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Differential coupling of 5-HT_1 receptors to G proteins of the G_i family

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1 Since all 5-HT_1 receptors couple to G_i – type G proteins and inhibit adenylyl cyclase, the functional significance of five distinct subtypes of 5-HT_1 receptors has been unclear.

2 In previous studies we have used transfected cells to demonstrate that 5-HT_{1B} receptors can couple more efficiently than 5-HT_{1A} receptors to activation of extracellular signal-regulated kinase (ERK) and to inhibition of adenylyl cyclase. These findings suggested the possibility that individual 5-HT_1 receptors differentially couple to isoforms of $G_{i\alpha}$.

3 In the present study we utilized a model system in which pertussis toxin resistant forms of human $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ were used to directly compare the coupling of human 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} receptors to each $G_{i\alpha}$ in transfected human HeLa cells.

4 5-HT_{1A} receptors displayed a preference for $G_{i\alpha1}$ and $G_{i\alpha2}$, relative to $G_{i\alpha3}$. Pertussis toxin resistant forms of $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ rescued 73%, 76%, and 44%, respectively, of the ERK activation stimulated by 5-HT in the absence of pertussis toxin.

5 In contrast, pertussis toxin resistant forms of $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ rescued 32%, 118%, and 35% of 5-HT_{1B} receptor-stimulated activity, respectively, indicating that 5-HT_{1B} receptors coupled primarily through $G_{i\alpha2}$. A similar preference for $G_{i\alpha2}$ was found in studies of the 5-HT_{1D} receptor, where toxin resistant $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ rescued 30%, 70%, and 40% of activity, respectively.

6 In conclusion, the observed differential coupling of 5-HT_1 receptors to isoforms of $G_{i\alpha}$, provides additional evidence for our previous findings that the subtypes of 5-HT_1 receptors exhibit similar, but distinct, functions.

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Abbreviations: 5-HT, 5-hydroxytryptamine or serotonin; ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase

Introduction

At least 16 types of mammalian receptors for serotonin (5-HT) have been identified and classified within seven families (Hoyer *et al*., 1994; Scalzitti & Hensler, 1996). The physiological significance of such a large number of receptors is currently unclear. This is especially true regarding those receptors classified as 5-HT_1 receptors, which have been postulated to play a role in the treatment and pathophysiology of a number of disorders including depression, anxiety, and migraine headaches. All of the 5-HT_1 receptors, designated 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F}, couple to G proteins of the G_i class and inhibit adenylyl cyclase. Although these receptors have clear distinctions in pharmacology and structure, there is little currently known about differences in coupling to cellular signals. However, we have previously demonstrated that 5-HT_{1B} receptors couple more effectively than 5-HT_{1A} receptors to activation of the mitogen-activated protein (MAP) kinase ERK and to inhibition of adenylyl cyclase in Chinese Hamster Ovary (CHO) cells (Mendez *et al*., 1999). In those studies we directly compared the intrinsic activity of each receptor subtype in stably transfected cells expressing receptors at the same densities. The observed differences in receptor function suggested the possibility that the receptors differentially couple to isoforms of $G_{i\alpha}$.

However, binding studies utilizing membranes from infected *Spodoptera frugiperda* (Sf9) insect cells over-expressing receptors and G proteins, suggest that differential receptor/G protein coupling, in fact, might not occur. All three isoforms of $G_{i\alpha}$ have been reported to reconstitute high-affinity binding by 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} receptors (Butkerait *et al*., 1995; Clawges *et al*., 1997; Brys *et al*., 2000). Nevertheless, it cannot be assumed that these findings translate into similar non-preferential utilization of G proteins in the coupling of receptors to cellular signals in mammalian cells. In fact, Garnovskaya *et al*. (1997) found that pertussis toxin resistant forms of $G_{i\alpha1}$ were ineffective in rescuing coupling of 5-HT_{1A} receptors to Na^+/H^+ exchange in transfected CHO cells.

In the present studies we examined the coupling of human 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} receptors to the MAP kinase ERK. The ERK pathway is known to enhance cell survival, and is required for normal neuronal functioning (Encinas *et al*., 1999; Erhardt *et al*., 1999). In particular, our studies were

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aimed at uncovering differences in the coupling of 5-HT_1 receptors to $G_{i\alpha}$. Since $G_{i\alpha}$ is selectively ADP-ribosylated by pertussis toxin, we utilized toxin resistant mutants of human $G_{i\alpha}$ to 'rescue' receptor/G protein-coupling from pertussis toxin-catalyzed inhibition of 5-HT_1 receptor/G protein coupling. In this way, the efficacy of receptor coupling to each subtype of $G_{i\alpha}$ could be studied in isolation. An advantage to this approach, over some other methods, is that such studies directly address functional specificity in a cell type-independent manner. Since pertussis toxin prevents the coupling of receptors to endogenous $G_{i\alpha}$, the relative endogenous expression of $G_{i\alpha}$ isoforms in the particular cell type used does not alter the observed results.

