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Transfection of Human 5-Hydroxytryptamine$_{1A}$ Receptors in NIH-3T$_3$ Fibroblasts: Effects of Increasing Receptor Density on the Coupling of 5-Hydroxytryptamine$_{1A}$ Receptors to Adenylyl Cyclase

ANNIE VARRAULT, LAURENT JOURNOT, YVES AUDIGIER, and JOËL BOCKAERT
Centre CNRS-INSERM de Pharmacologie-Endocrinologie, 34094 Montpellier Cedex 5, France
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SUMMARY

Human serotonin [5-hydroxytryptamine (5-HT)$_{1A}$] receptors have been transfected in NIH-3T$_3$ cells, and their pharmacology and coupling to adenylyl cyclase have been analyzed. Three cellular preparations were used, 1) monoclonal cell lines (clones 6, 2B, and 4B), expressing 45, 280, and 500 fmol of 5-HT$_{1A}$ receptors/mg of protein, respectively; 2) clones 6, 2B, and 4B in which the concentration of 5-HT$_{1A}$ receptors was increased after stimulation of the glucocorticoid-inducible promoter with dexamethasone; and 3) polyclonal cell lines that expressed an increasing amount of 5-HT$_{1A}$ receptor as a function of cell passage. The transfected 5-HT$_{1A}$ receptors inhibited basal, forskolin-stimulated, and isoproterenol-stimulated adenylyl cyclase. The inhibition was dependent on the receptor density expressed, increasing from 60% at low density (45 fmol/mg) to 90% at a density higher than 280 fmol/mg. The pharmacology of the 5-HT$_{1A}$ receptor was studied, with particular attention being paid to the behavior of some agonists. These pharmacological characteristics are similar to those of 5-HT$_{1A}$ receptors in hippocampus but different from those of 5-HT$_{1A}$ in cerebral cortex. Analysis of the potencies and efficacies of the full agonist 5-HT and the partial agonist ipsapirone, as a function of receptor density in the three cellular populations used, revealed that 1) the efficacies of the full and partial agonists increased with the receptor density; 2) the EC$_{50}$ values of the full and partial agonists were not shifted to the left when the receptor density was increased (based on the increase in efficacy and considering the classical pharmacological models of receptor-drug action, a 9–10-fold shift was expected); and 3) the ratio between the efficacies of the full agonist 5-HT and the partial agonist ipsapirone was not modified when the receptor concentration was increased or when the GTP-binding protein availability was decreased. The results indicate that neither the classical nor the operational model of drug-receptor action can be used to describe the coupling of 5-HT$_{1A}$ receptors to adenylyl cyclase in transfected NIH-3T$_3$ cells. One of the explanations could be that 5-HT$_{1A}$ receptors and GTP-binding proteins are coupled in functional domains (almost precoupled), rather than distributed in homogeneous compartments in which they are free to diffuse.

In vertebrates, serotonin (5-HT), like several other neurotransmitters, triggers its physiological and behavioral functions by interacting with both G protein-coupled receptors (5-HT$_1$, 5-HT$_2$, and 5-HT$_4$ receptor types) (1, 2) and ionic channel receptors (5-HT$_3$) (3, 4). Based on binding studies, 5-HT$_1$ receptors have been divided into four subtypes, 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1C}$, and 5-HT$_{1D}$ (1). Recently, human 5-HT$_{1A}$ (5, 6), rat 5-HT$_{1A}$ (7), rat 5-HT$_{1C}$ (8), rat 5-HT$_2$ (9), rat 5-HT$_{1B}$ (10), and rat (11) and human 5-HT$_{1D}$ (12) and 5-HT$_3$ (4) receptors have been cloned. Cloning of 5-HT$_{1A}$ receptors has already helped investigators to understand one intriguing pharmacological particularity of the 5-HT$_{1A}$ receptor subclass, its ability to interact with β-adrenergic antagonists (13). This is easily understood when the amino acid sequences of the central part of transmembrane domain VI of both β_2-adrenergic receptors and 5-HT$_{1A}$ receptors are compared; 18 of 20 amino acid residues are identical (6). However, several other pharmacological properties of 5-HT$_{1A}$ receptors remain to be explained. 1) Classically, receptor stimulation results in the inhibition of vasoactive intestinal peptide- and/or forskolin-stimulated adenylyl cyclase (1, 14). However, in rat and guinea pig hippocampus, it has been shown that 5-HT$_{1A}$ receptors stimulate basal adenylyl cyclase activity (15, 16). Therefore, the problem is to determine whether there are one or two different 5-HT$_{1A}$ receptors responsible for these two opposing couplings to adenylyl cyclase. 2) 5-HT$_{1A}$ receptors inhibiting cAMP in hippocampal

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; 8-OH-DPAT, 8-hydroxy-(2-(*N*,*N*-di)propylamino)-1,2,3,4-tetrahydronaphthalene; 5-CT, 5-carboxamidotryptamine; 5-MeOT, 5-methoxytryptamine; 5-MeO-*N*,*N*-DMT, 5-methoxy-*N*,*N*-dimethyltryptamine; *N*-MT, *N*-methyltryptamine; *N*,*N*-DMT, *N*,*N*-dimethyltryptamine; *d*-LSD, *d*-lysergic acid diethylamide; IBMX, isobutylmethyl xanthine; EC$_{50}$, half-maximal efficacy; G protein, GTP-binding protein.

neurons and those inhibiting cAMP in cortical neurons possess different pharmacological properties (14). In particular, metergoline and methysergide are agonists in hippocampal neurons and competitive antagonists in cortical neurons. In hippocampal neurons 8-OH-DPAT is a full agonist with a better potency than 5-HT, whereas it is a partial agonist in cortical neurons (14). Similarly, the activities of ipsapirone, a new anxiolytic drug, and 8-OH-DPAT as full agonists, partial agonists, or even antagonists of 5-HT_{1A} responses have been reported in many electrophysiological studies (17–21). Do these pharmacological properties rely on differences in the primary structure of 5-HT_{1A} receptors, in the stoichiometry between 5-HT_{1A} receptors and G proteins, or in the ionic environment of the receptors? Few studies have been published concerning the influence of increasing the number of receptors expressed in a given cell line on the behavior of a partial agonist. Generally, this problem is examined after a decrease in the number of receptors obtained after desensitization or alkylation of receptors. We have transfected human 5-HT_{1A} receptors under the control of a glucocorticoid-inducible promoter in NIH-$3T_3$ fibroblasts. This report deals with the influence of increasing the number of 5-HT_{1A} receptors on full and partial agonist dose-inhibition curves for adenylyl cyclase. Cells expressing different amounts of 5-HT_{1A} receptors were obtained several ways, i.e., isolation of clones expressing different densities of receptors, stimulation of receptor expression in these clones by dexamethasone, and, finally, increase in receptor concentration in a polyclonal cell population after different numbers of cell passages.

