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Modulation by μ-Opioid Agonists of Guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate Binding to Membranes from Human Neuroblastoma SH-SY5Y Cells

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SUMMARY

The ability of μ-opioid agonists to activate G proteins has been demonstrated by studying the binding of the GTP analogue guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) to membranes from the human neuroblastoma SH-SY5Y cell line. The potent opioid agonist fentanyl caused an approximate doubling of basal [^{35}S]GTPγS binding in a naloxone-sensitive manner, confirming this to be an opioid receptor-mediated process. The presence of GDP was necessary to observe this effect. Pretreatment of the cells with pertussis toxin (100 ng/ml, for 24 hr) completely prevented the fentanyl-stimulated increase in [^{35}S]GTPγS binding and lowered the basal binding of [^{35}S]GTPγS. These latter data suggest an involvement of G_i and/or G_o proteins and their activation by added membrane-bound receptors even in the absence of agonist. The order of potency of a series of opioid agonists in stimulating the binding of [^{35}S]GTPγS was buprenorphine > cyclazocine = levallorphan > nalorphine > [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAMGO) > fentanyl > morphine > pentazocine. DAMGO, fentanyl, and morphine were full agonists but the remaining compounds showed decreasing levels of intrinsic activity in the order buprenorphine > pentazocine > cyclazocine = nalorphine > levallorphan. The opioid antagonist naloxone was without effect. Under the conditions of the [^{35}S]GTPγS assay, binding of agonists was to a high affinity site, indicating that a high agonist affinity state of the μ-opioid receptor is responsible for the observed stimulation of [^{35}S]GTPγS binding. The level of [^{35}S]GTPγS binding (597 fmol/mg of protein) stimulated by DAMGO was 2-fold greater than the maximal number of μ-opioid agonist binding sites (B_{max}) determined using [^{3}H]DAMGO (254 fmol/mg of protein). The opioid agonist-mediated stimulation of [^{35}S]GTPγS binding in SH-SY5Y cell membranes thus provides a "functional" measure of agonist occupation of μ-opioid receptors and offers a simple method for the determination of efficacy and intrinsic activity of μ-opioid agonists.

μ-Opioid receptors belong to the superfamily of seven-transmembrane domain receptors that couple to heterotrimeric G proteins (1). Agonist occupation of the μ-opioid receptor can lead directly to inhibition of adenylyl cyclase (2, 3), activation of an inwardly rectifying potassium conductance (4), and inhibition of Ca^{2+} conductance through N-type channels (5). All of these events are mediated by pertussis toxin-sensitive G proteins.

One of the first biochemical events after agonist occupation of seven-transmembrane domain, G protein-linked receptors is guanine nucleotide exchange (6). Thus, GDP, which is bound to the G_α subunit of the $G_{\alpha\beta\gamma}$ complex in the resting state, dissociates and is replaced by GTP. This is followed by dissociation of G_α-GTP from the βγ subunits. Both G_α-GTP (6) and βγ (7) have the potential to stimulate downstream events. The activity of the subunits is terminated by the intrinsic GTPase activity of G_α, which hydrolyzes the terminal phosphate bond of bound GTP to regenerate G_α-GDP, which reassociates with βγ subunits. Addition of the nonhydrolyzable GTP analogue GTPγS leads to the formation of G_α-GTPγS. However, this results in permanent activation of G_α, because GTPγS is only slowly susceptible to the intrinsic GTPase activity of G_α and thus G_α-GTPγS survives as a long-lived functional species (8). Using [^{35}S]GTPγS, the activation of pertussis toxin-sensitive G proteins after agonist occupation of muscarinic (9–11), formyl peptide (12), adenosine A_1 (13), and α_2-adrenergic (14) plasma membrane-bound receptors has been determined as an increase in the binding of the labeled nucleotide to the membranes. Lazareno and Birdsall (15) demonstrated the validity of [^{35}S]GTPγS binding as a functional measure of response and used the assay to estimate affinity constants and Schild slope factors for a wide range of muscarinic m1–m4 receptor antagonists.