In order to simulate the G protein-coupling of endogenous human 5-HT_1 receptors, as closely as possible, we utilized a model system in which the receptors, G proteins, and cell line were all human. Studies presented here demonstrate that 5-HT_{1B} and 5-HT_{1D} receptors couple more selectively than 5-HT_{1A} receptors to subtypes of $G_{i\alpha}$. The expression of multiple receptors that display similar, but distinct, coupling to G proteins and cellular signals provides insight into the large number of receptors (at least 16) required to mediate the diverse and highly complex actions of 5-HT in the central nervous system.

Methods

Materials

5-HT, tranylcypromine, and pertussis toxin were purchased from Sigma (St. Louis, MO, U.S.A.).

Cell culture

HeLa cells were obtained from American Type Culture Collection (Rockville, MD, U.S.A.), and were routinely cultured in Eagle's minimum essential medium supplemented with non-essential amino acids and 10% dialyzed foetal bovine serum (dialyzed in membranes with 1000 Dalton molecular weight cut-offs against a 100-fold greater volume of 150 mM NaCl to remove endogenous 5-HT), 100 units penicillin-100 μg streptomycin/ml (95% air, 5% CO_2).

Transient transfections of cells

cDNAs for the human 5-HT_{1B} and 5-HT_{1D} receptors were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). cDNA for the human 5-HT_{1A} receptor has been previously described (Fargin *et al*., 1989; Cowen *et al*., 1996). cDNAs for pertussis toxin resistant mutants of human $G_{i\alpha 1}$ (C351I), $G_{i\alpha 2}$ (C352I) and $G_{i\alpha 3}$ (C351I) were obtained from the Guthrie cDNA Resource Center (Sayre, PA, U.S.A.). Expression of all sequences was under the control of the CMV promoter. Transient transfections were performed 48 hours prior to cellular studies using the Profectin calcium phosphate procedure (Promega, Madison, WI, U.S.A.). For studies of receptor coupling, cells were cultured in 60 mm plates and co-transfected with 6 μg of receptor plasmid DNA plus 6 μg of G protein plasmid DNA or empty vector. For studies of $G_{i\alpha}$ expression, cells were cultured in 100 mm plates and transfected with 20 μg of receptor plasmid DNA.

Immunoblots

Monoclonal anti-phospho-ERK1/ERK2 (Thr202/Tyr204) was obtained from Cell Signalling (Beverly, MA, U.S.A.). Goat polyclonal anti-$G_{i\alpha}$ recognizing all $G_{i\alpha}$ subtypes, rabbit polyclonal total ERK1/ERK2, and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The day prior to use, cells were washed with phosphate-buffered saline and cultured overnight under serum- and geneticin free conditions. Cells were stimulated with the specified concentrations of agonists, and routinely lysed with a 26-gauge needle in (mM) HEPES 25 (pH 7.4), NaF 50, EDTA 5, sodium orthovanadate 1, 250 μM 4-(2-aminoethyl)-benzene-sulfonyl-fluoride hydrochloride, 0.1% aprotinin, and 10 $\mu g\ ml^{-1}$ leupeptin. In studies of $G_{i\alpha}$ expression, NaCl 150 mM, 1% Triton X-100, and β-glycerolphosphate 1 mM were included in the lysis buffer. Proteins were separated on 12% resolving gels (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and transferred to 0.45 μM Immobolin-P polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA, U.S.A.). Membranes were blocked overnight with 3% powdered milk before incubation with primary and secondary antibodies. Bound antibodies were visualized using Enhanced Luminol Chemiluminescence Reagent (NEN Life Sciences, Boston, MA, U.S.A.) and exposure to a Kodak Image Station 440CF with a cooled, full-frame-capture CCD camera (Kodak). Net intensity of bands was calculated directly from stored images using Kodak Digital Science 1D Image Analysis Software (version 3.5) on defined regions of interest.

Binding assays

Cells were transfected, as described above, with 6 μg of receptor plasmid DNA plus 6 μg of empty vector. The day prior to use, cells were washed with phosphate-buffered saline and cultured overnight under serum-free conditions. Receptor binding assays were performed using the radioligand [^{3}H]-5-HT (Veldman & Bienkowski, 1992), obtained from Perkin-Elmer Life Sciences (Boston, MA, U.S.A.). Assays contained 10–20 μg of membrane protein, 15 nM [^{3}H]-5-HT, and 1 μM tranylcypromine in a total volume of 100 μl. Displaceable binding of [^{3}H]-5-HT was determined in the presence of 10 μM 5-HT.

