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Activation of G protein by opioid receptors: role of receptor number and G-protein concentration

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Abstract

The collision-coupling model for receptor-G-protein interaction predicts that the rate of G-protein activation is dependent on receptor density, but not G-protein levels. C6 cells expressing μ- or δ-opioid receptors, or SH-SY5Y cells, were treated with β-funaltrexamine (μ) or naltrindole-5′-isothiocyanate (δ) to decrease receptor number. The time course of full or partial agonist-stimulated [^{35}S]GTPγS binding did not vary in C6 cell membranes containing <1–25 pmol/mg μ-opioid receptor, or 1.4–4.3 pmol/mg δ-opioid receptor, or in SHSY5Y cells containing 0.16–0.39 pmol/mg receptor. The association of [^{35}S]GTPγS binding was faster in membranes from C6μ cells than from C6δ cells. A 10-fold reduction in functional G-protein, following pertussis toxin treatment, lowered the maximal level of [^{35}S]GTPγS binding but not the association rate. These data indicate a compartmentalization of opioid receptors and G protein at the cell membrane.

Keywords: μ-Opioid receptor; δ-Opioid receptor; G-protein; [^{35}S]GTPγS binding; Collision-coupling model; Compartmentalization

1. Introduction

Opioid agonists alter intracellular events by binding to receptor leading to subsequent activation of inhibitory G proteins. The interaction of receptors and second messenger proteins in a cell membrane has been described by a collision coupling model, originally defined following study of the coupling of the β-adrenoceptor and adenylyl cyclase in turkey erythrocytes (Tolkovsky and Levitzki, 1978). Extension of this model to G-protein would describe activated receptors as mobile catalysts for G protein activation, with access to many G proteins per unit time. The consequences of this model are that the level of G protein activated is independent of the concentration of agonist-bound receptors, but the rate of G protein activation is proportional to the concentration of agonist-bound receptors. In contrast, if receptor and G proteins are closely associated then a reduction in receptor number would lead to a reduction in the number of G proteins activated but would not be expected to alter the rate of their activation. The collision coupling model has been more recently refined to include the effect of agonist occupancy turnover on G protein activation (Stickle and Barber, 1993, 1996). This shows that the rate of G protein activation is also dependent on the agonist binding frequency relative to the encounter time of G protein and receptor.

To gain further insight into the organization of opioid receptors and G proteins we have tested the hypothesis, based on the collision coupling model, that a reduction in receptor density would cause no reduction in maximal level of G protein activation but would reduce the rate of G protein activation. We have also studied the consequences of a reduction in G protein density. For these studies we have used membranes from C6 glioma cells stably expressing the rat μ-(C6μ) or rat δ-(C6δ) opioid receptors (Emmerson et al., 1996; Clark et al., 1997; Alt et al., 1998) and human neuroblastoma SH-SY5Y cells expressing endogenous μ-opioid receptors (Kasmi and Misra, 1987; Yu and Sadee, 1988). These systems provide an

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excellent model to evaluate the efficacy of agonists in vitro by measuring agonist-stimulated [^{35}S]GTPγS (guanosine-5′-*O*-[3-thiotriphosphate]) binding (Traynor and Nahorski, 1995). The findings demonstrate that the extent of agonist-stimulated [^{35}S]GTPγS binding is dependent on both receptor number and G-protein density. Studies in reconstituted and other systems have shown that the rate-limiting step in G protein activation is the dissociation of GDP (Ferguson et al., 1986; Florio and Sternweis, 1989; Breivogel et al., 1998). Consequently the rate of association of GTP analogues, such as [^{35}S]GTPγS, reflects this dissociation and so the rate of activation of G proteins (Ferguson et al., 1986; Mukhopadhyay and Ross, 1999). In the present experiments the association rate for [^{35}S]GTPγS binding was shown to be independent of either G-protein or receptor levels. Moreover, there appear to be differences between μ- and δ-opioid receptors regarding the kinetics of G protein activation.