The human μ-opioid receptor is endogenously expressed in

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ABBREVIATIONS: GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; DAMGO, [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TIPP, Tyr-Tic-Phe-Phe (where Tic is tetrahydroisoquinoline-3-carboxylic acid).

the neuroblastoma SH-SY5Y cell line (2, 3). The interaction of human μ-opioid receptors with G proteins in these cells has been directly demonstrated as an agonist-mediated increase in the binding of the photoaffinity GTP analogue [α-32P]GTP-azidoanilide to G proteins. These studies have indicated association with various subtypes of G_i and G_o proteins (16), which has generally been confirmed by others using selective antibodies (17). However, such methodology does not readily lend itself to quantitative pharmacological analysis. To date, such analysis has relied on measurement of the secondary event of increased activity of the intrinsic GTPase of G_α, measured as the hydrolysis of 32P-labeled GTP (18), which follows formation of the G_α-GTP species (19, 20). This assay is not very sensitive and suffers from the requirement for a GTP-regenerating system and the important complication that the stimulated GTPase activity has to be distinguished from high-K_m GTPase enzymic activity within the system, which contributes significantly to [32P]GTP hydrolysis (18–20).

In this work we show that opioid agonists stimulate the binding of [35S]GTPγS to membranes of SH-SY5Y cells. The increase in the binding of [35S]GTPγS stimulated by opioid agonists is a ready "functional" measure of agonist occupation of μ-opioid receptors, and thus the system is able to distinguish compounds of differing efficacy and intrinsic activity. Furthermore, activation of G proteins, measured as an increase in [35S]GTPγS binding, may represent an important amplification of the agonist-mediated signal. A preliminary account of parts of this work has been presented (21).

Materials and Methods

Chemicals and drugs. [35S]GTPγS (46.1–51.5 TBq/mmol) was from New England Nuclear, and [3H]DAMGO (1.49 TBq/mmol) and [3H]diprenorphine (1.78 TBq/mmol) were from Amersham International. Unlabeled DAMGO and naloxone were purchased from Sigma (Poole, UK). Other compounds were gifts, as follows: fentanyl (Janssen, Beerse, Belgium), morphine (MacFarlan Smith, Edinburgh, UK), buprenorphine (Reckitt and Colman, Hull, UK), pentazocine, levallorphan, cyclazocine, and nalorphine (Zeneca, Alderley Edge, UK), and TIPP (Dr. P. Schiller, Clinical Research Institute of Montreal, Canada). Minimal essential medium, fetal and newborn calf serum, fungizone, penicillin/streptomycin, and L-glutamine were from GIBCO. Pertussis toxin and all other chemicals were from Sigma and of analytical grade.

Membrane preparation. Undifferentiated human neuroblastoma SH-SY5Y cells (passages 75–90) were cultured in minimal essential medium supplemented with 10% newborn calf serum, 2% fetal calf serum, and antibiotics, as described previously (22). Briefly, cells were grown to confluency in monolayers at 37° in a humidified 5% CO_2 atmosphere. The cells were harvested in HEPES (20 mM, pH 7.4)-buffered saline containing 1 mM EDTA and were dispersed by agitation. After centrifugation at 500 × *g*, the cell pellet was suspended in a buffer of 20 mM HEPES, pH 7.4, 100 mM NaCl, and 10 mM $MgCl_2 \cdot 6H_2O$ (buffer A) and was homogenized using an Ultraturrax homogenizer. The resultant homogenate was centrifuged at 20,000 × *g* and the pellet was collected, washed in buffer A, and recentrifuged. The pellet was finally resuspended in buffer A to give a protein concentration of 100–200 μg/ml (23). All procedures were performed at 4°. In certain experiments to determine opioid binding, buffer A was replaced with 50 mM Tris·HCl buffer, pH 7.4, or Krebs-HEPES buffer, pH 7.4, in all procedures and the final pellet was resuspended to a protein concentration of approximately 100 μg/ml (23).

[35S]GTPγS binding assay. Unless otherwise stated, membranes (100–200 μg of protein), prepared as described above, were incubated in buffer A containing [35S]GTPγS (80 pM), GDP (3 μM), and varying concentrations of opioid (0.1–10,000 nM), in a total volume of 1 ml, for 60 min at 30°. Nonspecific binding was defined using unlabeled GTPγS (10 μM). Bound and free [35S]GTPγS were separated by vacuum filtration through GF/B filters and quantified by liquid scintillation counting.

Opioid binding assays. Membranes (100 μg of protein) prepared as described above were incubated in the Tris·HCl buffer with varying concentrations (0.04–10 nM) of the μ-opioid ligand [3H]DAMGO, in a total volume of 1 ml, for 40 min at 25°, as described previously (24). Competition assays using [3H]diprenorphine (0.8 nM) were performed as described above but in the presence of TIPP (100 nM) (25), to prevent labeling of δ-opioid receptors, in buffer A containing GDP (3 μM) and GTPγS (100 pM) or in Krebs-HEPES buffer (25 mM $NaHCO_3$, 11.8 mM NaCl, 4.7 mM KCl, 1.17 mM KH_2PO_4, 1.18 mM $MgSO_4 \cdot 7H_2O$, 2.52 mM $CaCl_2$, 11.7 mM glucose, 10 mM HEPES, pH 7.4). In all opioid binding experiments, nonspecific binding was defined with naloxone (10 μM), and bound ligand and free ligand were separated by vacuum filtration and quantified by liquid scintillation counting. For both opioid and [35S]GTPγS binding assays, total numbers of binding sites (B_{max}) and affinities (K_d) were determined using the program LIGAND (26).