Results

Activation of MAP kinase was assayed by measuring MAP kinase kinase (MEK)-dependent phosphorylation of ERK1 and ERK2 at threonine 202 and tyrosine 204 (Cobb & Goldsmith, 1995). When nontransfected HeLa cells were treated with 5-HT, no increase in the level of activated, phosphorylated ERK was detected (Figure 1A). Therefore, although HeLa cells were found in binding studies to apparently express endogenous receptors for 5-HT (Table 1), they did not express subtypes of 5-HT receptors that couple to activation of ERK. In contrast, 5-HT did stimulate phosphorylation of ERK in cells transfected with cDNA for 5-HT_1 receptors. When transfected cells

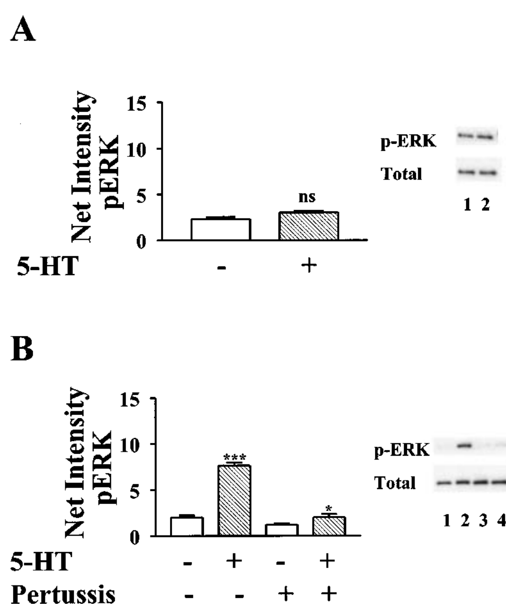

Figure 1 5-HT_{1A} receptors couple to activation of ERK through pertussis toxin sensitive G proteins. (A) Nontransfected HeLa cells were treated for 5 min with 10 μM 5-HT (lane 2), and then lysed. (B) HeLa cells transfected with cDNA for the human 5-HT_{1A} receptor were treated overnight in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 20 ng ml^{-1} pertussis toxin before treatment with 10 μM 5-HT (lanes 2 and 4) for 5 min, and subsequent lyses. Total lysate was analysed by immunoblotting with antibody to phospho-ERK1/ERK2 (p-ERK). Membranes were then stripped and analysed with antibody to total ERK1/ERK2 (Total). Net intensities of bands were calculated from three separate experiments, performed in duplicate, and expressed as the means$\pm$s.e.mean ($\times 10^3$). $*P<0.05$; $***P<0.001$; n.s., statistically not significant *vs* absence of 5-HT, two-sided paired Student *t*-test calculated separately for both the presence and absence of pertussis toxin. Representative immunoblots from one of the three experiments are shown to demonstrate that treatment with pertussis toxin does not alter the levels of total ERK.

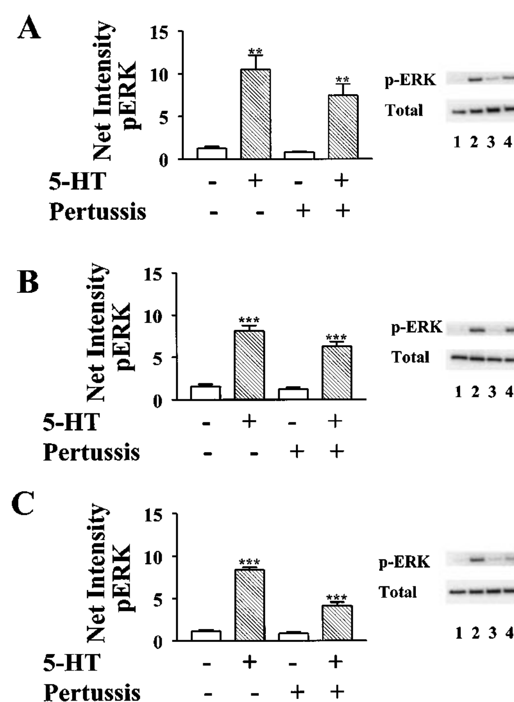

Figure 2 5-HT_{1A} receptors couple to activation of ERK through multiple subtypes of $G_{i\alpha}$. HeLa cells co-transfected with cDNA for the human 5-HT_{1A} receptor and pertussis toxin resistant forms of either (A) $G_{i\alpha1}$, (B) $G_{i\alpha2}$, or (C) $G_{i\alpha3}$ were treated overnight in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 20 ng ml^{-1} pertussis toxin before treatment with 10 μM 5-HT (lanes 2 and 4) for 5 min, and subsequent lyses. Total lysate was analysed by immunoblotting with antibody to phospho-ERK1/ERK2 (p-ERK). Membranes were then stripped and analysed with antibody to total ERK1/ERK2 (Total). Net intensities of bands were calculated from three separate experiments, performed in duplicate, and expressed as the means$\pm$s.e.mean ($\times 10^3$). $**P<0.01$; $***P<0.001$ *vs* absence of 5-HT, two-sided paired Student *t*-test calculated separately for both the presence and absence of pertussis toxin. Representative immunoblots from one of the three experiments are shown to demonstrate that treatment with pertussis toxin does not alter the levels of total ERK.

