor binding using equilibrium binding assays. Initial binding experiments using the procedure of Peroutka and Snyder (13), carried out with 10 nM [^{3}H]serotonin in the absence of antioxidants, gave results that did not correspond to the potencies obtained with assays of serotonin-activated cyclase. For example, LSD, a potent partial agonist in the adenylate cyclase assay, did not displace bound [^{3}H]serotonin, whereas the tetraline derivative 8-OH-DPAT did displace [^{3}H]serotonin binding (IC_{50} = 30 μM) but displayed no agonistic or antagonistic effect in the adenylate cyclase assay. These discrepancies were found to be due to rapid oxidation of the low concentrations of serotonin in the absence of antioxidants,[3] in agreement with results reported by others (20, 21). Binding data under these conditions represented those of the serotonin oxidation products. When ascorbic acid was used to stabilize serotonin, no specific binding of [^{3}H]serotonin was observed. When [^{3}H]LSD, a ligand with greater affinity for serotonin receptors, was used, the binding measured did not correspond pharmacologically to the serotonin-activated adenylate cyclase obtained. For example, the α-adrenergic agents clonidine and phentolamine competed for the [^{3}H]LSD binding site, indicating that most of the specific binding represented an association with an α-adrenergic receptor. Our inability to quantitate specific binding to the serotonin receptors may have been due to the low affinity of available ligands or to the low density of the receptors. This is consistent with the small degree of activation of adenylate cyclase by serotonin.

Desensitization of the receptor-mediated response. Pretreatment of intact cells with serotonin resulted in a time-dependent decrease in serotonin-stimulated adenylate cyclase activity in subsequently isolated membrane particles (Fig. 5). In these experiments, intact cells were incubated with 0 μM, 10 μM, or 1 mM serotonin and, at the indicated times, treated and control cells were harvested and washed and particles were prepared. Both basal and maximal (10 μM) serotonin-stimulated adenylate cyclase activities were determined. Pretreatment with serotonin had no significant effect on basal enzyme activity. The desensitization is relatively slow; 2–3 hr of preincubation with 10 μM serotonin are required for loss of half of the initial response. After 12 hr of pretreatment, only 20% of the original response remained. Treatment of the cells for periods as long as 24-hr did not desensitize the response significantly more than that seen after 12 hr. A higher concentration of serotonin accelerated the rate of desensitization. In Fig. 5, pretreatment of intact cells with 1 mM serotonin results in a time-dependent desensitization, with a $t_{1/2}$ = 0.5–1 hr. Desensitization at high serotonin concentrations appears to be specific for serotonin, because β-adrenergic agonist-stimulated activity was not affected (data not shown).

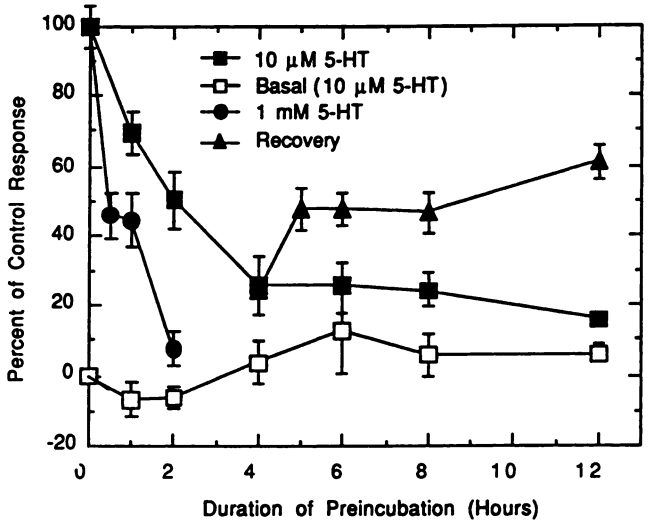

Fig. 5. Time- and concentration-dependent action of serotonin pretreatment of intact cells on basal and serotonin-stimulated adenylate cyclase activity. Intact cells were incubated with 10 μM serotonin (■) or 1 mM serotonin (●). At the indicated times, cell membranes were washed to remove residual serotonin and were assayed for basal cyclase activity (□) or activity with 10 μM serotonin. A fraction of the cells treated with 10 μM serotonin were washed after 4 hr and the incubation (without serotonin) was continued until the indicated times. Then the cells were harvested, particles were prepared, and adenylate cyclase activity was determined in the presence of 10 μM serotonin (▲). *Points*, mean ± standard error of values determined in three separate experiments using 10 μM serotonin and in two experiments for recovery from desensitization and mean ± standard deviation of triplicate determinations from a single representative experiment using 1 mM serotonin.