Experimental Procedures

Materials. Drugs were obtained from the following sources: GTP, cAMP, ATP, IBMX, NAD, phosphocreatine, 5-HT, *N*-MT, *N,N*-DMT, 5-MeO-*N,N*-DMT, and (−)-isoproterenol were from Sigma Chemical Company (St. Louis, MO); ipsapirone from J. Traber, Troponwerke GmbH and Co. (Cologne, Germany); buspirone from F. D. Yocca, Bristol Myers Co.; RU 24969 and RU 28253, from J. F. Pujol, Roussel-Uclaf (Romainville, France); 5-CT from P. P. A. Humphrey, Glaxo Group Research (Hertfordshire, UK); metergoline and methysergide from H. Gozlan, Faculté de Médecine, Pitié Salpêtrière (Paris, France); spiperone and ketanserine from J. Leysen, Janssen Pharmaceutica (Beerse, Belgium); haloperidol from Rhône-Poulenc Santé (Lyon, France); ICS 205 930, mesulergine, and *d*-LSD from G. Engel, Sandoz Ltd (Basel, Switzerland); mianserine from Organon (Oss, Holland); 8-OH-DPAT from Research Biochemical Inc. (Wayland, MA); oxymetazoline from Schering Corp. (Kenilworth, NJ); and pertussis toxin from List Biological Laboratories (Wayland, MA). Radioligands were purchased from Du Pont-New England Nuclear ([α-^{32}P]NAD, 800 Ci/mmol) or Amersham ([^{3}H]8-OH-DPAT, 110 Ci/mmol; [2-^{3}H]adenine, 24 Ci/mmol). Dulbecco's modified Eagle medium 01885 and fetal calf serum were from GIBCO BRL.

Expression vector and cell transfection. The *Xba*I-*Bam*HI fragment from the human 5-HT_{1A} receptor genomic clone G-21 (5), containing the complete coding sequence, was subcloned into the expression vector pMSG (Pharmacia). This vector contains the mouse mammary tumor virus long terminal repeat, which acts as a glucocorticoid-inducible promoter for the expression of the 5-HT_{1A} receptor gene, and the gene coding for xanthine-guanine phosphoribosyl transferase, under the control of SV40 early promoter.

The calcium phosphate method was used for permanent transfection of NIH-$3T_3$ cells (22). Cells were selected in Dulbecco's modified Eagle medium plus 10% dialyzed fetal calf serum, containing mycophenolic acid (78 μM), hypoxanthine (100 μM), xanthine (1.4 mM), thymidine (47 μM), and aminopterine (4.5 μM). Cells were selected and either cultured without subcloning or subcloned by limiting dilution. Three subclones were used in this study, referred as clones 6, 4B, and 2B.

Cell culture. The transfected NIH-$3T_3$ cells were grown as previously described (23).

Radioligand binding assays. Cells were washed with 9% NaCl and then with 50 mM Tris·HCl, pH 7.4, 1 mM $MgCl_2$ ($T_{50}M_1$) or another buffer, as indicated. They were scraped with a rubber policeman in $T_{50}M_1$ and homogenized 20 times with a glass-Teflon Potter homogenizer. The pellet (39,000 × *g*, 15 min, 4°) was resuspended in $T_{50}M_1$ and heated to 37° for 10 min (to eliminate the possible contamination with 5-HT that might be present in the serum). After another centrifugation, the pellet was washed once in $T_{50}M_1$ and resuspended in $T_{50}M_1$ at a final protein concentration of 200 μg/ml.

Incubation (40 min, 30°) of membranes (60 μg of protein) with the appropriate concentration of [^{3}H]8-OH-DPAT (0.15–20 nM), in a final volume of 100 μl, was terminated by addition of 900 μl of ice-cold $T_{50}M_1$, rapid vacuum filtration through Whatman GF/C filters, and three washes with 5 ml of ice-cold $T_{50}M_1$. Specific binding was defined as the amount of radioligand binding inhibited by 10 μM 5-HT. Determinations were performed in triplicate. Protein content was measured using the method described by Lowry *et al.* (24), with bovine serum albumin as a standard.

Whole-cell cAMP assay. The cAMP content of cells was measured by the prelabeling technique previously described (25). Cells were cultured in 12-well plates. When apparent confluency was reached, cells were incubated with 2 μCi/ml [^{3}H]adenine. After 2 hr, the cultures were washed and incubated with 0.75 mM IBMX, 10 μM forskolin, and the indicated drugs (all prepared in culture medium), in a final volume of 1 ml, for 6 min at 37°. The reaction was stopped by aspiration of the medium and addition of 1 ml of ice-cold 5% trichloracetic acid. Cells were scraped with a rubber policeman, and 100 μl of a solution of 5 mM ATP and 5 mM cAMP were added to the mixture. Cellular proteins were centrifuged at 5000 × *g*. [^{3}H]ATP and [^{3}H]cAMP were separated by sequential chromatography on Dowex and alumina columns (26). cAMP formation was expressed as percentage conversion:

$$\frac{[^3\text{H}]\text{cAMP}}{[^3\text{H}]\text{cAMP} + [^3\text{H}]\text{ATP}} \times 100$$

ADP-ribosylation with pertussis toxin. Membranes of control or transfected NIH-$3T_3$ cells (with or without dexamethasone treatment) were prepared as previously described (27) and stored at −80°, at a final protein concentration of 0.5–1.0 mg/ml. ADP-ribosylation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were conducted on aliquots of membranes containing 50 μg of protein, as previously described (27).

Data analysis. Estimates of EC_{50} (potency) and E_{max} (efficacy) were obtained by fitting the equation (28)

$$E = E_{max} \frac{L}{EC_{50} + L}$$

to the observed response E, as a function of drug concentration L, using nonlinear regression analysis (Grafit; Erithacus Software Ltd., Staines, UK).

Results

Permanent expression of functional 5-HT_{1A} receptors in NIH-$3T_3$ fibroblasts. NIH-$3T_3$ cells did not contain any detectable [^{3}H]8-OH-DPAT binding sites either before or after transfection with the pMSG vector alone (data not shown). In contrast, when transfected with the pMSG vector containing the *Xba*I-*Bam*HI fragment of the human 5-HT_{1A} receptor genomic clone, NIH-$3T_3$ cells expressed [^{3}H]-8-OH-DPAT binding sites of only one affinity, whatever the subclone used. Clones 6, 4B, and 2B expressed 45, 280, and 500 fmol/mg, respectively

(Fig. 1). The K_d of these binding sites was 0.80, 0.53, and 0.66 nM, respectively.