Table 1 Transfected 5-HT_1 receptors are all expressed at the same density

Expressed receptor	*DPM/20 µg membrane protein (mean ± s.e.mean)*
Nontransfected	225 ± 25*
5-HT_{1A}	430 ± 20
5-HT_{1B}	515 ± 66 (n.s.)
5-HT_{1D}	377 ± 44 (n.s.)

Binding assays were performed on membranes isolated from nontransfected HeLa cells or HeLa cells transfected with cDNA for 5-HT_{1A}, 5-HT_{1B}, or 5-HT_{1D} receptors. Results are expressed as mean$\pm$s.e.mean of DPM of selective [^{3}H]-5HT binding/20 μg membrane protein defined by displacement with 10 μM 5-HT. Since the K_D's for 5-HT for each receptor are similar (Hamblin & Metcalf, 1991; Veldman & Bienkowski, 1992), differences in binding are reflective of differences in B_{max}. $*P<0.05$; n.s., not significantly different *vs* binding by membranes from cells expressing 5-HT_{1A} receptors; ANOVA, Bonferroni analysis.

expressing human 5-HT_{1A} receptors were treated with 5-HT, a 3.8-fold activation of ERK was observed. This activation represented primarily ERK2, as the observed band (Figure 1B) migrated at a relative weight equal to the lower band of a band doublet of p44 ERK1/p42 ERK2 seen from PC12 cell lysate run on the same gel (not shown). Activation of ERK2 by 5-HT was mediated by G_i, as pretreatment with pertussis toxin caused almost complete inhibition.

In contrast, when cells were co-transfected with cDNA for the 5-HT_{1A} receptor and pertussis toxin resistant forms of human $G_{i\alpha1}$, $G_{i\alpha2}$, or $G_{i\alpha3}$, all three toxin-resistant subtypes were found to 'rescue' receptor-mediated activation of ERK from inhibition by pertussis toxin (Figure 2). However, the receptor demonstrated a preference for $G_{i\alpha1}$ and $G_{i\alpha2}$, as transfection with toxin resistant $G_{i\alpha3}$ caused the smallest activation of ERK. Pertussis toxin resistant forms of $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ rescued 73, 76 and 44%, respectively, of the increase in levels of activated ERK stimulated by 5-HT in the absence of pertussis toxin. While both $G_{i\alpha1}$ and $G_{i\alpha2}$ effectively coupled 5-HT_{1A} receptors to activation of ERK, concentration–response curves revealed somewhat more efficient coupling by $G_{i\alpha2}$. The EC_{50} (calculated by nonlinear regression analysis of the net intensities of bands) for 5-HT-stimulated activation was 12 nM for cells expressing toxin resistant $G_{i\alpha2}$, but 40 nM for cells expressing $G_{i\alpha1}$ (Figure 3A). The EC_{50} for cells transfected with $G_{i\alpha3}$ was similar to