[3] We did not rigorously demonstrate that the decomposition of [^{3}H]serotonin was a result of oxidation. We use the term oxidation because of the similarity of the decomposition to that referred to in the literature, where the oxidative nature of the decomposition had been determined (20, 21).

Recovery from desensitization occurred slowly. Fig. 5 also illustrates the time-dependent recovery after 4 hr of pretreatment with 10 μM serotonin. Nearly 8 hr were required for recovery of half of the lost response. The rate of recovery is similar after pretreatment for 12 hr with 10 μM serotonin. Full responsiveness of the system returns after several days.

Desensitization also was observed when intact cells were preincubated with the partial agonist BOL; however, a more stringent washing procedure was required to remove the BOL added during the preincubation.

The desensitization induced by serotonin is homologous with respect to stimulation of adenylate cyclase by prostaglandin E_1 and isoproterenol. Pretreatment of cells with 10 μM serotonin for 4 hr resulted in an attenuation of the serotonin response with no decrement of prostaglandin E_1- or isoproterenol-stimulated adenylate cyclase activity (data not shown). Like the effect of serotonin, the BOL-induced desensitization is specific to the serotonin receptor. Desensitization did not appear to be mediated by cAMP; pretreatment of intact cells with 1 mM dibutyryl cAMP or 10 μM prostaglandin E_1, which effectively raise intracellular cAMP levels, has no effect on serotonin-stimulated adenylate cyclase activity (data not shown).

Discussion

The results reported above indicate that the serotonin activation of adenylate cyclase in membrane particles from NCB.20 neuroblastoma cells is receptor mediated, through a G protein signalling system. The presence of a single receptor population coupled to adenylate cyclase is suggested by the observation that none of the agonists or antagonists tested produce biphasic or shallow titration curves. Furthermore, the slopes of the Hill plots of the agonist concentration-response data gave coefficients that varied from 0.9 to 1.2 GTP was required to obtain maximal stimulation of serotonin-activated cyclase. The nonhydrolyzable GTP analogs GTPγS and Gpp(NH)p activate cyclase without serotonin and potentiate the serotonin stimulation of the enzyme. Some cyclase activity was seen when serotonin was used without additional guanine nucleotide. This GTP-independent activity appears to be due to the presence of endogenous GTP in the membrane particles. This is indicated by the finding that GDP and GDPβS inhibited this activity. Both agents act as competitive inhibitors of GTP at the common guanine nucleotide binding site. Signal transduction from the serotonin receptors appears to be manifested through the α subunit of G_s. This is supported by the finding that pretreatment of the intact cells with cholera toxin activated adenylate cyclase and potentiated the effects of serotonin in particles prepared from the treated cells.

Serotonin receptors in intact NCB.20 cells can undergo desensitization and recovery. Desensitization occurs in a time- and concentration-dependent manner and is relatively slow; half of the response to serotonin is lost in 2 to 3 hr. The event observed appears to be analogous to slow desensitization of the β-adrenergic receptors ($t_{1/2}$ = 2.6 hr) (22). The slow desensitization to serotonin in NCB.20 cells is in contrast to what has been reported regarding serotonin receptors in the invertebrate *F. hepatica*. In this organism, rapid desensitization occurs in both intact organisms and isolated membrane preparations (23). Unlike in the report concerning guinea pig hippocampal membranes (6), desensitization of the serotonin response was not observed when particle preparations of NCB.20 cells were incubated with the amine.