In a parallel study, we used the polyclonal cell population. We noticed that the concentration of 5-HT$_{1A}$ binding sites increased as a function of the cell passage number. The concentration of receptors was 110 fmol/mg at passage 2 and 600 fmol/mg at passage 20. Thereafter, the concentration of receptors became almost stable until passage 30 (Fig. 2, A and B). Scatchard analysis indicated that the K_d did not vary during the cell passages and was similar to that obtained in the different subclones (K_d = 0.33 nM and 0.40 nM after 2 and 30 passages, respectively) (data not shown).

The inhibition of forskolin-stimulated cAMP accumulation was a function of the density of 5-HT$_{1A}$ receptors expressed, both in the subclones and in the polyclonal population (Fig. 2, C and D). Although the relationships between receptor density and cAMP inhibition were not superimposable, the maximal inhibitions were similar (80–90%) (Fig. 2, C and D). Indeed, one can notice that, when 280 fmol/mg levels of 5-HT$_{1A}$ receptors (clone 4B) were expressed, 80–90% inhibition of the adenylyl cyclase by 5-HT was obtained (Fig. 2D), whereas, for the same density of receptors expressed in the polyclonal cells, only a 60% inhibition was reached. This can be tentatively explained as follows: all the polyclonal cells expressing 280 fmol/mg or more of 5-HT$_{1A}$ receptors are limited to an adenylyl cyclase inhibition of 90%. In addition, they are mixed with cells expressing few or no 5-HT$_{1A}$ receptors. It appears that the mean density of receptors was 280 fmol/mg, whereas the mean inhibition of adenylyl cyclase was 60%. This is circumstantial.

In these transfected cells, 5-HT$_{1A}$ receptors also inhibited nonstimulated and β-adrenergic-stimulated adenylyl cyclase activity to a similar extent (Fig. 3). We did not observe any stimulation of basal cAMP production induced by 5-HT$_{1A}$ receptors in our system (data not shown).

Pharmacological characterization of the transfected 5-HT$_{1A}$ receptor. The binding characteristics of various 5-HT$_{1A}$ receptor-interacting drugs at human (6) and rat (7) 5-HT$_{1A}$ receptors transfected in eukaryotic cells have already been published. However, no detailed study has been reported

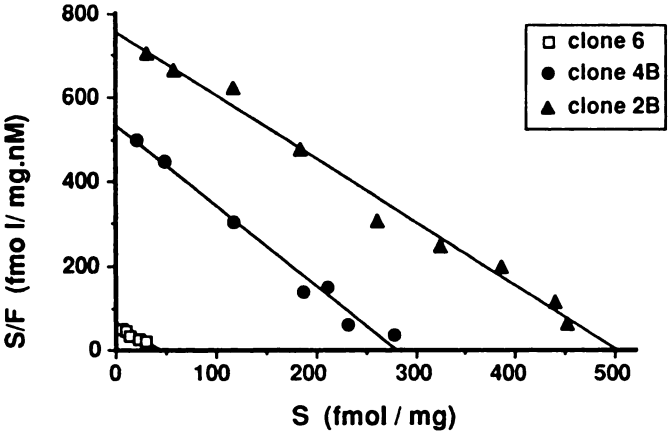

Fig. 1. Specific binding of [^{3}H]8-OH-DPAT to membranes from three clones of NIH-3T$_3$ cells transfected with the human 5-HT$_{1A}$ receptor. Scatchard analyses of binding of [^{3}H]8-OH-DPAT to clones 6, 4B, and 2B, in 50 mM Tris·HCl, pH 7.4, 1 mM $MgCl_2$, were carried out. The data are well fit by a one-site model, with K_d = 0.80, 0.53, and 0.66 nM and B_{max} = 45, 280, and 500 fmol/mg of protein for clones 6, 4B, and 2B, respectively.