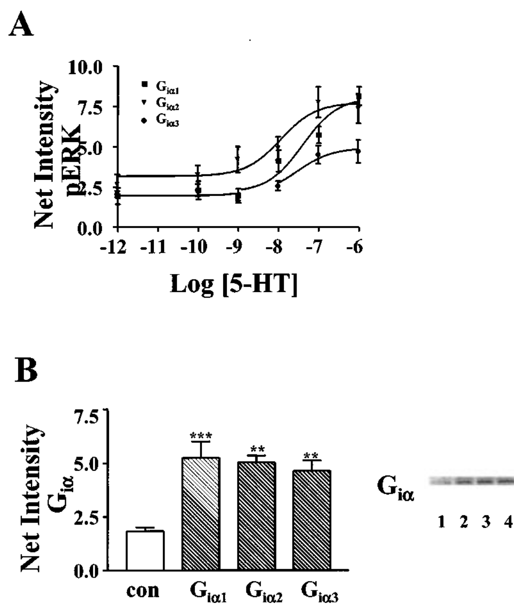

Figure 3 5-HT_{1A} receptors couple most efficiently to activation of ERK through $G_{i\alpha2}$ despite equal expression of all transfected $G_{i\alpha}$ subtypes. (A) HeLa cells co-transfected with cDNA for the human 5-HT_{1A} receptor and pertussis toxin resistant forms of either $G_{i\alpha1}$, $G_{i\alpha2}$, or $G_{i\alpha3}$ were treated overnight with 20 ng/ml pertussis toxin before treatment with the indicated concentrations of 5-HT for 5 min, and subsequent lyses. (B) HeLa cells were transfected with cDNA for pertussis toxin resistant forms of either $G_{i\alpha1}$ (lane 2), $G_{i\alpha2}$ (lane 3), or $G_{i\alpha3}$ (lane 4) and the density of expression of $G_{i\alpha}$ subunits were compared to nontransfected (con) cells (lane 1). (A) Total lysate was analysed by immunoblotting with antibody to phospho-ERK1/ERK2 (p-ERK) or (B) 20 μg of total lysate was analysed by immunoblotting with antibody recognizing all forms of $G_{i\alpha}$. Net intensities of bands were calculated from three separate experiments, performed in duplicate, and expressed as the means $\pm$ s.e.mean ($\times 10^3$) $^{**}P<0.01$; $^{***}P<0.001$ *vs* nontransfected (con) cells, ANOVA, Bonferroni analysis. A representative immunoblot from one of the three experiments is shown.

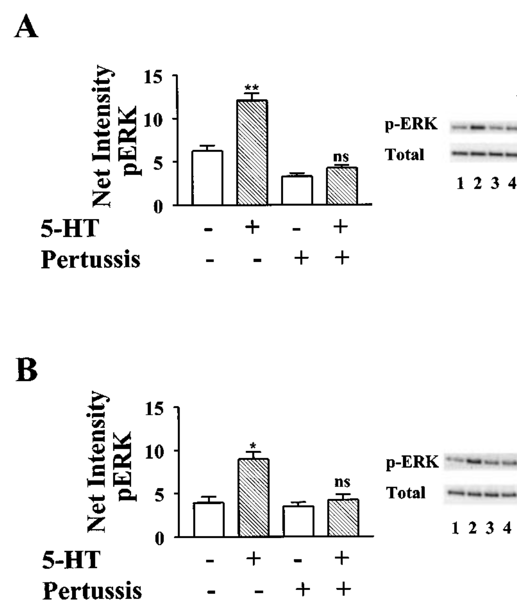

Figure 4 5-HT_{1B} and 5-HT_{1D} receptors couple to activation of ERK through pertussis toxin sensitive G proteins. (A) HeLa cells transfected with cDNA for the human 5-HT_{1B} receptor or (B) human 5-HT_{1D} receptor were treated overnight in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 20 ng ml^{-1} pertussis toxin before treatment with 10 μM 5-HT (lanes 2 and 4) for 5 min, and subsequent lyses. Total lysate was analysed by immunoblotting with antibody to phospho-ERK1/ERK2 (p-ERK). Membranes were then stripped and analysed with antibody to total ERK1/ERK2 (Total). Net intensities of bands were calculated from three separate experiments, performed in duplicate, and expressed as the means $\pm$ s.e.mean ($\times 10^3$). $^*P<0.05$; $^{**}P<0.01$; n.s., statistically not significant *vs* absence of 5-HT, two-sided paired Student *t*-test calculated separately for both the presence and absence of pertussis toxin. Representative immunoblots from one of the three experiments are shown to demonstrate that treatment with pertussis toxin does not alter the levels of total ERK.

that for $G_{i\alpha1}$, 32 nM, though the maximal effect was significantly reduced. Interestingly, the basal levels of activated ERK were higher in cells transfected with toxin resistant $G_{i\alpha2}$ than in cells transfected with toxin resistant $G_{i\alpha1}$ and $G_{i\alpha3}$.

The observed preferential coupling of 5-HT_{1A} receptors to $G_{i\alpha1}$ and $G_{i\alpha2}$, relative to $G_{i\alpha3}$, was not the result of differences in the levels of transfected $G_{i\alpha}$ subunits. Each subunit was expressed at an approximately 2.5-fold greater density than endogenously expressed $G_{i\alpha}$ subunits (Figure 3B).

As was seen with 5-HT_{1A} receptors, coupling of 5-HT_{1B} receptors to activation of ERK was almost completely inhibited by pertussis toxin (Figure 4A). However, the relative preference for coupling to subtypes of $G_{i\alpha}$ was different than that found for 5-HT_{1A} receptors. $G_{i\alpha2}$ much more effectively rescued receptor-mediated activation of ERK than $G_{i\alpha1}$ and $G_{i\alpha3}$. In contrast to the complete (118%) rescue by $G_{i\alpha2}$, pertussis toxin resistant forms of $G_{i\alpha1}$ and $G_{i\alpha3}$ rescued only 32 and 35%, respectively, of the activation of ERK stimulated by 5-HT in the absence of toxin (Figure 5). Significantly, the more selective G protein-coupling by 5-HT_{1B} receptors, relative to 5-HT_{1A} receptors, was not the result of expression of a lower density of transfected 5-HT_{1B} receptors. The level of displaceable binding of [^{3}H]5-HT to membranes prepared from cells transfected with cDNA for the 5-HT_{1B} receptor was similar to that from membranes prepared from cells transfected with cDNA for the 5-HT_{1A} receptor (Table 1).