2. Materials and methods

2.1. Chemicals and drugs

[^{35}S]GTPγS (1300 Ci/mmol), [^{3}H]naloxone (53 Ci/mmol), [^{3}H]naltrindole (NTI, 33 Ci/mmol), and [^{32}P]NAD were purchased from Dupont NEN (Boston, MA). Pertussis toxin was purchased from List Biochemicals (Campbell, CA). Nalbuphine, naltrindole, SIOM (7-spiroindinooxymorphone), β-funaltrexamine, β-chlornaltrexamine, and naltrindole-5′-isothiocyanate were obtained through the Opioid Basic Research Center at the University of Michigan (Ann Arbor, MI). Geneticin was purchased from Fisher Scientific (Itasca, IL). Protogel was purchased from National Diagnostics (Atlanta, GA). Tyr-D-Ala-Gly-(Me)Phe-Gly-ol (DAMGO), [D-Ser^{2},L-Leu^{5}]enkephalyl-Thr (DSLET), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, Tris–HCl, NAD, GDP and other biochemicals were purchased from Sigma (St. Louis, MO).

2.2. Cell culture

C6 Glioma cells were stably transfected with the μ-opioid receptor as described in Emmerson et al. (1996) or with the δ-opioid receptor as described in Clark et al. (1997). Cells were grown to confluence at 37°C under 5% CO_2 in DMEM (C6μ and C6δ) or Minimal Essential Medium (MEM, SH-SY5Y) containing 10% fetal bovine serum and, in the case of C6 cells, either with 0.5 mg/ml Geneticin (for subculture) or without Geneticin (for harvest). Pertussis treatment of cells was carried out by addition of pertussis toxin (2–20 ng/ml) with complete media replacement 4 or 20 h before harvesting. For treatment with β-funaltrexamine, or naltrindole-5′-isothiocyanate cell culture media was replaced with appropriate medium without fetal bovine serum at 37°C. For treatment with β-chlornaltexamine, cell culture media was replaced with physiological buffer containing 128 mM NaCl, 2.4 mM KCl, 1.3 mM $CaCl_2$, 2 mM $NaHCO_3$, 3 mM $MgSO_4$, 10 mM Na_2HPO_4, and 10 mM glucose at 37°C. β-funaltrexamine (4–20 nM final concentration), β-chlornaltrexamine (100 nM) or vehicle (water) was added to C6μ or SH-SY5Y cells or naltrindole-5′-isothiocyanate (100–300 nM) or vehicle (water) was added to C6δ cells. After 1 h at 37°C (3 h at 25°C for β-chlornaltrexamine) cells were washed four times with DMEM at 37°C before harvesting for the preparation of membranes.

2.3. Membrane preparation

Plasma membranes were prepared by lysis of cells in isotonic sucrose. Cells were washed twice with ice-cold phosphate-buffered saline (0.9% NaCl, 0.61 mM Na_2HPO_4, pH 7.4) and detached from culture flasks by incubation in lifting buffer (5.6 mM glucose, 5 mM KCl, 5 mM HEPES, 137 mM NaCl, 1 mM EGTA, pH 7.4) at room temperature, then collected by centrifugation at 200 $\times g$ for 3 min. The cell pellet was resuspended in 10 volumes of ice-cold 0.32 M sucrose (pH 7.4 with 1mM Tris–HCl) with a Teflon-glass homogenizer mounted to a Tri-R Stir-R motor at 1000 rpm. The suspension was centrifuged for 10 min at 1000 $\times g$ at 4°C, and the supernatant removed and kept on ice. The resuspension and centrifugation was repeated with the remaining pellet an additional three times, storing the supernatant from each spin on ice. The combined supernatants were then centrifuged at 15,000 $\times g$ for 20 min at 4°C. The resulting supernatant was discarded and the upper pellet separated by gently washing with ice-cold 0.32 M sucrose, then homogenized with a glass–glass homogenizer and centrifuged at 15,000 $\times g$ for 20 min at 4°C. The upper pellet was resuspended in 50 mM Tris–HCl buffer (pH 7.4) and centrifuged 20 min at 20,000 $\times g$ and 4°C. The final pellet was resuspended in 50 mM Tris buffer and frozen at −80°C in 0.25 ml aliquots (1–2 mg/ml). Protein concentration was determined by the method of Bradford (1976) with a bovine serum albumin standard. Samples were dissolved with 1 N NaOH for 30 min at room temperature and neutralized with 1 M acetic acid before protein determination.