Results

Stimulation of the binding of [35S]GTPγS (80 pM) to membranes from SH-SY5Y cells by the μ-opioid agonist fentanyl at a maximal concentration of 3 μM was observed only in the presence of GDP (Fig. 1). A minimal level of 300 nM GDP was necessary to observe stimulation, and an optimal signal-to-noise ratio was obtained with 3 μM GDP. Stimulation of [35S]GTPγS was optimal at temperatures between 25° and 30° and linear up to 400 μg of protein. The agonist-induced increase in [35S]GTPγS binding was observed after 5 min under these conditions but did not reach a maximal effect until 120 min (Fig. 2). Assays were routinely performed at 30° with 150–200 μg of membrane protein for 60 min, to afford approximately 100% stimulation of [35S]GTPγS (80 pM) binding over controls in the presence of a maximal concentration of fentanyl. This represented an increase of 34.4 ± 3.2 fmol of [35S]GTPγS bound/mg of protein (23 experiments).

The increase in binding of [35S]GTPγS, over basal levels,

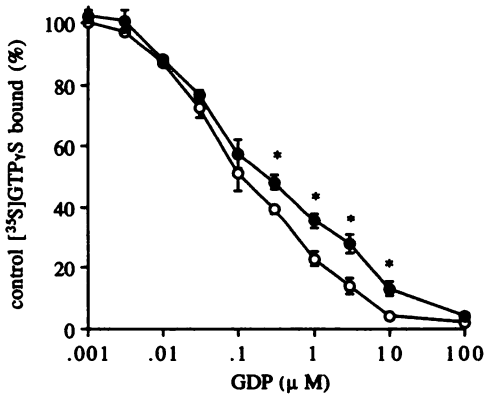

Fig. 1. Effect of GDP concentration on the binding of [35S]GTPγS (80 pM) to membranes of SH-SY5Y cells in the absence (○) and presence (●) of fentanyl (3 μM). Assays were performed as described in Materials and Methods, for 60 min at 30°. *Points*, means ± standard errors from three separate experiments performed in duplicate. *, $p < 0.05$ (Student's *t* test).

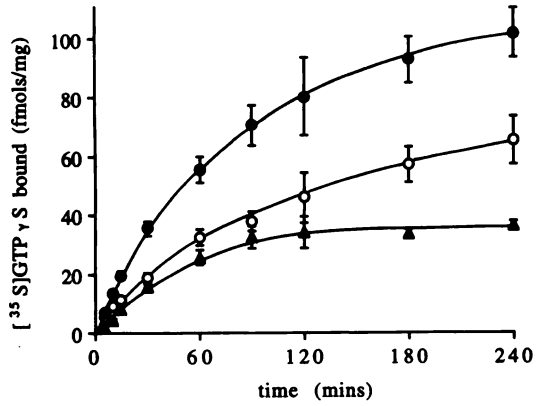

Fig. 2. Time course for the binding of [^{35}S]GTPγS (80 pM) to membranes of SH-SY5Y cells in the absence (○) and presence (●) of fentanyl (3 μM). ▲, Degree of [^{35}S]GTPγS binding stimulated by fentanyl (3 μM). Assays were performed in the presence of 3 μM GDP, as described in Materials and Methods, at 30°. *Points*, means ± standard errors from four separate experiments performed in duplicate.

induced by the μ-opioid agonist fentanyl was concentration dependent (Fig. 3), affording an EC_{50} of 15 nM (Table 1). This stimulation was antagonized by naloxone (30 nM), which shifted the concentration-effect curve for fentanyl to the right by approximately 12-fold (Fig. 3), to afford an apparent pA_2 for naloxone of 8.6, indicating μ-opioid receptor involvement (27).

The fentanyl-mediated stimulation of [^{35}S]GTPγS binding to membranes from SH-SY5Y cells was completely blocked by incubation of the cells with pertussis toxin (100 ng/ml) for 24 hr before preparation of the membranes (Fig. 4). Indeed, pertussis toxin treatment also lowered the control binding in these cells by approximately 30%, from a level of 31 fmol/mg of protein to 21 fmol/mg of protein. No change in this level was observed even in the presence of 10 μM fentanyl.