5-HT_{1D} receptors were found to be similar to 5-HT_{1B} receptors in their coupling to $G_{i\alpha}$. As was found with 5-HT_{1B} receptors, treatment with pertussis toxin almost completely uncoupled endogenous $G_{i\alpha}$ from 5-HT_{1D} receptors (Figure 4B). Pertussis toxin resistant $G_{i\alpha2}$ rescued 70% of receptor-mediated activation of ERK. In contrast, toxin resistant forms of $G_{i\alpha1}$ and $G_{i\alpha3}$ rescued only 30 and 40%, respectively, of the activity stimulated by 5-HT in the absence of pertussis toxin (Figure 6).

Discussion

Our studies demonstrate that human 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} receptors exhibit clear differences in coupling to subtypes of $G_{i\alpha}$. 5-HT_{1B} and 5-HT_{1D} receptors were found to much more efficiently utilize $G_{i\alpha2}$ relative to $G_{i\alpha1}$ and $G_{i\alpha3}$. In contrast, 5-HT_{1A} receptors demonstrated less selectivity, particularly with regards to $G_{i\alpha1}$ *vs* $G_{i\alpha2}$. These findings represent a progression of our earlier studies in which we used transfected CHO cells to demonstrate more efficient

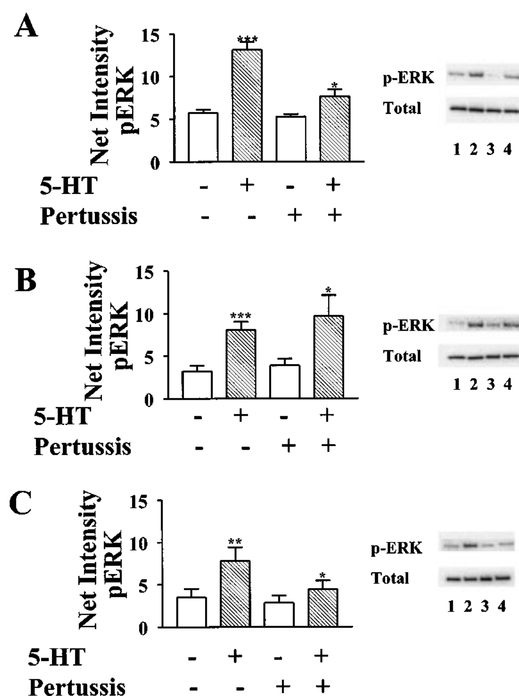

Figure 5 5-HT$_{1B}$ receptors couple preferentially to G$_{i\alpha 2}$. HeLa cells co-transfected with cDNA for the human 5-HT$_{1B}$ receptor and pertussis toxin resistant forms of either (A) G$_{i\alpha 1}$, (B) G$_{i\alpha 2}$, or (C) G$_{i\alpha 3}$ were treated overnight in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 20 ng ml^{-1} pertussis toxin before treatment with 10 μM 5-HT (lanes 2 and 4) for 5 min, and subsequent lyses. Total lysate was analysed by immunoblotting with antibody to phospho-ERK1/ERK2 (p-ERK). Membranes were then stripped and analysed with antibody to total ERK1/ERK2 (Total). Net intensities of bands were calculated from three separate experiments, performed in duplicate, and expressed as the means$\pm$s.e.mean ($\times 10^3$). *$P<0.05$; **$P<0.01$; ***$P<0.001$ *vs* absence of 5-HT, two-sided paired Student *t*-test calculated separately for both the presence and absence of pertussis toxin. Representative immunoblots from one of the three experiments are shown to demonstrate that treatment with pertussis toxin does not alter the levels of total ERK.