2.4. Receptor binding

Ligand binding was carried out as described previously (Fischel and Medzihradsky, 1981). The binding of [^{3}H]naloxone (0.7–27 nM) or [^{3}H]naltrindole (0.004–1.5 nM) was determined in the presence of 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$ and 5–15 μg membrane protein in a total volume of 0.1 ml or 2.0 ml, respectively. Non-specific binding was defined using 10

μM naloxone or 1 μM naltrindole, respectively. Membranes were preincubated for 15 min at 25°C in assay buffer and ligand binding initiated by the addition of radiolabeled ligand. After incubation for 90 min at 25°C, the samples were quickly filtered through glass fiber filters (Schleicher and Schuell no. 32, Keene, NH) mounted in a Brandel cell harvester (Biomedical Research and Development Laboratories, Gaithersburg, MD) and rinsed 3 times with ice-cold 50 mM Tris–HCl (pH 7.4). Each filter was removed and placed in a 5-ml polypropylene scintillation vial with 0.4 ml ethanol, and 4 ml Ultima Gold scintillation cocktail was added. The samples were subjected to liquid scintillation counting. Results from 2–3 assays, carried out in duplicate, were combined and fitted to a one-site binding hyperbola using GraphPad Prism (GraphPad, San Diego, CA) to determine K_d and B_{max} values.

2.5. [^{35}S]GTPγS binding

Opioid-stimulated [^{35}S]GTPγS was determined in the presence of 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (freshly prepared), 50 μM GDP, 80–960 μg membrane, a maximal concentration of opioid agonist or vehicle (water), and 0.05 nM [^{35}S]GTPγS in a total volume of 8.0 ml. Membranes and assay buffer were preincubated for 10 min in a shaking water bath at 37°C then agonist or vehicle was added for an additional 10 min at 37°C before addition of [^{35}S]GTPγS to initiate the reaction. The association rate for [^{35}S]GTPγS binding was measured by removing 0.5 ml samples at 2–10 min intervals up to 100 min and filtering these through glass fiber filters (Schleicher and Schuell no. 32) as described above. The filters were quickly rinsed four times with 2 ml ice-cold 50 mM Tris–HCl (pH 7.4) containing 100 mM NaCl, and 5 mM $MgCl_2$. Filters were placed in polypropylene vials with 0.4 ml ethanol, and 4 ml Ultima Gold scintillation cocktail was added and subjected to liquid scintillation counting. Agonist-stimulated [^{35}S]GTPγS binding was determined as the difference between [^{35}S]GTPγS binding with and without agonist at each time point. The data was fitted to a one-phase exponential association curve using GraphPad Prism to determine the rate and maximum agonist stimulated [^{35}S]GTPγS binding. Each experiment was repeated two to three times. Concentration–response relationships for DAMGO in control and β-funaltrexamine treated cell membranes and for [Met^5]enkephalin and [Leu^5]enkephalin were determined in a 30 min assay at 25°C, as previously described (Clark et al., 1997).

2.6. Pertussis toxin catalyzed [^{32}P]ADP-ribosylation of G protein

The amount of G protein remaining after pertussis toxin treatment was determined by pertussis toxin catalyzed [^{32}P]ADP-ribosylation as described by Neubig et al. (1985). Membranes were thawed, centrifuged for 1 min in an Eppendorf table top centrifuge, and resuspended to 10 mg/ml in 20 mM Tris–HCl, 1 mM EDTA, and 1 mM dithiothreitol at pH 8.0 (TED). G proteins were extracted from the membranes by adding sodium cholate to a final concentration of 2% and incubating for 60 min on ice with frequent vortexing. The extracts were diluted 20 fold with 0.1% Lubrol PX in TED, frozen in liquid nitrogen, and stored at −80°C for up to 2 weeks. Ribosylation was initiated by adding 10 μl of thawed extract to 26 μl pertussis reaction mixture resulting in a final concentration of 2 μM [^{32}P]NAD (5000–10,000 cpm), 10 μg/ml pertussis toxin, 10 mM dithiothreitol, 100 mM Tris–HCl, 10 mM thymidine, 2.5 mM $MgCl_2$, 1 mM Na EDTA, 1 mM ATP, 0.1 mM GTP, and 0.61 mg/ml dimyristoylphosphatidylcholine at pH 8. After incubating for 60 min at 30°C the reaction was quenched by adding 10 μl electrophoresis sample buffer containing 6.25 mM Tris–HCl, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, and 0.05% bromophenol blue at pH 6.8. Samples were heated 5 min at 95°C before running on a 12% polyacrylamide gel according to the method of Laemmli (1970). The gel was dried and exposed to X-ray film at −80°C for 16 h. Each band was cut out and counted by liquid scintillation counting in 4 ml EconoSafe scintillation cocktail. After subtracting the blank value, the sample cpms (run in duplicate) were divided by the average cpm/pmol of the standards (0.5 and 1.0 pmol G_o/G_i purified from bovine brain generously provided by Dr. Richard R. Neubig, The University of Michigan) to determine the amount of [^{32}P]ADP ribosylated G protein.