The stimulatory effect of fentanyl on [^{35}S]GTPγS binding was also mimicked by the opioid receptor agonists DAMGO and morphine, which gave similar maximal responses. In contrast, buprenorphine and pentazocine acted as partial agonists in the system (Fig. 5). The maximal stimulation of

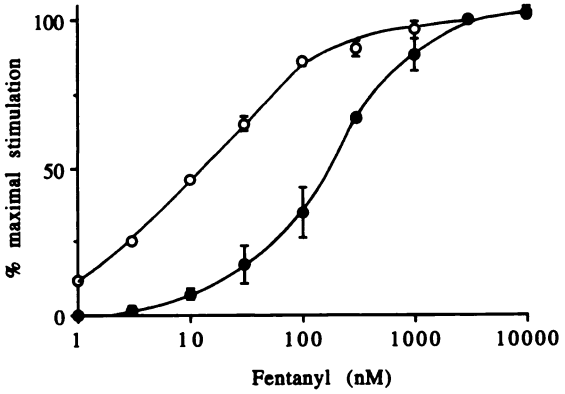

Fig. 3. Stimulation of [^{35}S]GTPγS (80 pM) binding to membranes of SH-SY5Y cells by various concentrations of fentanyl in the absence (○) and presence (●) of naloxone (30 nM). Assays were performed in the presence of 3 μM GDP for 60 min at 30°. *Points*, means ± standard errors from three separate experiments performed in duplicate. Binding of [^{35}S]GTPγS was 33.00 ± 4.03 fmol/mg of protein in the absence of fentanyl and 56.25 ± 3.76 fmol/mg of protein in the presence of fentanyl (3 μM).

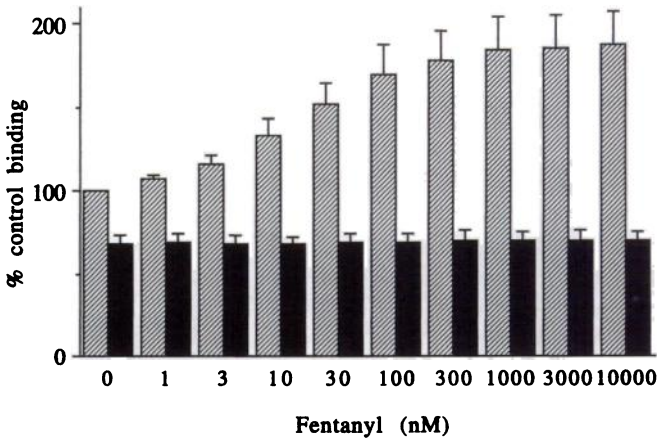

Fig. 4. Effect of pertussis toxin (100 ng/ml) pretreatment (24 hr) of SH-SY5Y cells on the fentanyl-mediated stimulation of [^{35}S]GTPγS (80 pM) binding to membranes. ▨, Membranes prepared from control cells; ■, membranes prepared from pertussis toxin-treated cells. Assays were performed in the presence of 3 μM GDP for 60 min at 30°. Values represent means ± standard errors from three separate experiments performed in duplicate. Control binding (32.1 ± 4.1 fmol/mg of protein) was significantly higher ($p < 0.005$, Student's *t* test) than all pertussis toxin-treated values (21.4 ± 0.5 fmol/mg of protein).

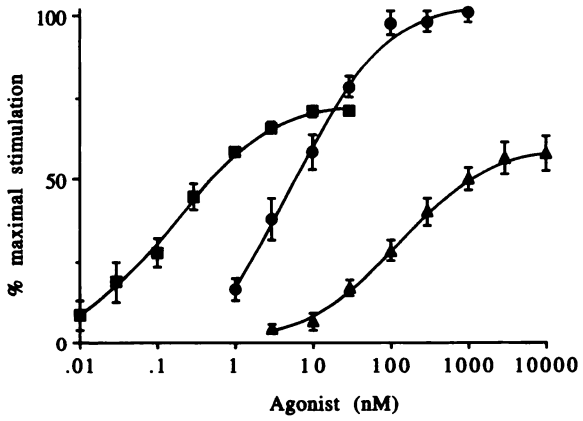

Fig. 5. Comparison of the stimulation of [^{35}S]GTPγS (80 pM) binding to membranes of SH-SY5Y cells by DAMGO (●), buprenorphine (■), and pentazocine (▲). Assays were performed in the presence of 3 μM GDP for 60 min at 30°. Values represent percentage of the maximal response to fentanyl (3 μM) and are given as means ± standard errors from three separate experiments performed in duplicate. Control binding of [^{35}S]GTPγS was 47.2 ± 4.13 fmol/mg of protein in the absence of fentanyl and 81.0 ± 7.95 fmol/mg of protein in the presence of fentanyl (3 μM).