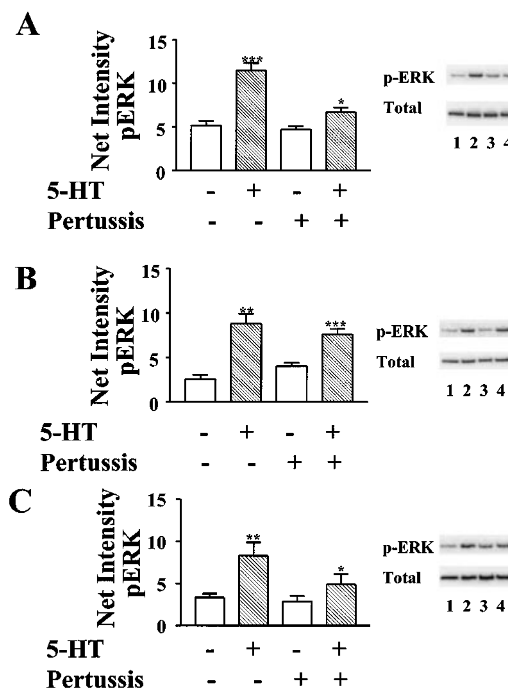

Figure 6 5-HT$_{1D}$ receptors are similar to 5-HT$_{1B}$ receptors in preferentially coupling to G$_{i\alpha 2}$. HeLa cells co-transfected with cDNA for the human 5-HT$_{1D}$ receptor and pertussis toxin resistant forms of either (A) G$_{i\alpha 1}$, (B) G$_{i\alpha 2}$, or (C) G$_{i\alpha 3}$ were treated overnight in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 20 ng ml^{-1} pertussis toxin before treatment with 10 μM 5-HT (lanes 2 and 4) for 5 min, and subsequent lyses. Total lysate was analysed by immunoblotting with antibody to phospho-ERK1/ERK2 (p-ERK). Membranes were then stripped and analysed with antibody to total ERK1/ERK2 (Total). Net intensities of bands were calculated from three separate experiments, performed in duplicate, and expressed as the means$\pm$s.e.mean ($\times 10^3$). *$P<0.05$; **$P<0.01$; ***$P<0.001$ *vs* absence of 5-HT, two-sided paired Student *t*-test calculated separately for both the presence and absence of pertussis toxin. Representative immunoblots from one of the three experiments are shown to demonstrate that treatment with pertussis toxin does not alter the levels of total ERK.

regulation of ERK and adenylyl cyclase by 5-HT$_{1B}$ receptors relative to 5-HT$_{1A}$ receptors (Mendez *et al*., 1999). Significantly, in each study the coupling by receptor subtypes was compared in an individual cell line, under identical conditions. Therefore, the difficulties inherent in comparing receptors expressed in different cell types was avoided. Together, these studies provide evidence that although all 5-HT$_1$ receptors, to some degree, negatively regulate adenylyl cyclase, they differentially couple to G proteins and consequently exhibit differences in coupling to cellular signals.

CHO cells were not utilized in the present study because they express, at low density, endogenous 5-HT$_{1B}$ receptors that couple to activation of ERK (Mendez *et al*., 1999). That low level of expression was useful in our earlier studies in which the coupling of receptors to cellular signals was studied at various receptor densities. However, interpretation of results from studies of receptor coupling to G proteins is facilitated when all receptors are activated with the physiological agonist (i.e. 5-HT, in the present studies). In this manner, problems resulting from the use of different receptor-selective agonists are avoided. For example, it has been reported that the relative preference of 5-HT$_{1A}$ receptors for subtypes of G$_{i\alpha}$ is modulated by the particular receptor agonist studied (Gettys *et al*., 1994). It is quite possible that the same also occurs with 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors.

Significantly, our finding that 5-HT$_{1A}$ receptors more effectively utilized G$_{i\alpha 1}$ than did 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors, cannot be attributed to differences in expression of receptors or G proteins. Binding studies demonstrated that the density of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors was similar to the density of 5-HT$_{1A}$ receptors. Similarly, the poor coupling by all 5-HT$_1$ receptor subtypes to G$_{i\alpha 3}$, was not the result of lower levels of expression of the G$_\alpha$ subunit relative to G$_{i\alpha 1}$ and G$_{i\alpha 2}$. Immunoblot analysis demonstrated similar levels of expression of all three G$_{i\alpha}$ subunits.

Our finding that all three subtypes of 5-HT$_1$ receptors effectively utilize G$_{i\alpha 2}$ to activate ERK is consistent with findings from studies of other G$_i$-coupled receptors. Winitz *et al*. (1994) demonstrated that a dominant-negative form of G$_{i\alpha 2}$ inhibited thrombin and ATP receptor-coupling to activation of ERK. Conversely, pertussis toxin resistant forms of G$_{i\alpha 2}$ have been reported to rescue activation of

ERK by A1-adenosine receptors (Pace *et al*., 1995), and constitutively active forms independently stimulate activation of the MAP kinase (Edmastsu *et al*., 1998). However, our results additionally demonstrate that some G_i-coupled receptors can also, to varying degrees, utilize $G_{i\alpha1}$ and $G_{i\alpha3}$ to stimulate activation of ERK. Interestingly, in our studies of G protein coupling to $5\text{-}HT_{1A}$ receptors, we found that transfection with $G_{i\alpha2}$ resulted in increased basal activity relative to that seen with transfection of $G_{i\alpha1}$ and $G_{i\alpha3}$. This may represent an enhancement of receptor constitutive activity by $G_{i\alpha2}$.