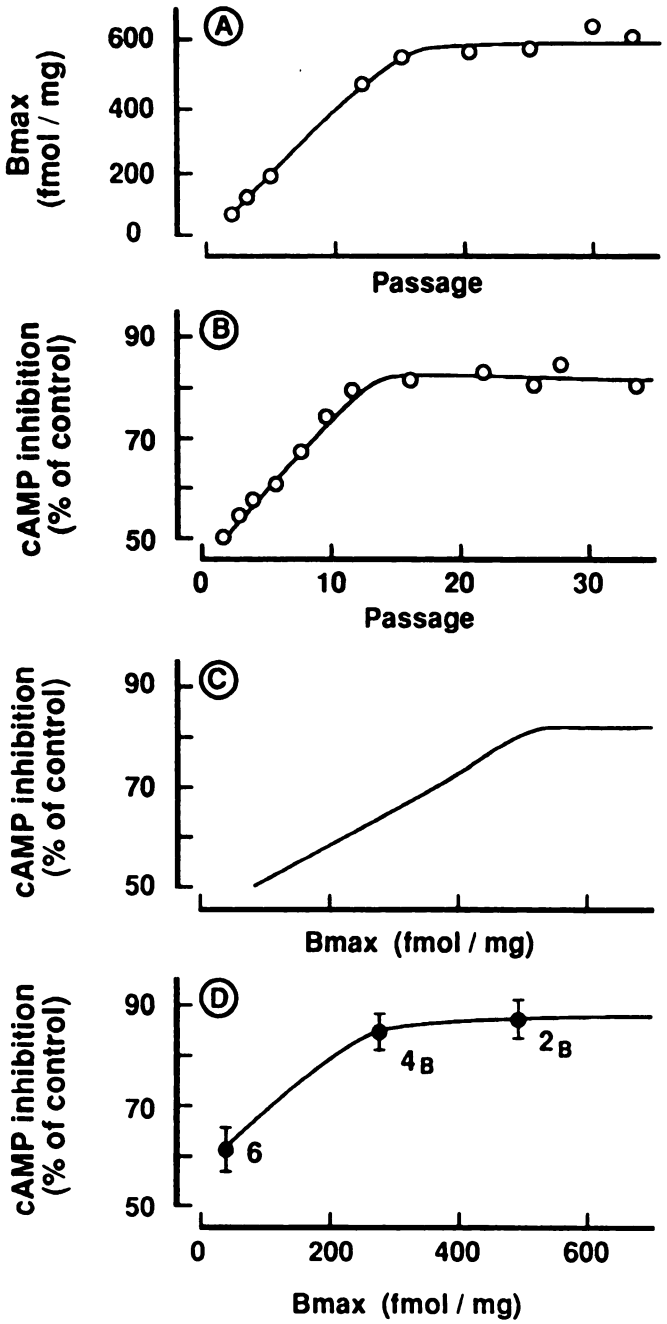

Fig. 2. Evolution of 5-HT$_{1A}$ receptor expression and cAMP inhibition with the number of passages of polyclonal NIH-3T$_3$/5-HT$_{1A}$ cells and for the three clones under study. A, The concentrations of 5-HT$_{1A}$ receptors (B_{max}) were determined by Scatchard analysis of [^{3}H]8-OH-DPAT binding to membranes of polyclonal cells, as indicated in Fig. 1, and plotted against the number of passages. B, The maximal cAMP (10 μM forskolin) inhibition induced by 5-HT (10 μM) was determined with polyclonal cells at different passages. C, The maximal cAMP inhibition (10 μM forskolin) induced by 10 μM 5-HT was plotted against the concentration of 5-HT$_{1A}$ receptors in the polyclonal cells at different passages; this curve is derived from A and B. D, The maximal cAMP inhibition induced by 5-HT was plotted against B_{max} for each monoclonal cell line tested (clones 6, 4B, and 2B) (values are the means ± standard errors of four separate experiments).

on the agonist, partial agonist, or antagonist properties of some of these drugs for adenylyl cyclase. In the present study, we have used clone 4B, expressing 280 fmol/mg of protein. The cAMP production was measured during a 6-min incubation

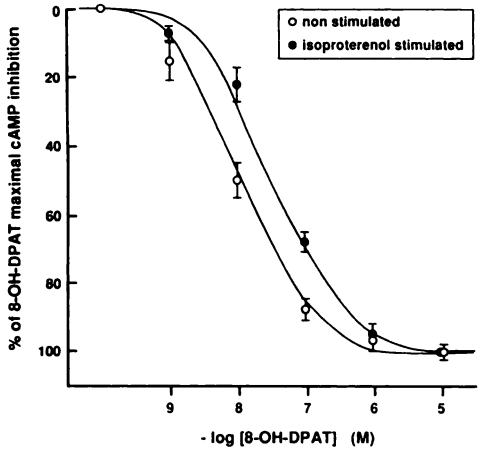

Fig. 3. Inhibition of nonstimulated or isoproterenol-stimulated adenylyl cyclase by 8-OH-DPAT. Cells were incubated in the absence or presence of 1 μM isoproterenol and increasing concentrations of 8-OH-DPAT. Conversion of [^{3}H]ATP to [^{3}H]cAMP was determined after a 6-min incubation period at 37°. In the absence of 8-OH-DPAT, the percentage of conversion was 0.100 ± 0.008 and 9.9 ± 0.3% (three experiments) for unstimulated and isoproterenol-stimulated cAMP production, respectively. Data are expressed as the percentage of 8-OH-DPAT maximal inhibitory effect; 100% corresponded to 70 ± 4 and 80 ± 3% (three experiments) inhibition of cAMP production for unstimulated and isoproterenol-stimulated cAMP formation, respectively. The values are the means ± standard errors of three separate experiments, each performed in duplicate.

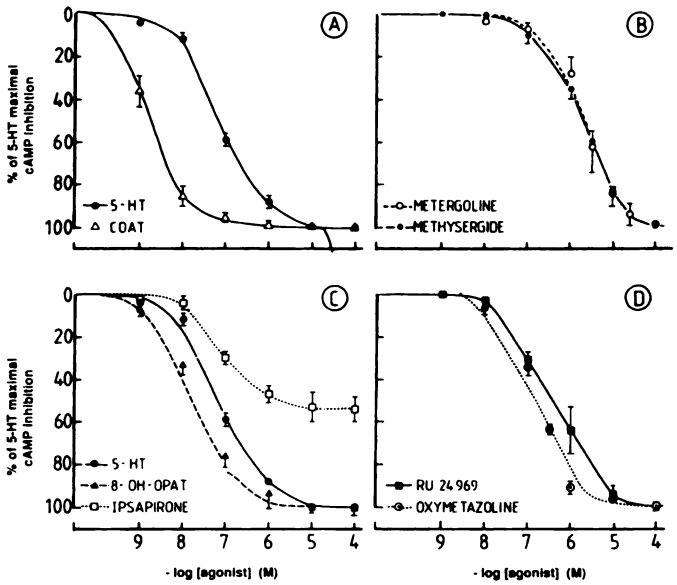

Fig. 4. Pharmacological characteristics of 5-HT$_{1A}$ receptors in transfected NIH-3T$_3$ cells. Effects of different agonists on forskolin-stimulated cAMP formation in NIH-3T$_3$/5-HT$_{1A}$ cells (clone 4B). Cells were incubated in the presence of 10 μM forskolin and increasing concentrations of agonists. Conversion of [^{3}H]ATP to [^{3}H]cAMP was determined after a 6-min incubation period at 37°. In the absence of 5-HT, the percentage of conversion was 8.7 ± 0.2% (60 experiments). Data are expressed as a percentage of the maximal inhibitory effect of 5-HT; 100% corresponded to 85 ± 2.5% (15 experiments) inhibition of cAMP production. The values are the means ± standard errors of three to five separate experiments, each performed in duplicate. *COAT*, 5-CT.

period. The response was linear until 15 min (data not shown), and we did not observe any homologous desensitization of the response during that period (data not shown). As expected from binding studies in rat brain (29), as well as from cAMP measurement in mouse hippocampal and cortical neurons (14), 5-CT and 8-OH-DPAT were more potent than 5-HT in inhibiting adenylyl cyclase in transfected NIH-3T$_3$ cells (Fig. 4, A and C). As in mouse and guinea pig hippocampal neurons (14, 30), two ergolines (methysergide and metergoline) were full agonists (Fig. 4B). This was interesting, because these ergolines are pure antagonists of 5-HT$_{1A}$ receptors in mouse cortical neurons (14).

Similarly, arylpiperazine derivatives, such as buspirone and ipsapirone, were partial agonists on hippocampal neurons (14, 31) but were antagonists on cortical neurons (14). Here, ipsapirone behaved as a potent partial agonist (Fig. 4C). Finally, two classical (RU 24969, Fig. 4D; RU 28253, data not shown) (32) and a nonclassical (oxymetazoline, Fig. 4D) (33) 5-HT$_{1A}$ agonist were also full agonists on human 5-HT$_{1A}$ receptors transfected in NIH-3T$_3$ cells.

A good correlation was obtained between the pEC$_{50}$ values for 10 5-HT$_{1A}$ receptor-interacting drugs to inhibit cAMP production in hippocampal neurons and their respective pEC$_{50}$ values to inhibit forskolin-stimulated cAMP accumulation in transfected NIH-3T$_3$ cells ($r = 0.882$, $p < 0.01$) (Table 1). Similarly, the pK_i values of a series of 10 antagonists that inhibit the 5-HT-induced inhibition of cAMP production in hippocampal neurons and NIH-3T$_3$ transfected cells were very close (Table 1).

