The signal transduction pathways of the cloned human 5-HT1A receptor have been examined in two mammalian cell lines transiently (COS-7) or permanently (HeLa) expressing this receptor gene. In both systems, 5-hydroxytryptamine (5-HT, serotonin) mediated a marked inhibition of β2-adrenergic agonist-stimulated (80% inhibition in COS-7 cells) or forskolin-stimulated cAMP formation (up to 90% inhibition in HeLa cells). This serotonin effect (EC50 = 20 nM) could be competitively antagonized by mepipetine and spiperone (Kᵢ = 81 and 31 nM, respectively) and could also be blocked by pretreatment of cells with pertussis toxin. In both cell types, 5-HT failed to stimulate adenyl cyclase through the expressed receptors. In HeLa cells, 5-HT also stimulated phospholipase C (~40–75% stimulation of formation of inositol phosphates). Again, this effect was inhibited by mepipetine. However, the EC50 of 5-HT was considerably higher (~3.2 μM) than that found for inhibition of adenyl cyclase. Both pathways were demonstrated to be similarly affected by pertussis toxin. These findings indicate that like the 5-HT1A receptor can couple to multiple transduction pathways with varying efficiencies via pertussis toxin-sensitive G-proteins. The lack of stimulation of cAMP formation by this 5-HT1A receptor may suggest the existence of another pharmacologically closely related receptor.

Multiple types of serotonin receptors have been described in both the peripheral and central nervous systems. Bradley et al. (1) and the same vector, in which the cDNA of G-protein coupled receptors was inserted (20), was also used in transient expression experiments. For transient expression, COS-7 cells were transfected using the DEAE-dextran method (19). Cells were allowed to grow to confluence. Transformed cells were selected for their resistance to the antibiotic G418 (0.8 mg/ml) and then pooled to check their ability to express the 5-HT1A receptor. The same vector, in which the cDNA of G-protein coupled receptors was inserted (20), was also used in transient expression experiments. For transient expression, COS-7 cells were transfected using the DEAE-dextran method (19). Cells were allowed to grow 60 h before assays were performed. Constitutive gene expression was obtained by cotransfecting the 5-HT1A receptor construct with pRSVNeo into HeLa cells, using the calcium phosphate procedure (19). Both placids were linearized at a unique PvuI restriction site prior to transfection. Transformed cells were selected for their resistance to the antibiotic G418 (0.8 mg/ml) and then pooled to check their ability to demonstrate specific [3H]8-OH-DPAT binding. Pooled cells were then subjected to single cell dilution cloning and each clone was tested for [3H]8-OH-DPAT specific binding activity.

Assessment of cAMP Formation—The effects of agonists and antagonists on cAMP formation were assessed in both COS-7 and HeLa cells. Briefly, adherent cells (~80% confluent) were incubated twice

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**Experimental Procedures**

**Methods**

**Cell Culture**—COS-7 and HeLa cells were grown as monolayers in DMEM (Gibco) supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml), in 5% CO₂ at 37 °C. Radioligand Binding Studies—5-HT1A receptor binding studies were carried out in cell membranes as previously described (3), except for the lysis buffer (5 mM Tris-HCl, 2 mM EDTA, pH 7.4). [3H]8-OH-DPAT was used as the radioligand (110 Ci/mmol) and 10 μM serotonin (5-HT) was used to determine nonspecific binding. After incubation with ligands, samples were filtered through GF/F filters.