3. Results

3.1. Effect of receptor density

The prediction of the collision-coupling model is that despite a decrease in receptor concentration the maximal extent of G-protein activation should be unchanged but the rate of activation should be altered. To examine this, membranes from C6μ and C6δ cells, with different receptor densities were employed. Receptor density was varied by treatment of cells with the irreversible ligands β-funaltrexamine or β-chlornaltrexamine (for μ receptors) or with naltrindole-5′-isothiocyanate (for δ receptors). The number of receptors was determined by Scatchard analysis of [^{3}H]naloxone or [^{3}H]naltrindole binding, respectively. The mean affinities of these ligands in the controls were not significantly different from those treated with alkylating agent in membranes from C6μ cells ($K_d = 3.56 \pm 0.36$ nM and 2.79 ± 0.54 nM, respectively) or from C6δ cells ($K_d = 0.136 \pm 0.076$ nM and 0.188 ± 0.085 nM, respectively).

Maximal G protein activation was determined by the degree of binding of [^{35}S]GTPγS stimulated by 1 μM DAMGO. Maximal G protein activation in control C6μ cells and β-funaltrexamine treated cells did not vary significantly (0.126 ± 0.017 pmol/mg and 0.124 ± 0.032 pmol/mg, respectively; Fig. 1). The maximal G protein activation by 1 μM nalbuphine, a partial agonist, was lower in membranes from β-funaltrexamine treated cells (0.039 ± 0.007 pmol/mg) than in control membranes (0.079 ± 0.013 pmol/mg). In membranes from C6δ cells, maximal G protein activation by 5 μM DSLET was not significantly different between membranes from control and naltrindole-5′-isothiocyanate treated cells (0.104 ± 0.022 pmol/mg and 0.090 ± 0.019 pmol/mg, respectively). The average number of receptors were 3.6 ± 1.1 pmol/mg in control and 1.5 ± 0.1 pmol/mg in membranes from naltrindole-5′-isothiocyanate treated cells, as measured by [^{3}H]naltrindole binding. Maximal activation by 1 μM SIOM was lower than that of DSLET, but not significantly different between membranes from control and naltrindole-5′-isothiocyanate treated cells (0.024 ± 0.006 pmol/mg and 0.020 ± 0.005 pmol/mg, respectively).

The binding of [^{35}S]GTPγS to G proteins is dependent on the agonist-mediated dissociation of GDP and so provides a measure of G protein activation. The association rate for agonist-stimulated [^{35}S]GTPγS binding did not significantly change over a 4-to 5-fold range of receptor concentration (5–23 pmol/mg protein) (Fig. 2A). The association rate for [^{35}S]GTPγS binding stimulated by the partial agonist nalbuphine was lower than that seen with DAMGO, but also showed no change with varying receptor number (Fig. 2A). Similar experiments using membranes from C6δ cells indicated no relationship between the association rate stimulated by the full agonist DSLET and receptor number over a fourfold receptor concentration range (1.4 to 4.3 pmol/mg) (Fig. 2B). The association rate stimulated by the partial agonist SIOM was not significantly different from the full agonist DSLET (Fig. 2B). The rate of association of basal [^{35}S]GTPγS binding in