Effect of receptor density on the dose-response curves of 5-HT and ipsapirone. In order to investigate the mechanisms of partial agonism, we modulated the stoichiometry between receptors and G proteins, either by varying receptor concentration at constant G proteins density or by varying the concentration of functional G proteins at constant receptor amounts. We had three possible ways to modify the 5-HT$_{1A}$ receptor number in the transfected NIH-3T$_3$ cells. The first was to use different subclones (clones 6, 4B, and 2B), the second was to stimulate in these clones the mouse mammary tumor virus long terminal repeat, which acts as a glucocorticoid-inducible promoter for the transfected 5-HT$_{1A}$ receptor gene, and the last was to take polyclonal cells at different passages.

When the density of 5-HT$_{1A}$ receptors increased from 45 to 500 fmol/mg (clones 6 and 2B, respectively), the efficacy of 5-HT in inhibiting cAMP production increased from 60 to 90% (Fig. 5A). This was not accompanied by any increase in potencies (EC$_{50}$ = 110 ± 10 nM and 80 ± 10 nM for clones 6 and 2B, respectively) (Fig. 5, A and B). The efficacy of ipsapirone in inhibiting cAMP production also increased from 30 to 58% when the density of 5-HT$_{1A}$ receptors increased from 45 to 500 fmol/mg (Fig. 5A), with no significant change in potencies (EC$_{50}$ = 80 ± 30 nM and 130 ± 30 nM for clones 6 and 2B, respectively) (Fig. 5, A and B). As seen in Fig. 5B, the ratio between the potencies of 5-HT and ipsapirone was similar in the two clones. Similar qualitative results were obtained when two populations of polyclonal cells (taken at passages 4 and 20), expressing 150 and 600 fmol of 5-HT$_{1A}$ receptors/mg, were used (Fig. 5, C and D).

Dexamethasone increased receptor density by 3-, 1.6-, and 1.2-fold in clones 6, 4B, and 2B, respectively (Fig. 6). Measurement of the relative efficacies of 5-HT and ipsapirone in inhibiting cAMP production indicated that they were not significantly modified by increasing the cellular density of 5-HT$_{1A}$ receptors (Fig. 6).

Fig. 7 shows the efficacy of ipsapirone, relative to that of 5-HT, as a function of cellular concentrations of 5-HT$_{1A}$ receptors

TABLE 1

pK_i values of different 5-HT antagonists and pEC$_{50}$ values for agonists in primary cultures of mouse hippocampal neurons and in NIH-3T$_3$/5-HT$_{1A}$ cells (clone 4B)

Values for hippocampal neurons are from Dumuis *et al.* (14).

	pK_i			pEC$_{50}$	
	Mouse hippocampal neurons	NIH-3T$_3$ cells transfected with human 5-HT$_{1A}$		Mouse hippocampal neurons	NIH-3T$_3$ cells transfected with human 5-HT$_{1A}$
(±)-Pindolol	7.9	7.2	5-HT	7.3	7.2
Spiperone	7.6	7.5	5-CT	7.0	8.7
Methiothepin	7.5	6.3	5-MeO-*N*,*N*-DMT	7.3	7.0
Propranolol	6.4	6.2	5-MeOT	7.0	7.2
Haloperidol	6	5.3	*N*-MT	6.3	5.7
ICS 205 930	<5	<5	8-OH-DPAT	8.1	7.8
Mesulergine	6.2	<5	Ipsapirone	7.1	7.1
Ketanserine	5.5	<5	Buspirone	6.9	6.7
Mianserine	5.8	<5	RU 28253	6.8	7.4
Prazosin	Inactive	Inactive	RU 24969	7.1	6.4
Methysergide	Agonist	Agonist	*d*-LSD	7.4	7.5
Metergoline	Agonist	Agonist	Methysergide	6.3	5.6

in the different cellular models used. Although there was a slight increase in the ratio when the cellular densities of 5-HT$_{1A}$ receptors increased from 45 to 680 fmol/mg, it was not significant. Increase in receptor concentration never shifted the dose-response curves leftward for ipsapirone, in any of the cellular models used.

Absence of variation in G$_i$ density in the different cellular preparations of NIH-3T$_3$ used. It was important to verify that the densities of G$_i$ proteins, known to couple 5-HT$_{1A}$ receptors to adenylyl cyclase, were the same in the different cellular preparations used. Fig. 8 indicates that 40-kDa (G$_{i2}$) and 41-kDa (G$_{i1}$ or G$_{i3}$) ADP-ribosylated pertussis toxin substrates were similar in clones 6, 4B, and 2B before and after dexamethasone treatment, as well as in the polyclonal population at different passages.

Lack of modification of the partial agonism of ipsapirone by decreasing cellular density of G$_i$ proteins. In these experiments, we used clone 4B and the polyclonal population. The results with clone 4B are described in Fig. 9. Decreasing the density of G$_i$ proteins coupled to 5-HT$_{1A}$ receptors by increasing the concentrations for pertussis toxin treatment did not modify the efficacy of ipsapirone, relative to 5-HT.

Discussion

We transfected the human 5-HT$_{1A}$ receptor in NIH-3T$_3$ fibroblasts by using the pMSG vector, which contains a glucocorticoid-inducible promoter. This was done to obtain a cell line in which the 5-HT$_{1A}$ receptor concentration could be increased, in order to study its influence on agonist- and partial agonist-induced adenylyl cyclase inhibition. We used three different populations of transfected cells. The first was composed of three clonal cell lines, 6, 4B, and 2B, expressing 45, 280, and 500 fmol/mg of protein, respectively. The second consisted of the same clones treated with dexamethasone to increase 5-HT$_{1A}$ receptor expression. Finally, the third consisted of different polyclonal populations of cells expressing different densities of 5-HT$_{1A}$ receptors. We knew that interpreting the results with these multiclonal populations would be more difficult. However, we thought that it would be interesting to compare them, although with caution, with the clonal cell lines. Indeed, it is likely that, *in vivo*, a heterogeneity of cellular receptor density does exist.