Expression Vector and Cell Transfection—The HindIII-BamHI fragment from the human 5-HT1A receptor genomic clone “G-21” (4), containing the complete coding sequence, was subcloned into the expression vector pBCE21 (19). This vector contains the SV40 origin of replication and the Rous sarcoma virus long terminal repeat which acts as the promoter for the expression of the 5-HT1A receptor gene. The same vector, in which the cDNA of β2-adrenergic receptor was inserted (20), was also used in transient expression experiments. For transient expression, COS-7 cells were transfected using the DEAE-dextran method (19). Cells were allowed to grow 60 h before assays were performed. Constitutive gene expression was obtained by cotransfecting the 5-HT1A receptor construct with pRSVNeo into HeLa cells, using the calcium phosphate procedure (19). Both plasmids were linearized at a unique PvuI restriction site prior to transfection. Transformed cells were selected for their resistance to the antibiotic G418 (0.8 mg/ml) and then pooled to check their ability to demonstrate specific [3H]8-OH-DPAT binding. Pooled cells were then subjected to single cell dilution cloning and each clone was tested for [3H]8-OH-DPAT specific binding activity.
for 10 min with DMEM, 10 mM Hepes. Medium, containing 100 μM 3-isobutyl-1-methylxanthine and various concentrations of test drugs were then added to the cells. At the end of this treatment (10-min incubation), the reaction was stopped by aspiration of the media and addition of 2 ml of 100 mM HCl. Cells were scraped and centrifuged for 10 min at 1000 × g. Aliquots of the supernatant, corresponding to approximately 1000-2000 cells, were used to perform cAMP measurements by radioimmunoassay.

Assessment of Inositol Phosphate Formation—Cells grown in 6-well dishes (Falcon), were equilibrated for 24 h in regular medium containing 5 μCi/ml of myo-[3H]inositol (14.6 Ci/mmol). After washing with phosphate-buffered saline (30 min, 37 °C), cells were incubated for 30 min in phosphate-buffered saline containing 20 mM LiCl. This medium was then replaced by fresh medium containing different concentrations of various drugs. Reactions were stopped by aspiration and addition of 1 ml of 0.4 M perchloric acid. Lysates were then used for the measurement of [3H]inositol phosphates as described by Martin (21), using ionic exchange chromatography with Dowex 1-X8 (0.8 ml, 100-200 mesh) in the formate phase.

Materials

Drugs were obtained from the following sources: 8-OH-DPAT, Research Biochemicals Inc. (RBI; Natick, MA); metitepine, Hoffmann-La Roche; 5-HT, spiperone, isoproterenol, and 3-isobutyl-1-methylxanthine, Sigma. myo-[3H]inositol and cAMP Radioimmuno Assay kit were from Du Pont-New England Nuclear.

RESULTS

Transient and Permanent Expression of the 5-HT1A Receptor—COS-7 and HeLa cells were chosen for transfection of the 5-HT1A receptor since these cells appear to be devoid of serotonin response. No specific binding of [3H]8-OH-DPAT, [3H]HT, and [3H]ketanserin was detected and serotonin (up to 10−3 M) did not induce stimulation or inhibition of cAMP formation, or activation of PI turnover. These findings indicate that none of the subtypes of 5-HT1 or 5-HT2 receptor could be detected in either of these cell lines.

As shown in Fig. 1, when varying amount of G21 expression vector were used to transfect COS-7 cells, a gradual increase in the transient expression of receptor binding activity was observed. Expression levels reached a maximum of 10−15 pmol/mg protein when 10 μg of plasmid DNA/1.5 × 106 cells were used for the transfection while higher amounts of plasmid DNA did not lead to a further increase.

For permanent expression of the 5-HT1A receptor, clonal HeLa cell lines were obtained as described under "Methods." As shown in Table I, four clones were selected for study which displayed receptor levels varying from 0.5 to 2.8 pmol/mg protein. Binding studies performed with the agonist [3H]8-OH-DPAT on clone HA6, which showed the highest level of expression, demonstrated classical agonist saturation binding isotherms. The receptor displayed high and low affinity binding components for [3H]8-OH-DPAT with Kd and Kc, respectively, of 0.4 and 1.89 nM (data not shown).

Stimulation of cAMP Levels—Wild type COS-7 cells contain a low level (50–100 fmol/mg protein) of β2-adrenergic receptors which can mediate a 5–8-fold stimulation of cAMP formation by 2 μM isoproterenol. Transfection of COS-7 cells with the β2-adrenergic receptor expression vector, however, led to a further isoproterenol-induced increase in cellular cAMP levels (80–120%) (Fig. 2). Thus, this transient system can be used to investigate the positive coupling of an expressed receptor to adenylyl cyclase. Fig. 2 also shows results obtained when cells were transfected with the 5-HT1A receptor expression vector.