The experiments carried out with different serotonin agonists and antagonists were done with the intent of classifying the NCB.20 serotonin receptors within the known serotonin receptor subtypes in mammals (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}, 5-HT_{1D}, 5-HT_2, and 5-HT_3) (24). Table 4 summarizes pharmacological data from the present study and from reports in the literature describing the pharmacological properties of mammalian and trematode receptors. Correlation between potencies of different agonists and antagonists at serotonin-activated adenylate cyclase and their binding affinities to different receptor subtypes was examined. Only those compounds that had detectable effects on the NCB.20 serotonergic response are included in Table 4. Different methodologies were used to determine the potencies of these compounds for the NCB.20 serotonin receptors (adenylate cyclase assay) and the other mammalian receptors (equilibrium binding assay). The conclusions presented here regarding the pharmacological properties of the NCB.20 receptors are based on the rank order of agonists and antagonists, as well as the response to effectors that are selective for certain receptor types. The 5-HT_{1A} receptor type is the only well characterized mammalian serotonin receptor that is reported to activate adenylate cyclase (6). Fig. 6, A and B, shows that there is a very poor correlation of the rank order of potencies of agonists (r^2 = 0.08) or antagonists (r^2 = 0.07) between the NCB.20 receptor and the 5-HT_{1A} receptor. The 5-HT_{1B} receptor is thought to be an autoreceptor (25). The correlation of potencies of agonists (r^2 = 0.02) and antagonists (r^2 = 0.35) between the NCB.20 receptor and the 5-HT_{1B} receptor was also found to be poor (plots not shown). These results indicate that there is no pharmacological similarity between NCB.20 serotonin receptors and either 5-HT_{1A} or 5-HT_{1B} receptors. The ineffectiveness of more specific agents such as 8-OH-DPAT, buspirone, and pindolol supports this conclusion.

The 5-HT_{1C} receptor subtype is known to stimulate the production of inositol phosphates through activation of phospholipase C (30). The correlation between affinities of different agonists and antagonists for serotonin receptors obtained using adenylate cyclase activity as an assay in NCB.20 cells and those obtained using radioligand binding techniques in brain tissue that is rich in 5-HT_{1C} receptors is relatively good (r^2 = 0.83 for agonists; r^2 = 0.82 for antagonists (Fig. 6, C and D). However, the lack of effect of the 5-HT_{1C} receptor antagonist mesulergine (31) suggests that these receptors are not identical. This is supported by the low potency of ketanserin and the inability of cinanserin to antagonize the serotonin effect. Furthermore, experiments utilizing neuroblastoma cells prelabeled with [^{3}H] inositol showed a small increase of inositol phosphate production in response to carbachol but no effect caused by serotonin (data not shown). Thus, differences between the 5-HT_{1C} receptor and the serotonin receptor in NCB.20 cells are also based on the use of different signalling systems.

With regard to the 5-HT_{1D} receptor (26), there is a reasonable correlation of antagonist potencies (r^2 = 0.62), yet no correlation of agonist potencies (r^2 = 0.01) (figures not shown). As observed for the other 5-HT_1 sites (except 5-HT_{1C}), 5-carboxamidotryptamine has greater potency than does serotonin. Unlike the case with the NCB.20 5-HT receptor, LSD is less potent than serotonin at the 5-HT_{1D} binding site.

TABLE 4

Comparison between potencies for serotonin agonists and antagonists determined in NCB.20 serotonin-activated adenylate cyclase and in other types of serotonin receptors

The K_i values for compounds displacing radioligands from different mammalian serotonin receptors were taken from the literature: for 5-HT_{1A} 5-HT_{1B}, 5-HT_{1C}, and 5-HT_2 receptors, the reports by Engel *et al.* (25) and Hoyer *et al.* (17, 18) and for 5-HT_{1D} by Heuring and Peroutka (26); 5-HT_{1B}, 5-HT_{1C} and 5-HT_2. The K_i and K_{act} values for compounds inhibiting or activating adenylate cyclase were from the data of Estey and Mansour (27) for *S. mansoni* (column designated Schisto) and by Northup and Mansour (28) and McNall and Mansour (29) for *F. hepatica* (column designated Fluke).