An interesting observation was made with these polyclonal cells; increasing the number of subcultures increased the 5-HT$_{1A}$ receptor density. Seuwen *et al.* (34) demonstrated that, in the hamster lung fibroblast line CCL39, stimulation of native 5-HT$_{1B}$ receptors or transfected α_2-adrenergic receptors (35) induced cell division. 5-HT$_{1B}$, α_2-adrenergic, and 5-HT$_{1A}$ receptors are known to inhibit adenylyl cyclase. Because the serum used to culture NIH-3T$_3$ cells contained 5-HT, it is possible that cells expressing the highest concentration of 5-HT$_{1A}$ receptors are selected because of their highest rate of division. We recently observed a potentiation between epidermal growth factor and 5-HT$_{1A}$ receptors to increase [^{3}H]thymidine incorporation in transfected NIH-3T$_3$ cells.[1]

In all the monoclonal or polyclonal cells used, [^{3}H]8-OH-DPAT labeled only one population of binding sites having a high affinity (0.5 ± 0.2 nM, 15 experiments). A low affinity state could be generated in the presence of GTP (data not shown). The presence of only one binding site for [^{3}H]8-OH-DPAT in the absence of GTP has also been found in hippocampus (29). The interpretation is probably that all the [^{3}H]8-OH-DPAT binding sites are associated with a G protein, i.e., the G protein concentration is not limiting even when 680 fmol/mg are expressed. Such an interpretation has been given recently to explain the presence of binding sites of only one affinity for the transfected 5-HT$_{1D}$ receptors in Chinese hamster ovary cells (12). Human and rat 5-HT$_{1A}$ receptors have already been transfected in COS-7, HeLa, and Ltk cells (6, 7). When very high 5-HT$_{1A}$ receptor concentrations were expressed (19 and 1.9 pmol/mg), high and low affinity [^{3}H]8-OH-DPAT binding sites could be detected (6, 7).

The pharmacology of the human 5-HT$_{1A}$ receptor was analyzed in terms of agonist, partial agonist, and antagonist responses, by measuring the inhibition of cAMP production. It is interesting to note that this pharmacology is similar to that found for 5-HT$_{1A}$ receptors in mouse hippocampus but significantly different from that for 5-HT$_{1A}$ in cortical neurons (14). In particular, 8-OH-DPAT was a full agonist and more potent

[1] A. Varrault, J. Bockaert, and C. Waeber. Activation of 5-HT$_{1A}$ receptors expressed in NIH-3T$_3$ cells induces focus formation and potentiates EGF effect on DNA synthesis. Manuscript in preparation.

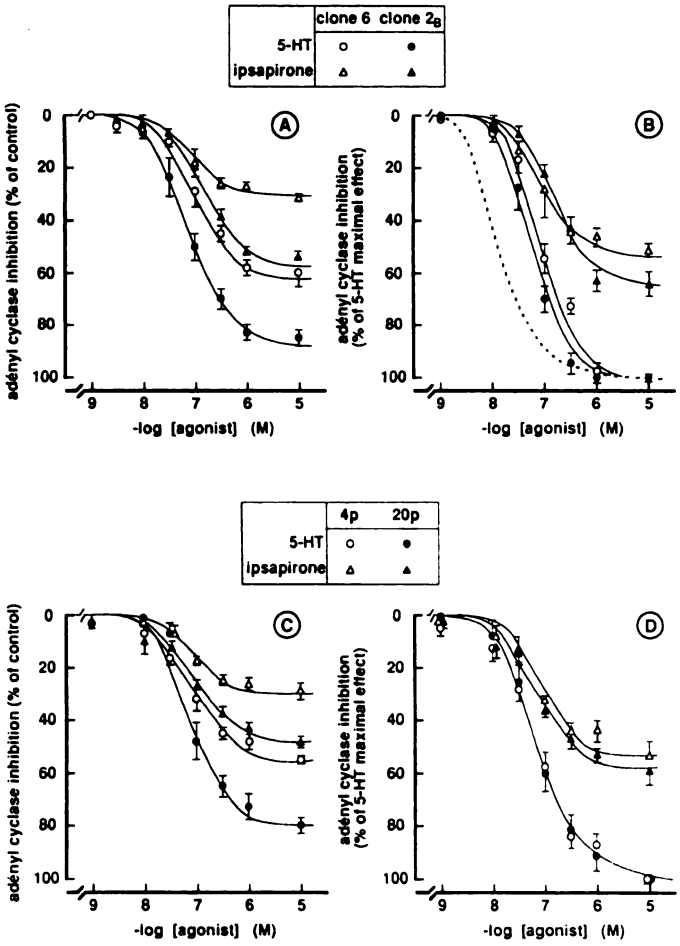

Fig. 5. Effect of the receptor densities of the polyclonal and monoclonal cells on the dose-response curves for 5-HT and ipsapirone. Polyclonal cells at passages 4 and 20 (C and D) and monoclonal cells of clones 6 and 2B (A and B) were incubated with 5-HT or ipsapirone at different concentrations, in the presence of 10 μM forskolin. After 6 min at 37°, cAMP production was measured. The results are expressed as the percentage of cAMP production in control cells (agonist concentration = 0) in A and C or as the percentage of 5-HT maximal inhibitory effect in B and D. – – – (B), theoretical dose-response curve for 5-HT in clone 2B, calculated as indicated in the Discussion and according to classical pharmacological models. The values are the means ± standard errors of three separate experiments, each performed in duplicate.

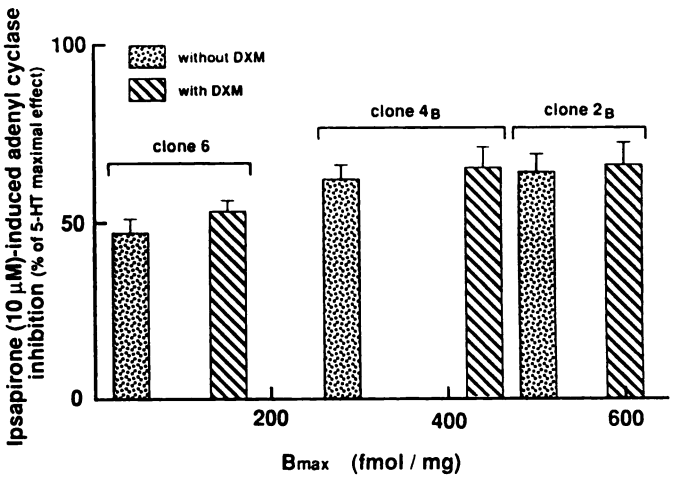

Fig. 6. Effect of increasing 5-HT$_{1A}$ receptor densities in cloned cells on the efficacy of ipsapirone, relative to 5-HT. Cells from clones 6, 4B, and 2B were treated for 15–20 hr with 0.50 μM dexamethasone (*DXM*) or with vehicle alone. The cAMP inhibition induced by increasing 5-HT and ipsapirone concentrations was measured. The maximal inhibitory effects of ipsapirone were determined by fitting the concentration-response curves, as indicated in Experimental Procedures, and are represented as a percentage of the maximal 5-HT effects for the different cell populations. The maximal 5-HT effects were 61 ± 5, 78 ± 5, 86 ± 3, 88 ± 4, 87 ± 4, and 89 ± 3% inhibition of cAMP production for clone 6, clone 6 treated with dexamethasone, clone 4B, clone 4B treated with dexamethasone, clone 2B, and clone 2B treated with dexamethasone, respectively. Values are the means ± standard errors of three separate experiments, each performed in duplicate.