Table I

Properties of the expressed 5-HT1A receptor in clonal HeLa cells

| Cell line         | 5-HT1A receptor % Inhibition of forskolin-stimulated cAMP levels |
|-------------------|---------------------------------------------------------------|
|                   | pmol/mg protein                                               |
|                   |                                      open bars | closed bars | stippled bars |
| HA4               | 0.98 ± 0.30 (n = 3) | 53.0       |              |
| HA6               | 2.76 ± 0.77 (n = 6) | 82.8       |              |
| HA7               | 0.49 ± 0.27 (n = 3) | 72.8       |              |
| HB24              | 2.29 ± 0.54 (n = 6) | 90.3       |              |
Cloned 5-HT1A Receptor

Inhibition of cAMP Levels—To study the action of an inhibitory receptor on adenyl cyclase, receptor-mediated inhibition of forskolin-stimulated cAMP levels is commonly used. However, in the transient COS-7 cell expression system, this paradigm cannot be effectively utilized. Forskolin stimulates the total cell population, whereas studies indicate that only 5-10% of COS-7 cells are competent and can be transfected (19). Thus, it would be difficult to demonstrate a significant inhibition occurring in only the transfected cells. Accordingly, we utilized a co-transfection protocol in which the vectors expressing both the 5-HT1A receptor and β2-adrenergic receptors were co-transfected. Again, it was found that in the transfected COS-7 cells, isoproterenol was able to further stimulate cAMP production by 90 ± 18% (n = 11) compared to nontransfected cells. As shown in Fig. 3, under optimal co-transfection conditions (see legend), 5-HT (10 μM) caused an 80% inhibition of the further isoproterenol-stimulated cAMP levels. In order to further document the ability of the 5-HT1A receptor to inhibit adenyl cyclase, we selected four HeLa clonal cell lines which permanently express different levels of the 5-HT1A receptor ranging from 0.5 to 2.8 pmol/mg of protein (Table I). Since these cells are clonal, the ability of 5-HT to inhibit forskolin-stimulated cAMP levels can be assessed without the complications alluded to above for COS-7 cells. Forskolin in these cells increased cAMP from 2- to 5-fold over basal levels. 5-HT induced a decrease in forskolin-stimulated cAMP levels in all four cell lines, however, the extent of this reduction did not appear to correlate with the levels of expression of the 5-HT1A receptor (Table I).

![Graph showing Graph 3. 5-HT inhibition of β2 agonist-stimulated cAMP formation in transfected COS-7 cells. Cells were transfected with a constant amount of the β2-adrenergic receptor expression vector (3 μg/1.5 × 10⁶ cells) while the amount of the 5-HT1A receptor expression vector was progressively raised from 3 to 20 μg of DNA. Under all conditions, 5-HT was found to significantly inhibit isoproterenol-induced stimulation of cAMP formation. The experiment shown is representative of results obtained using the optimal transfection conditions (plasmid ratio β2-adrenergic receptor/5-HT1AR, 3:10 μg). Transfected cells were used for determination of basal cAMP levels (open bars) or exposed to 2 μM isoproterenol alone (closed bars) or together with 10 μM 5-HT (stippled bars). Values are the mean of triplicate determinations ± S.E. and are representative of four experiments.](image)

![Graph showing Graph 4. Effects of the antagonists metitepine (Met) and spiperone (Spip) on 5-HT-induced inhibition of forskolin-stimulated cAMP formation in HA7 cells. Cells were exposed to 100 μM forskolin and increasing concentrations of 5-HT in the absence (○) or presence of 1 μM metitepine (●) or 1 μM spiperone (■). Results are expressed as the percent of maximal stimulation of cAMP formation obtained with forskolin alone. This experiment is representative of three independent experiments performed in triplicate. Values are means of triplicate determinations.](image)