[^{35}S]GTPγS binding by opioid ligands decreased in the order DAMGO = fentanyl = morphine > buprenorphine > pentazocine > cyclazocine = nalorphine > levallorphan (Table 1). The potencies of the compounds did not relate to the maximal stimulation of [^{35}S]GTPγS binding that could be obtained. Thus, the most potent and least potent compounds were the partial agonists buprenorphine (EC_{50}, 0.2 nM) and pentazocine (EC_{50}, 90 nM), respectively. The nonselective opioid antagonist naloxone, over the concentration range of 1–10,000 nM, did not alter the basal level of [^{35}S]GTPγS binding.

Displacement of the binding of the antagonist [^{3}H]diprenorphine under the conditions used for the [^{35}S]GTPγS binding assay revealed high affinity binding for the agonist DAMGO and the partial agonist pentazocine (Fig. 6), affording K_i values of 20.4 ± 0.4 nM (three experiments) and 51.9 ±

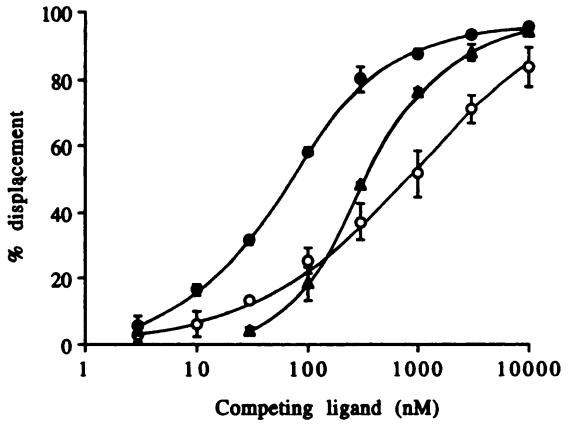

Fig. 6. Displacement of the binding of [3H]diprenorphine (0.8 nM) from membranes of SH-SY5Y cells by DAMGO (●) and pentazocine (▲) in [35S]GTPγS binding buffer (buffer A; see Materials and Methods) and DAMGO in Krebs-HEPES buffer (○). Assays were performed as described in Materials and Methods. *Points*, means ± standard errors from three separate experiments performed in duplicate.

TABLE 1

Stimulation of [35S]GTPγS binding to membranes from SH-SY5Y cells by opioids of varying efficacy and comparison with μ-opioid binding site affinity.

Membranes from SH-SY5Y cells were incubated with [35S]GTPγS (80 pM) and GDP (3 μM) for 60 min at 30, with increasing concentrations of drugs. Maximal effect was measured as the stimulation of binding in response to fentanyl (3 μM). Values are means ± standard errors from three or more separate experiments performed in duplicate. Opioid binding data are taken from Ref. 28 and were determined in rat brain homogenates in Krebs-HEPES buffer, pH 7.4, containing 10 μM guanosine-5′-(β, γ-imido)triphosphate.

Compound	[35S]GTPγS binding		Opioid binding, K_i
	EC_{50}	Maximal effect	
	nM		nM
Fentanyl	15.2 ± 3.0	1.00	214
Morphine	26.7 ± 6.3	0.82 ± 0.05	416
DAMGO	6.40 ± 1.0	1.10 ± 0.11	966
Pentazocine	90.2 ± 29.8	0.60 ± 0.05	270
Buprenorphine	0.18 ± 0.06	0.73 ± 0.03	NT[a]
Levallorphan	1.78 ± 0.75	0.32 ± 0.02	1.68
Cyclazocine	1.70 ± 0.50	0.40 ± 0.03	2.5
Nalorphine	2.78 ± 1.30	0.40 ± 0.02	19.5
Naloxone	No effect		3.0

[a]NT, not tested.