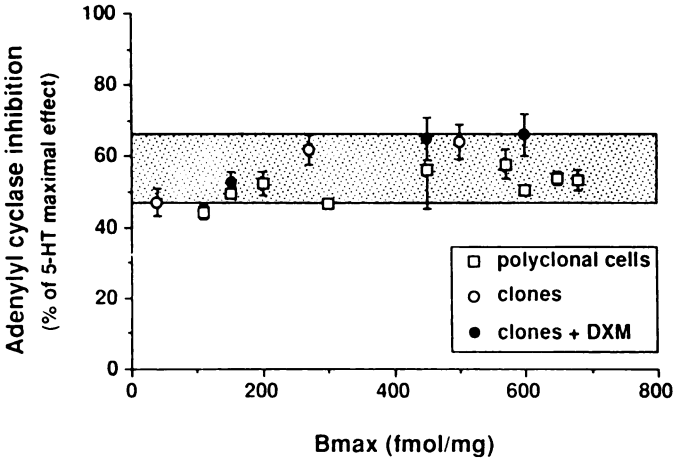

Fig. 7. Effect of increasing 5-HT$_{1A}$ receptor densities on the efficacy of ipsapirone, relative to 5-HT, in different models. □, Polyclonal cells at different passages; ○, clones 6, 4B, and 2B; ●, clones 6, 4B, and 2B treated with dexamethasone. Methods were as described in the legend to Fig. 6.

than 5-HT in transfected NIH-3T$_3$ cells and in hippocampus. In contrast, this drug was less potent than 5-HT and a partial agonist in cortical neurons (14). Similarly, metergoline and methysergide were full agonists in transfected cells and in hippocampus but were antagonists in cortical neurons (14, 30, 31). Finally, ipsapirone was a partial agonist in hippocampus and transfected NIH-3T$_3$ cells (31). Different hypotheses could explain these discrepancies between the pharmacological characteristics of 5-HT$_{1A}$ receptors in different tissues, as follows. 1) There are distinct molecular species of 5-HT$_{1A}$ receptors, one type expressed in cortical neurons, which is negatively coupled to adenylyl cyclase, and one or two types expressed in hippocampal neurons (one that is negatively coupled to adenylyl cyclase, the other positively). 2) there is only one 5-HT$_{1A}$ receptor molecule, and its 5-HT$_{1A}$ ligand binding profile is identical whatever the tissue or cell in which it is expressed, but, depending on the G protein(s) to which it is coupled or other environmental factors, 5-HT$_{1A}$ agonists can stimulate or inhibit adenylyl cyclase activity, as well as being partial or full agonists. There are not enough cells transfected with 5-HT$_{1A}$ receptors for which the pharmacological analysis has been done to answer this question. The possible cloning of other 5-HT$_{1A}$ receptors could also help to solve this problem.

The pharmacological characterization of a receptor is generally based on several criteria, i.e., the rank order of potencies and efficacies of agonists and the rank orders of antagonist potencies. However, the G protein-coupled receptors raise several interesting questions, which have been recently analyzed by Kenakin and Morgan (36). What is the evolution of agonist potencies and efficacies when the concentration of receptors

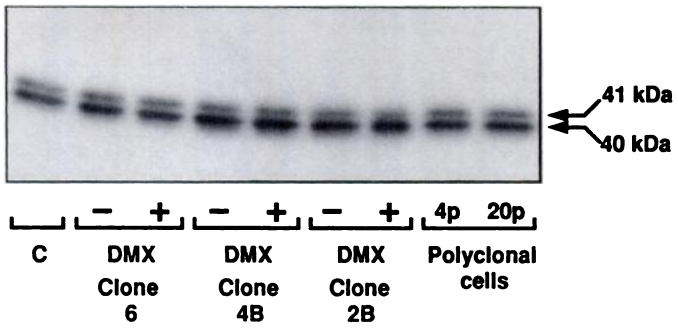

Fig. 8. Pertussis toxin-catalyzed ADP-ribosylation of membrane preparations from transfected cells, with or without dexamethasone (*DMX*) treatment. The reactions were conducted on 50 μg of protein for each sample, as described (27). *Arrows*, apparent molecular weights. *C*, untransfected NIH-3T$_3$ cells.

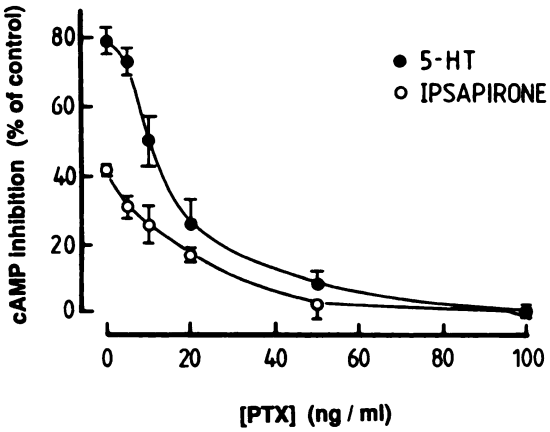

Fig. 9. Effect of pertussis toxin (*PTX*) treatment on the efficacy of 5-HT and ipsapirone in inhibiting cAMP formation in intact clone 4B cells. Cells were treated for 2 hr with increasing concentrations of pertussis toxin. The inhibition of forskolin (10 μM)-stimulated cAMP formation induced by 10 μM 5-HT or ipsapirone was measured as indicated for Fig. 4. Values are the means ± standard errors of three separate experiments, each performed in duplicate.