The observed differences in $5\text{-}HT_1$ receptor-coupling to isoforms of $G_{i\alpha}$ provide an explanation for our previous demonstration of differential coupling of receptors to cellular signals (Mendez *et al*., 1999). While all subtypes of $G_{i\alpha}$, by definition, inhibit the activity of adenylyl cyclase in *in vitro* studies, there is increasing evidence that the particular isoforms differentially regulate cellular pathways in intact cells. This may result, in part, from their expression in different cellular locations. For example, in LLC–PK1 renal epithelial cells, $G_{i\alpha2}$ is localized to the basolateral membrane where it negatively couples to adenylyl cyclase, while $G_{i\alpha3}$ is expressed both in the apical membrane where it stimulates Na^+ channel activity and in the Golgi (Ercolani *et al*., 1990). Interestingly, Garnovskaya *et al*. (1997) found that pertussis toxin resistant forms of $G_{i\alpha2}$ and $G_{i\alpha3}$, but not $G_{i\alpha1}$, rescued coupling of $5\text{-}HT_{1A}$ receptors to activation of Na^+/H^+ exchange in CHO cells. Our findings suggest that this likely reflected a lack of coupling of $G_{i\alpha1}$ to Na^+/H^+ exchange rather than a lack of coupling of $G_{i\alpha1}$ to $5\text{-}HT_{1A}$ receptors. Perhaps, in CHO cells, the expression of $G_{i\alpha1}$ is localized such that it cannot modulate Na^+/H^+ exchange. Our demonstration that $5\text{-}HT_{1A}$, but not $5\text{-}HT_{1B}$ and $5\text{-}HT_{1D}$, receptors efficiently utilize $G_{i\alpha1}$ relative to $G_{i\alpha2}$, is significant in that the differential coupling was observed in studies of the same cellular signal (ERK), in the same cells, under identical conditions.

Interestingly, our findings differ from those obtained in binding studies utilizing membranes from infected Sf9 cells. In those studies $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$, each were found to reconstitute high-affinity binding by all three $5\text{-}HT_1$ receptors (Butkerait *et al*., 1995; Clawges *et al*., 1997; Brys *et al*., 2000). However, our studies were different in that we attempted to more closely duplicate the coupling of endogenous receptor to endogenous G protein that would occur in human cells. While Sf9 cells are non-mammalian cells, our studies utilized human HeLa cells. Similarly, the previous studies utilized rat and mouse G proteins, while our studies examined coupling to human G proteins. These differences, as well as the much higher levels of protein expression achieved in Sf9 cells, relative to mammalian cells, could account for our different results.

Significantly, since our study utilized toxin resistant mutants of human $G_{i\alpha}$ to ‘rescue’ receptor/G protein-coupling, we were able to study the efficacy of receptor coupling to each subtype of $G_{i\alpha}$ in isolation. In that pertussis toxin prevents the coupling of receptors to endogenous $G_{i\alpha}$, the relative endogenous expression of $G_{i\alpha}$ isoforms in the particular cell type studied does not alter the observed results. Although HeLa cells express endogenous $G_{i\alpha3}$ and $G_{i\alpha1}$ in a 10:1 ratio, with little $G_{i\alpha2}$ (Raymond *et al*., 1993), our results are relevant to any type of cell expressing $5\text{-}HT_1$ receptors, regardless of the composition of expressed $G_{i\alpha}$.

Our studies, in that they model the coupling of $5\text{-}HT_1$ receptors to G proteins in human cells, may therefore be relevant to human central nervous system (CNS) neurons, and consequently to understanding the etiology of mood disorders. There is increasing evidence that patients with depression and bipolar disorder exhibit alterations in the expression of G proteins (Young *et al*., 1994; Avissar *et al*., 1997). Our findings suggest that changes in the expression of $G_{i\alpha2}$ could have an effect on the activation of ERK stimulated by each of the three $5\text{-}HT_1$ receptors. In contrast, alterations in the level of $G_{i\alpha1}$ would impact primarily the activity elicited by $5\text{-}HT_{1A}$ receptors. Such changes in G protein expression could be postulated to contribute to the pathophysiology of mood disorders since the ERK pathway is required for normal neuronal functioning, and is known to enhance cell survival (Encinas *et al*., 1999; Erhardt *et al*., 1999).

Our findings that subtypes of $5\text{-}HT_1$ receptors display similar, but distinct, patterns of coupling to G proteins and cellular signals (Mendez *et al*., 1999) is consistent with the hypothesis that a large number of receptors, with subtle differences, are required to mediate the actions of 5-HT. Serotonergic medications are known to have diverse and complex actions on the central nervous system. They are used to treat such complicated disorders as depression, anxiety, eating disorders, obsessive–compulsive disorder, and schizophrenia. As the functions of individual 5-HT receptors continue to be elucidated, it may become possible to design medications that act more selectively at the specific receptor/receptors relevant to particular disorders.

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