6.0 nM (three experiments), respectively, similar to the EC_{50} values for the stimulation of [35S]GTPγS binding by these two compounds (Fig. 5; Table 1). In contrast, the occupancy curve for the opioid peptide agonist DAMGO at low affinity states of the μ-opioid receptor, measured as the ability to displace the antagonist [3H]diprenorphine from binding sites in SH-SY5Y cell membranes in Krebs buffer (28) (Fig. 6), was approximatley 2 log units to the left of the dose-response curve for stimulation of [35S]GTPγS binding (Fig. 5). Comparison of these data with those generated in rat brain membranes under similar conditions but with the addition of the GTP analogue guanosine-5′-(β,γ-imido)triphosphate (28) showed a comparable affinity for DAMGO (Table 1). However, the relationship between the EC_{50} values obtained for the stimulation of [35S]GTPγS binding and the affinity (K_i) of other ligands for the low agonist affinity state of the μ-opioid binding site varied (Table 1). Thus, whereas the EC_{50} values for morphine, fentanyl, and DAMGO were considerably to the left of the occupancy curves, the EC_{50} values and K_i values for the partial agonists were comparable.

To determine the maximal binding of [35S]GTPγS that could be stimulated by opioid agonists in the SH-SY5Y cell membranes, the homologous displacement of [35S]GTPγS by unlabeled GTPγS was carried out over 3 hr in the absence and presence of a maximal (10 μM) concentration of DAMGO, to ensure that all relevant G proteins were labeled (Fig. 7). The stimulation of [35S]GTPγS binding by agonists was to a high affinity (nanomolar) site and was not observed at concentrations of GTPγS exceeding 10 nM. Analysis of the difference between the two displacement curves using the curve-fitting program LIGAND (Fig. 7, *inset*) afforded a K_d for GTPγS at this site of 0.72 ± 0.09 nM and a B_{max} of 597 ± 58.9 fmol/mg of protein. Analysis of the saturation binding of [3H]DAMGO to SH-SY5Y cell membranes in Tris·HCl buffer indicated a B_{max} of 254 ± 19 fmol/mg of protein and a single high affinity site (K_d, 0.74 ± 0.24 nM) (Fig. 8).

Discussion

The results demonstrate that μ-opioid receptor agonists are able to stimulate the binding of [35S]GTPγS to membranes from SH-SY5Y human neuroblastoma cells and that this is a concentration-dependent, receptor-mediated event. This provides an assay, using a simple membrane preparation, that distinguishes the ability of agonists to activate G proteins and provides a functional correlate of ligand-binding experiments.

To observe a stimulation of [35S]GTPγS binding by opioid agonists, it was necessary to include GDP in >1000-fold excess over [35S]GTPγS. The GDP reduced control [35S]GTPγS binding more than μ-opioid-stimulated binding, resulting in a measurable difference. This critical role for GDP has also been observed in studies of the stimulation of [35S]GTPγS binding by the chemotactic peptide formyl-Met-Leu-Phe in HL-60 cell membranes (12), by adenosine A_1 agonists in bovine brain membranes (13), and by muscarinic agonists in porcine cardiac membranes (9) but not by α_2-adrenergic agonists in PC-12 cells (14). Similarly, high levels

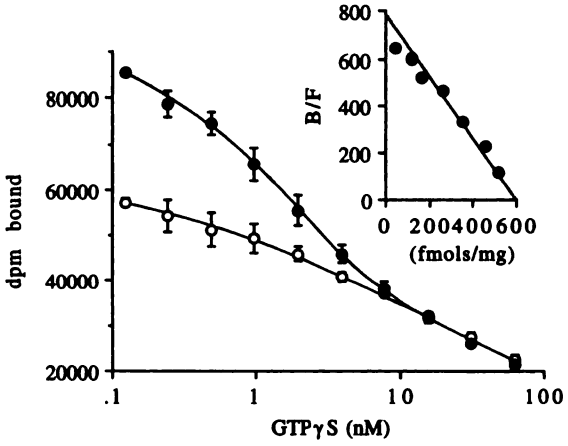

Fig. 7. Homologous displacement by GTPγS of [35S]GTPγS (80 pM) binding to membranes of SH-SY5Y cells in the absence (○) and presence (●) of DAMGO (10 μM). Assays were conducted for 180 min at 30° in the presence of 3 μM GDP. *Points*, means ± standard errors from three separate experiments performed in duplicate. *Inset*, mean Scatchard plot of the difference between the two curves obtained using the LIGAND program.