increases? What happens when one receptor is coupled to one effector through different G proteins, especially if the receptor has different affinities for the different G proteins, when occupied by different agonists? The possibility of obtaining cell lines expressing increasing amounts of receptors with a fixed concentration of G proteins or decreasing the availability of G proteins at a fixed concentration of receptors available using transfection have not yet really been used to solve some of these questions. Here we describe such an approach, and we found two unexpected results. The first one was the observation that, when the concentration of total receptor increased 12-fold or more, the EC$_{50}$ of the dose-response curve for 5-HT was not shifted to the left as predicted by the classical pharmacological model (36, 37) and the operational model (37, 38). If one supposes that agonist (L) interacts with receptors (R) following a simple mass action kinetic:

$$RL = R_T \frac{L}{K_d + L} \quad (1)$$

and a chain of hyperbolic coupling steps (including the interaction of RL with G proteins, RL + G ⇌ RLG occurs between the formation of RL and the final response, then the final response is expected to be a hyperbolic function of the ligand concentration L, as observed generally (39) and, in particular, in the present study. All the dose-inhibition curves could be fitted with a single mass action kinetic by computerization (see Experimental Procedures). Therefore, the final response (E) (adenylyl cyclase inhibition) is a hyperbolic function of RL:

$$E = E_T \frac{RL}{C_t + RL} \quad (2)$$

From eqs. 1 and 2, we obtain the relationship between the final response (E) and the agonist concentration (L):

$$E = \frac{E_T}{1 + C_t/R_T} \times \frac{L}{\frac{K_D}{1 + R_T/C_t}} + L \quad (3)$$

where R_T is the total concentration of receptor, C_t is the coupling constant, E_T is the maximal response that could be obtained if $R_T \gg C_t$, K_d is the dissociation constant of the agonist, $K_d/(1 + R_T/C_t)$ is the potency (EC$_{50}$ of the response), and $E_T/(1 + C_t/R_T)$ is the efficacy.

As shown by eq. 3 and discussed by Kenakin and Morgan (36), the EC$_{50}$ $[K_d/(1 + R_T/C_t)]$ should decrease as a function of R_T. This was not the case for our system. As seen in Fig. 5, both in individual clones and in the polyclonal population the EC$_{50}$ for 5-HT was unchanged by increasing receptor density. It can be argued that the coupling is poor (C_t very high) and that, consequently, R_T/C_t is very low, compared with 1, even with a 10–15-fold increase in R_T. However, because the efficacy of 5-HT increased from 60 to 90% inhibition of cAMP production when the concentration of R_T increased from 45 to 500 fmol/mg, C_t can be calculated, from the efficacy values (see eq. 3), as $C_t = 25$ fmol/mg. C_t provides a way of calculating the leftward shift of the EC$_{50}$. When R_T is increased from 45 to 500 fmol/mg, the shift should be 9-fold (Fig. 5B). Therefore, the classical model of coupling did not fit in NIH-3T$_3$ cells transfected with 5-HT$_{1A}$ receptors. Two hypotheses could be given for such an observation, but they are only speculative. The first is based on the fact that the 5-HT$_{1A}$ receptor does not diffuse freely in the cell membrane. The classical pharmacological models only apply if the ligand-receptor complexes, as well as the G proteins, are free to diffuse in the membrane, which is considered to be a homogeneous system (36). However, the membrane is not such a homogeneous system and the diffusion could depend not only on the receptor but also on the nature of the agonist and the membrane analyzed. Therefore, it is possible that in our system the classical pharmacological model applies only in a restricted cellular area in which such free diffusion occurs. Therefore, considering the whole cell means considering the sum of restricted domains in which the classical models occur. In addition, increasing the number of receptors could simply result in an increase in the number of domains within the cell; the efficacy of the drug will increase but the EC$_{50}$ will not (the EC$_{50}$ will be the same in all areas). The second hypothesis, which is the extreme limit of the first, considers that the 5-HT$_{1A}$ receptor is precoupled, i.e., associated with only one G protein. In this case, increasing the receptor number, again, will increase the agonist efficacy only if the G protein number is not limiting. Agonist potency will not change, because this parameter is an intrinsic property of each agonist-activated receptor. Recently, Yocca *et al.* (40) showed that alkylation of 5-HT$_{1A}$ receptors with *N*-ethoxycarbonyl-2-ethoxy-1,2 dihydroquinoline *in vitro* did not change the EC$_{50}$ for 8-OH-DPAT-

induced adenylyl cyclase inhibition, whereas there was a decrease in the maximal inhibition obtained. The nonclassical behavior (in terms of relative changes in EC_{50} and efficacies) of the dose-response curves for muscarinic inhibition of adenylyl cyclase has recently been reviewed (37).

The second observation was also unexpected; the partial agonist ipsapirone remained a partial agonist, having always 50–60% of the effect of 5-HT whatever the concentration of receptors expressed. This was not predicted by the classical pharmacological models. Indeed, in these models and as experimentally observed *in vitro* with muscarinic receptors, the difference between a partial and a full agonist resides in the affinity of RL for the G protein. Therefore, a lower C_t is expected for a partial agonist than for a full agonist. However, as shown by eq. 3, increasing R_T should reduce the ratio between the efficacies ($E_T/1 + C_t/R_T$) of full versus partial agonists. However, the ratios between the efficacies of the full (5-HT) and the partial (ipsapirone) agonists in cells expressing 45 and 600 fmol of receptor/mg were not significantly different (Fig. 5). Similarly, if the affinity of RL for the G protein is lower when a partial agonist is bound, then decreasing the concentration of G proteins should increase the ratio between the efficacies of full and partial agonists. This was not observed. We observed, however, a constant ratio between the efficacies of 5-HT and ipsapirone whatever the stoichiometry between receptors and G proteins. Here again, the potencies of ipsapirone did not vary with the concentration of receptors (data not shown).

The hypotheses mentioned above could also explain the results obtained with ipsapirone. 1) Within the restricted membrane areas in which free diffusion occurs, there is no major increase in receptor density and, therefore, no possibility of reducing the partial agonism of ipsapirone. Again, the increase in receptor density could result in an increase in the number of domains rather than in the number of receptors in those domains. 2) If the receptor is precoupled, then the characteristics of a partial agonist will not change, because these parameters are relative to the ability of the partial agonist-activated receptor to modulate the G protein to which it is coupled.

In conclusion, this is the first time we have used cell lines in which the number of receptors expressed is increased rather than decreased, in order to test some of the coupling problems raised by classical pharmacology. We observed nonclassical behavior, in terms of potency and efficacy, for full and partial agonists. Although we are far from having a definitive explanation for such behavior, a simple hypothesis would be to propose that, in these transfected NIH-3T_3 cells, 5-HT_{1A} receptors are not free to diffuse within the entire membrane plane. Therefore, if the areas of diffusion are small, the overall behavior of the system will be close to that observed with precoupled receptors, with the final response of the cell being the sum of the individual responses in these areas. It is possible that the size of the domains depends on the cell considered or on the agonist that occupies the receptor and, therefore, it is possible that, for the same receptor, the leftward shift of the dose-response curves will or will not be observed when an increase in receptor expression is produced.

Acknowledgments

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Send reprint requests to: Dr. Annie Varrault, Centre CNRS-INSERM de Pharmacologie-Endocrinologie, Rue de la Cardonille, 34094 Montpellier Cedex 5, France.