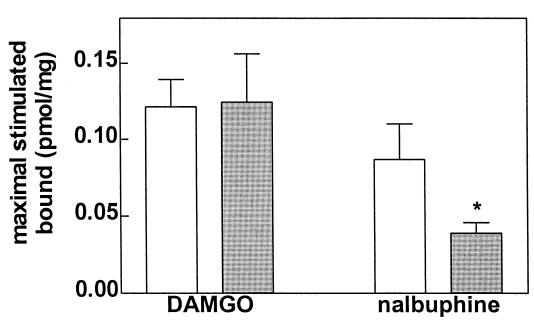

Fig. 1. Maximal stimulation of [^{35}S]GTPγS binding in membranes prepared from β-funaltrexamine treated and control C6μ cells. Cells were treated for 1 h at 37°C without (control) or with 15 or 20 nM β-funaltrexamine in the absence of serum, washed and membranes prepared as described in Section 2. Agonist-stimulated [^{35}S]GTPγS binding was determined at 37° with 1 μM DAMGO or 1 μM nalbuphine in control (open bars) or β-funaltrexamine treated (shaded bars) membranes as described in Section 2. β-funaltrexamine treatment reduced the receptor concentration from an average 15.0 ± 2.6 pmol/mg in control to 5.4 ± 0.1 pmol/mg as measured by [^{3}H]naloxone binding. Shown is the data from six to seven assays (error bars represent S.E.M.), each carried out in duplicate except for basal and complete displacement carried out in triplicate. The asterisk (*) indicates significantly different maximal nalbuphine-stimulated [^{35}S]GTPγS binding in membranes from control and β-funaltrexamine treated C6μ cells ($P < 0.05$ in a two-tailed, unpaired *t*-test). The average EC_{50} for DAMGO stimulation from two experiments is 80 ± 11 nM for control and 105 ± 5 nM for β-funaltrexamine treated membranes (data not shown).

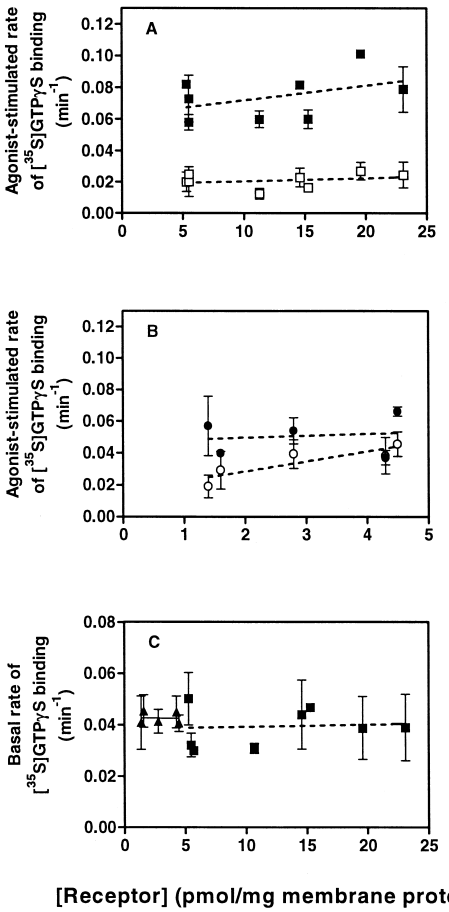

Fig. 2. Rate of agonist-stimulated [^{35}S]GTPγS binding in membranes prepared from β-funaltrexamine treated and control C6μ cells and from naltrindole-5′-isothiocyanate treated and control C6δ cells. Cells were treated for 1 h at 37°C without or with 4, 15 or 20 nM β-funaltrexamine (C6μ) or without or with 100 or 300 nM naltrindole-5′-isothiocyanate (C6δ) in the absence of serum, washed and membranes prepared as described in Section 2. The agonist-stimulated rate of [^{35}S]GTPγS binding was determined at 37°C with (A) 1 μM DAMGO (■), 1 μM nalbuphine (□), and (B) 5 μM DSLET(●), or 1 μM SIOM (○) as described in Section 2. (C) depicts the basal rate of [^{35}S]GTPγS binding in membranes prepared from control and alkylating agent-treated C6μ (■) and C6δ (▲) cells. Shown is the average rate (error bars represent standard error) from two to three assays of treated and control membranes from four separate treatments. The receptor number in the membrane preparations was determined by averaging [^{3}H]naloxone (μ) or [^{3}H]naltrindole (δ) saturation binding data from two to three experiments. The lines shown are the result of linear regression.