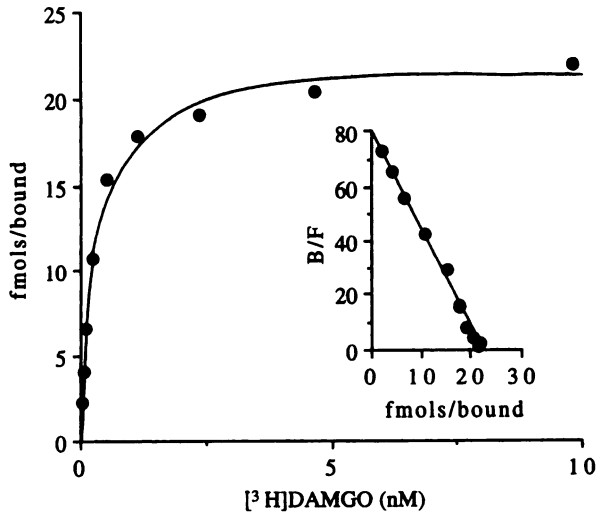

Fig. 8. Saturation binding of [^{3}H]DAMGO to membranes (92.1 μg of protein) from SH-SY5Y cells in Tris buffer (50 mM, pH 7.4) for 40 min at 25°. *Inset*, Scatchard transformation of the data. This is a representative experiment, which was replicated three times.

of GDP are needed for optimal [^{35}S]GTPγS binding stimulation by muscarinic agonists acting at m2 and m4 receptors expressed in Chinese hamster ovary (10) or human embryonic kidney (11) cells, which like opioid receptors are coupled to inhibition of adenylyl cyclase, but not for agonists acting at m1 and m3 receptors expressed in the same cells and presumably retaining identical G protein profiles (10, 11). The requirement for GDP may therefore relate to the different subtypes of heterotrimeric G proteins with which the various receptors interact.

The stimulation of [^{35}S]GTPγS binding by the μ-opioid agonist fentanyl was completely blocked by pretreatment of the cells with pertussis toxin, confirming that the event is mediated entirely through pertussis toxin-sensitive G proteins. This agrees with findings using the photoreactive affinity label [α-^{32}P]GTP-azidoanilide, which indicate a preference for μ-opioid receptors in these cells to couple to G_{i3} (16), and studies with selective antibodies to G proteins, which suggest that μ-opioid receptors couple to adenylyl cyclase via G_o (17). In addition, opioid-mediated inhibition of Ca^{2+} influx (5) reported in these cells is similarly pertussis toxin sensitive. However, pertussis toxin treatment of cells also lowered basal [^{35}S]GTPγS binding in subsequently prepared membranes. Because the toxin only uncouples G proteins from receptors and does not alter other properties of G proteins (29), this suggests that non-agonist-occupied receptors expressed in SH-SY5Y cells are actively coupled to pertussis toxin-sensitive G proteins and stimulate the binding of [^{35}S]GTPγS to G_α. This could indicate some form of constitutive activity within the SH-SY5Y cell membranes, such as has been observed with mutants of G_s-coupled β_2-adrenergic receptors, G_q-coupled α_1-adrenoceptors, and G_i-linked α_2-adrenergic receptors (for review, see Ref. 30).

The ability of pertussis toxin to lower basal [^{35}S]GTPγS binding, thus implying the presence of constitutive activity, has also been reported in membranes prepared from HL-60 cells (12) and Chinese hamster ovary cells (11) but not cardiac sarcolemma (9). It is likely that the preparation of SH-SY5Y cell membranes, and indeed of membranes reported in other studies, in some way releases a proportion of the endogenous receptor population from the constraints that prevent expression of constitutive activity under normal resting circumstances and that an intact intracellular environment is needed to prevent spontaneous receptor-G protein association (30). Such an effect could underlie the concept of inverse agonism (31) observed in several receptor-effector systems, where antagonists can decrease the ability of receptors to assume an active state. A recent report provides evidence that continual narcotic agonist stimulation of retinoic acid-differentiated SH-SY5Y cells causes the gradual formation of constitutively active μ-opioid receptors, as measured by the activity of adenylyl cyclase, and that this is an important stage in the development of opiate tolerance (32). In this system naloxone acts as an inverse agonist at constitutively activated μ-opioid receptors, causing an increase in adenylyl cyclase activity. However, in the present system naloxone had no effect on basal [^{35}S]GTPγS binding. This may suggest that naloxone is not an inverse agonist in the undifferentiated SH-SY5Y cells used in this study. This agrees with findings for the neuroblastoma × glioma NG108–15 cell line, which expresses δ-opioid receptors where naloxone is a neutral antagonist, with neither positive nor negative intrinsic activity (33). Alternatively, the activation of G proteins by SH-SY5Y cell membranes in the absence of agonist may involve nonopioid receptors. For example, SH-SY5Y cells express α_2-adrenergic (34), muscarinic m1, m2, and m3 (35), vasoactive intestinal peptide (36), bradykinin (37), and prostaglandin E (38) receptors. Any or all of these receptors could contribute to the observed constitutive activity.