Compound	−Log [potency] for the indicated receptors							
	NCB.20	5-HT_{1A}	5-HT_{1B}	5-HT_{1C}	5-HT_{1D}	5-HT_2	Fluke	Schisto
Antagonists								
1. Methiothepine	7.9	7.1	7.3	8.8	7.4	8.8	6.1	7.0
2. BOL	7.8						7.5	8.3
3. Mianserin	7.5	5.9	5.3	8.1	6.3	8.1	5.1	
4. LSD	7.5	8.6	6.8	7.9	7.6	8.6	7.5	6.6
5. Cyproheptadine	6.7	6.4	5.3	7.9	5.9	8.5	5.5	6.2
6. Methysergide	6.4				6.9		5.2	5.3
7. Haloperidol	5.6						3.4	5.0
8. Ketanserin	5.3	6.0	5.7	7.1	5.1	8.9	4.5	5.1
9. Spiroperidol	5.3	7.2	5.3	5.9	5.8	8.8	4.0	5.8
Agonists								
10. LSD	7.5	8.6	6.8	7.9	7.6	8.6	7.3	
11. 5-Methoxytryptamine	6.7	8.0	6.4	7.4	8.6	5.6	4.7	
12. 5-Methoxy-dimethyltryptamine	6.7						5.3	
13. Serotonin	6.5	8.5	7.6	7.5	8.5	5.3	5.7	
14. *N,N*-Dimethyserotonin	6.3	7.6	6.0	7.2		6.4		
15. *N*-Methyltryptamine	6.3						4.9	
16. Tryptamine	5.9	6.8	5.0	7.3	7.4		4.8	
17. *N,N*-Dimethyltryptamine	5.8				6.7		5.0	
18. 5-Carboxamidotryptamine	5.4	9.7	8.3	6.2	9.1	4.8		
19. 5,6-Dihydroxytryptamine	5.4	6.0	5.2	6.2		5.0		

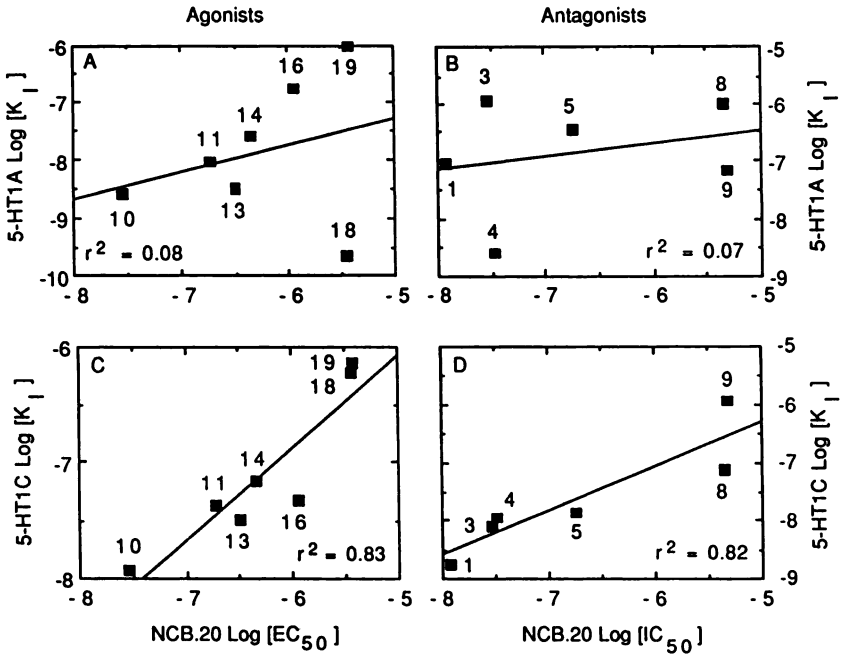

Fig. 6. Correlation between affinities of various serotonin agonists and antagonists for 5-HT_{1A} and 5-HT_{1C} receptors with EC_{50} and IC_{50} values for the adenylate cyclase system from NCB.20 cells. Affinities (K_i) for 5-HT_{1A} and 5-HT_{1C} receptors were obtained from data published by Engel *et al.* (25) and Hoyer *et al.* (17, 18), as summarized in Table 4. The *number* beside each *point* corresponds to the identifying number in Table 4. For the 5-HT_{1A} receptor, a correlation of agonist potencies is presented in A and of antagonist potencies in B. Similarly, for the 5-HT_{1C} receptor, a correlation of agonist potencies is presented in C and of antagonist potencies in D. Correlation lines were determined by nonweighted linear regression.