Clone HA7, which expresses levels of receptors comparable to those reported in hippocampus, was chosen to pursue the characterization of this activity. As shown in Fig. 4, serotonin was able to elicit a reduction of forskolin-stimulated cAMP levels in a concentration-dependent fashion, with an EC₅₀ of 20 ± 3 nM (n = 3). Maximal inhibition of ~75% was routinely achieved. In this system, metitepine and spiperone were able to induce a parallel and comparable rightward shift of the serotonin dose-response curve without affecting the maximal effect (Fig. 4). This is the classical pattern of competitive inhibition. Dissociation constants of 81 and 31 nM (mean of two experiments) for metitepine and spiperone, were, respectively, derived from these shifts and are in good agreement with those reported in the literature (12–14).

5-HT1A Receptor Coupling to Phosphoinositol Hydrolysis—Because serotonin, seemingly through a pharmacologically characterized 5-HT1A response, has been reported to couple
Cloned 5-HT1A Receptor

**Fig. 6.** Effect of 5-HT on phosphatidylinositol hydrolysis in HA7 cells. Accumulation of inositol phosphates was determined after treatment (15 min) with increasing concentrations of 5-HT. Results obtained from two independent experiments performed in triplicate are expressed as percent of stimulation over the basal IP3 level.

**Fig. 7.** Effect of pertussis toxin on 5-HT-induced inhibition of cAMP formation and stimulation of PI hydrolysis. Cells were treated for 4.5 h with increasing concentrations of pertussis toxin (0–100 ng/ml) before being assayed for 5-HT-induced inhibition of forskolin-stimulated cAMP levels (●) and 5-HT-induced accumulation of inositol phosphates (■). For the latter assay, cells were prelabeled ~20 h with 10 μCi of myo[3H]inositol and pertussis toxin was directly added to the medium for the last 4.5 h of incubation. Each data point is the average of two independent experiments performed in triplicate.

To different second messenger systems (7–18), it was of interest to examine the ability of the cloned 5-HT1A receptor to affect hydrolysis of phosphatidylinositol. In the stable cell line HA7, a modest but significant increase of total inositol phosphates (IP3, 40–75% over basal) was observed upon stimulation of the cells with 10 μM 5-HT for 15 min, in the presence of 20 mM LiCl. It is interesting that a similar pattern of response has been reported for the M2 and M3 muscarinic receptor subtypes which preferentially couple to inhibition of adenylyl cyclase, suggesting that it is mediated through a Gi protein.

A dose-response curve for the ability of 5-HT to stimulate inositol phosphate (total) production is shown in Fig. 6. The EC50 (3.2 ± 0.8 μM, n = 3) is considerably higher than that for the 5-HT1A receptor-mediated inhibition of adenylyl cyclase. In contrast, 5-HT1A receptors transiently expressed in COS-7 cells did not seem to couple efficiently to this pathway under conditions where α1-adrenergic receptors can mediate a large increase in production of inositol phosphates (not shown; Ref. 25).

**Effect of Pertussis Toxin on 5-HT1A Receptor-mediated Responses**—HA7 cells, pretreated with pertussis toxin for 4.5 h, were used to assess the effect of 5-HT (10 μM) on both forskolin-induced cAMP formation and accumulation of inositol phosphates. As shown in Fig. 7, both pathways appeared to be similarly sensitive to pertussis toxin, with 50% inhibition of activity observed at 20 ng/ml of toxin.

**DISCUSSION**

The cloning of various neurotransmitter receptors has opened new approaches to the study of their biology and regulation. In particular, expression of such receptor proteins in a variety of systems provides a powerful tool for assessing the biochemical mechanisms by which their signals are transduced. The situation for the serotonin receptor is particularly complex since at least 6 well-defined receptor subtypes have been described. Moreover, for the 5-HT1A receptor, seemingly contradictory reports of coupling to various effector systems, including both stimulation and inhibition of adenylyl cyclase in different systems have appeared in the literature (7–17).