The extent of stimulation of [^{35}S]GTPγS binding observed depended upon the opioid agonist used. Thus, DAMGO, fentanyl, and morphine all afforded similar maximal levels of stimulation, whereas buprenorphine > pentazocine > cyclazocine = nalorphine > levallorphan had progressively lower relative intrinsic activity and thus acted as partial agonists in this system, as in many other *in vitro* and *in vivo* systems (39), including retinoic acid-differentiated SH-SY5Y cells. In those cells pentazocine afforded 64% of the maximal morphine-induced inhibition of prostaglandin E_1-stimulated adenylyl cyclase (3). However, whereas the IC_{50} values for morphine (60 nM) and DAMGO (10 nM) in inhibiting cAMP production are similar to those seen here against [^{35}S]GTPγS binding, the potency of pentazocine is reduced (IC_{50}, 800 nM) (3).

The low agonist affinity conformation of μ-opioid receptors is likely to be the predominant form under physiological conditions (28). Comparison of the agonist concentration needed to occupy 50% of the μ-opioid receptors (K_i), under conditions promoting low agonist affinity states, with the EC_{50} for stimulation of [^{35}S]GTPγS binding showed a marked difference between compounds. Thus, the K_i/EC_{50} ratio for DAMGO was approximately 100, indicating a considerable receptor reserve. This is supported by the similarity between the EC_{50} values for the potent full agonists DAMGO and fentanyl and their reported ability to inhibit adenylyl cyclase in whole SH-SY5Y cells, affording values for half-maximal inhibition of 11 nM (19) and 27 nM (40), respectively. On the other hand, with the partial agonists the K_i and EC_{50} concentrations were similar, such that full occupation of receptors would be needed. However, under conditions identical to those used for the [^{35}S]GTPγS binding assay,

binding of ligands was to a higher affinity site, indicating that a high affinity state of the μ-opioid receptor is the form responsible for coupling to and activation of G proteins (6), rather than the low agonist affinity form, as suggested by Carroll *et al.* (28). The low affinity form would then represent receptors not associated with G proteins (6).

The number of [35S]GTPγS binding sites that can be occupied after agonist stimulation (597 fmol/mg) is approximately twice the total number of high affinity μ-opioid binding sites determined with the agonist [3H]DAMGO (254 fmol/mg). This is approximately the same as the ratio between the number of muscarinic receptors (1.4 pmol/mg) and the number of [35S]GTPγS binding sites (3–4 pmol/mg) under receptor control in porcine cardiac membranes (9). In contrast, in HL-60 cells the ratio of formyl-Met-Leu-Phe receptors to pertussis toxin-sensitive G proteins is 1:20, indicating a potential for receptors to activate a number of G proteins (12). These findings therefore apparently provide no evidence for amplification of the signal at this level of the receptor-effector pathway nor any explanation for the differences between full and partial agonists. However, an amplification of the full agonist signal could arise if one activated receptor was capable of stimulating many molecules of G protein. Thus, once G_α-[35S]GTPγS is formed after formation of an agonist-receptor-G protein ternary complex, the activated receptor could be released to activate a second G protein. In this way a single receptor could sequentially activate many G proteins, resulting in a large amplification as the number of G proteins activated increases with time (41), although with time the number of effective collisions would be reduced as available unactivated G protein becomes scarce (42).

An alternative explanation, which would help to clarify the difference between the partial and full opioid agonist responses, relies on the differences in the affinity of the agonist for the state of the receptor involved in the ternary complex (agonist-receptor-G protein) (high affinity) and the non-G protein-coupled receptor (low affinity). After dissociation of G protein subunits from the ternary complex, the agonist rapidly dissociates from the free receptor, which is thus available to be recycled and is able to interact with agonist and G protein to form a second ternary complex. Thus, the situation exists where a large number of receptors are activated for a small period of time, resulting in greater stimulation (42). In contrast, the partial agonists would not dissociate rapidly from the free receptor and thus the receptor would not be effectively available for recycling. For this latter hypothesis to be accepted, one has to include the caveat that the ligand-bound receptor, after dissociation of the G protein, is in a conformational state where it is not able to activate additional G protein molecules.

In conclusion, the findings demonstrate that μ-opioid agonists are able to stimulate the binding of [35S]GTPγS to pertussis toxin-sensitive G proteins in membranes of SH-SY5Y cells. Furthermore, the assay provides a simple functional response for opioid agonists in membrane preparations that distingushes compounds of differing intrinsic activity and that, in combination with conventional radioligand binding assays, can reveal variable efficacy of opioid agonists.

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