The 5-HT_2 binding site has been shown to modulate phosphatidylinositol turnover (32, 33). Our results show no correlation of antagonist potencies at the NCB.20 cyclase system with binding affinities reported in the literature ($r^2 = 0.23$), yet there is a relatively good correlation with the agonist potencies ($r^2 = 0.72$) (figure not shown). The inability of the potent 5-HT_2 receptor antagonists such as spiroperidol and cinanserin to antagonize the NCB.20 serotonin response strongly suggests that the two receptors are not identical.

The lack of effect of the 5-HT_3 receptor antagonist MDL 72222 and the piperazine derivative 1-(*m*-chlorophenyl)piperazine indicates that the NCB.20 receptor is probably unlike peripheral 5-HT_3 receptors or the recently identified central 5-HT_3-like binding site (34, 35). The high potency of the ergolines for the NCB.20 receptor is also uncharacteristic of 5-HT_3 receptors.

Atypical serotonin receptors positively coupled to adenylate cyclase have been described in guinea pig hippocampal membranes (6) and in mouse embryo colliculi neurons in primary culture (36, 37). These so-called 5-HT_4 receptors share several

pharmacological properties with the NCB.20 receptor. 5-methoxytryptamine and serotonin are equipotent, whereas 5-carboxamidotryptamine is a less potent agonist. All are relatively insensitive to 8-OH-DPAT, spiroperidol, mesulergine, ketanserin, pindolol, and MDL 72222. However, these receptors were shown to be insensitive to the potent NCB.20 receptor effectors LSD and methiothepine.

A conclusion can be made that, although the serotonin receptors in NCB.20 cells share many of the pharmacological characteristics of 5-HT_{1C} receptors and of the recently described atypical serotonin receptors (5-HT_4) (36, 37), they are not identical to any of the known serotonin receptor subtypes. The possibility should be considered that the novel pharmacological properties of the receptor are the result of a mutation or a product of additional cellular components uniquely present or absent in the NCB.20 cell. This is an inherent problem associated with an established clonal cell culture system and is difficult to address until a receptor analog is identified in another tissue.

Serotonin stimulates adenylate cyclase in the parasitic trematodes *S. mansoni* and *F. hepatica* (1, 27–29). The response is receptor mediated and mechanistically appears to be similar to the activation of adenylate cyclase by β-adrenergic receptors in mammals. The relative potency of agonists ($r^2 = 0.63$) and antagonists ($r^2 = 0.67$) for the *F. hepatica* receptor, also determined using the adenylate cyclase assay, is similar to their potency for the NCB.20 receptor. The relative potency of antagonists for the *S. mansoni* receptor, determined with the adenylate cyclase assay, is similar to their potency for the NCB.20 receptor ($r^2 = 0.73$). The common tryptamine derivatives tested showed little differences in their affinities for either the *F. hepatica* receptor or the NCB.20 receptor; LSD was considerably more potent in both. The pharmacological similarity of NCB.20 serotonin receptors to serotonin receptors in parasitic helminths and the fact that both receptor types use cAMP as a second messenger suggest an evolutionary conservation of the properties and function of these receptors.

Because the NCB.20 cells are derived from mouse neuroblastoma cells and hamster brain (9), it is relevant here to discuss similarities between some biochemical effects of serotonin on the brain and in the parasitic trematodes, where the serotonin receptors linked to cyclase are abundant. Serotonin was found to stimulate glycogenolysis (38), phosphorylation of a neuronal protein (39), and activity of adenylate cyclase (2–4) in different parts of the brain. In the trematodes *F. hepatica* and *S. mansoni*, serotonin-linked adenylate cyclase appears to play a role in the stimulation of glycolysis and glycogenolysis and in the activation and phosphorylation of glycolytic enzymes (1, 40–42). These results point to a novel serotonin receptor that is different from those that were identified on the basis of binding data. Although more work is needed to investigate the physiological role of serotonin-activated adenylate cyclase in mammalian cells, the possibility should be considered that these novel serotonin receptors, like the trematode receptors, may be linked to control mechanisms involved in glycolysis and protein phosphorylation. The NCB.20 cell line provides a system in which receptor-specific probes may be developed to address questions relevant to the physiological functions of these receptors in mammalian tissues.

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