Accordingly, having cloned the gene for the human 5-HT1A receptor, we were particularly interested in exploring the biochemical mechanisms of its coupling in transfected cells expressing the receptor. In these studies we have utilized two different expression systems and have examined three different responses. Our results clearly indicate that, of the responses studied, the most important coupling of the 5-HT1A receptor is to adenylyl cyclase inhibition. This response was demonstrated both in transiently and permanently transfected cell lines and could be blocked by pertussis toxin, suggesting that it is mediated through a Gi protein.

In contrast, we have no evidence that the 5-HT1A receptor can couple through Gs to stimulate adenylyl cyclase, as had been claimed based on reports using rat and guinea pig hippocampus, as well as rat brain cortex preparations (8–10). One possible explanation for the discrepancy is that the "5-HT1A" receptor binding studied in those preparations is in fact due to another very closely related sub-subtype of this receptor. This concept has recently been discussed by Dumuis et al. (12). Given currently emerging concepts relating the structure of G-protein coupled receptors to effector coupling specificity, it would be difficult indeed to postulate a mechanism for coupling a single receptor to both Gs and Gi. In fact to our knowledge, no single receptor which both stimulates and inhibits adenylyl cyclase has been discovered.

Our data also indicate that in the HeLa cell clone HA7, the expressed 5-HT1A receptor (0.5 pmol/mg protein) is also able to couple to activation of phospholipase C. The serotonin-induced stimulation can be blocked by the antagonist metipene, which confirms that the effect is mediated by the expressed serotonin receptor. However, the EC50 of 5-HT is about 100-fold higher than that calculated for the inhibition of adenylyl cyclase, suggesting that cyclase inhibition is the primary transduction pathway of the 5-HT1A receptor. A comparable response was obtained for the clone HB24, expressing a higher concentration of receptors (2.3 pmol/mg protein) suggesting that the extent of this effect does not increase at higher receptor levels (not shown). Furthermore, 5-HT1A receptor-induced phosphatidylinositol hydrolysis was not observed in COS-7 cells, which might indicate that...
the 5-HT1A receptor can elicit distinct biochemical responses in different cellular systems.

An interesting and important issue is the nature of the G protein(s) through which the various biological actions of the expressed 5-HT1A receptors are mediated. On the one hand, the identical pertussis toxin sensitivity of the two responses might be taken to suggest that the same, or at least very similar, G proteins mediate both effects. On the other hand, the very divergent dose-response relationships for 5-HT activation of the two pathways is more suggestive of distinct G proteins mediating the two effects. The pertussis toxin-sensitive G proteins thus far described include Go, Gi, and Gs. In addition, "Gp" which is a candidate for coupling to phospholipase C seems to be composed of at least two types of G proteins, a pertussis toxin-sensitive and a pertussis toxin-insensitive one (26). Further supporting the existence of distinct Gp is the differential pertussis toxin sensitivity of the cholecystokinin receptor- and M2 and M3 muscarinic receptor-mediated effects on PI turnover (24). It is thus possible that HeLa cells express a Gp protein displaying a pertussis toxin sensitivity similar to that of Gi and to which 5-HT1A receptors can couple.

It is interesting to compare the characteristics of the responses mediated by the 5-HT1A, muscarinic cholinergic receptors (22-24), and the alpha1- and alpha2-adrenergic receptors2 which apparently fall into two groups. The M1 and M4 muscarinic receptors and alpha1-adrenergic receptors are all primarily coupled to the phospholipase C system via a pertussis toxin-insensitive or poorly sensitive G-protein. These receptors mediate strong PI turnover responses. In contrast, M2 and M3 muscarinic receptors as well as alpha2-adrenergic receptors and the 5-HT1A receptor, are primarily linked to adenylyl cyclase inhibition but are also capable of mediating weak stimulation of the phospholipase C system via a pertussis toxin-sensitive G-protein. Whether the same G-protein mediates both the cyclase inhibition as well as PI stimulatory effects, whether the same phospholipase C mediates both the pertussis toxin-sensitive and -insensitive responses, and whether the weak PI responses mediated by 5-HT1A and similar receptors are physiologically relevant all remain subjects for future studies.

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