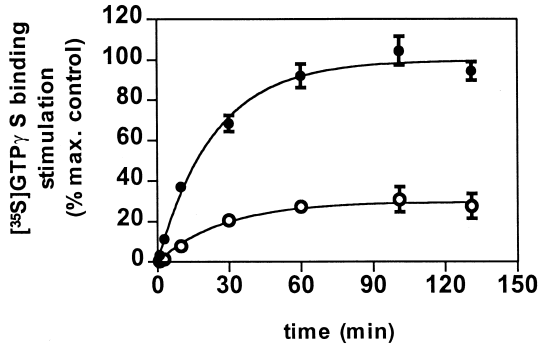

Fig. 3. Agonist-stimulated [^{35}S]GTPγS binding in membranes prepared from control and β-chlornaltrexamine treated-C6μ cells. Cells were incubated in physiological buffer for 3 h at 25°C in either the absence (●) or presence (○) of 100 nM β-chlornaltrexamine prior to membrane preparation as described in Section 2. Membranes from cells treated with β-chlornaltrexamine retained 11±2% of the receptors seen in control membranes, as measured by [^{3}H]naltrexone binding (data not shown). The effect of reduced receptor concentration on the ability of DAMGO (10 μM) to stimulate [^{35}S]GTPγS binding over time was then measured as described in Section 2. The data were fit to a one-phase exponential association equation using GraphPad Prism. Data shown are mean and S.E.M. from three experiments.

C6μ or C6δ membranes showed no correlation with varying receptor density (Fig. 2C).

A concern regarding these findings is that they were not gained using a cell line endogenously expressing opioid receptors, and from a cell line stably expressing particularly high levels of receptor. Therefore, two further experiments were performed. Firstly, membranes from C6μ cells expressing 2.6 ± 0.3 pmol/mg μ-opioid receptor were treated with β-chlornaltrexamine such that only 11 ± 2% of the receptors seen in control membranes remained. Membranes from these cells showed a reduction in the maximum level of [^{35}S]GTPγS binding stimulated by DAMGO giving only 24 ± 4% of that seen in control cells (Fig. 3). In contrast, the apparent association rates were unchanged: 0.042 ± 0.005 and 0.037 ± 0.009 min^{-1} corresponding to half-times of 16.6 min and 18.8 min in control and β-chlornaltrexamine treated membranes, respectively. Secondly, DAMGO-stimulated G protein activation was examined in membranes prepared from control and β-funaltrexamine treated SH-SY5Y membranes displaying 0.39 ± 0.12 and 0.16 ± 0.02 pmol receptor/mg membrane protein respectively as determined with [^{3}H]naloxone. The association rate of DAMGO-stimulated [^{35}S]GTPγS binding was again not significantly different between control and alkylated membranes (Fig. 4). However, a 60% decrease in the maximum level of [^{35}S]GTPγS binding was observed.

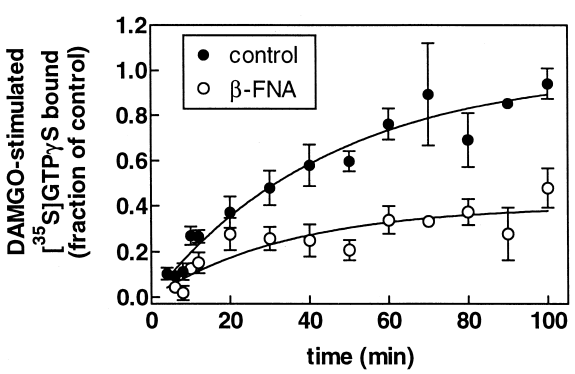

Fig. 4. Rate of agonist-stimulated [^{35}S]GTPγS binding in membranes prepared from β-funaltrexamine treated and control SH-SY5Y cells. SH SY5Y cells were treated for 1 h at 37°C with 0 (●) or 30 nM β-funaltrexamine (○), washed and membranes prepared as described in Section 2. Shown is the combined and normalized data from three to five experiments, measuring the rate of 1 μM DAMGO-stimulated [^{35}S]GTPγS binding at 37°C as described in Section 2. The curve shown is a result of a one component exponential association equation. The halftime of association is 25±6 min for control and 21±7 min for β-funaltrexamine treated membranes. The maximum amount of [^{35}S]GTPγS bound in the alkylated membranes is 40±6% of that in control membranes. The maximum [^{3}H]naloxone binding was 0.39 pmol/mg for control and 0.16 pmol/mg for β-funaltrexamine treated membranes (data not shown).

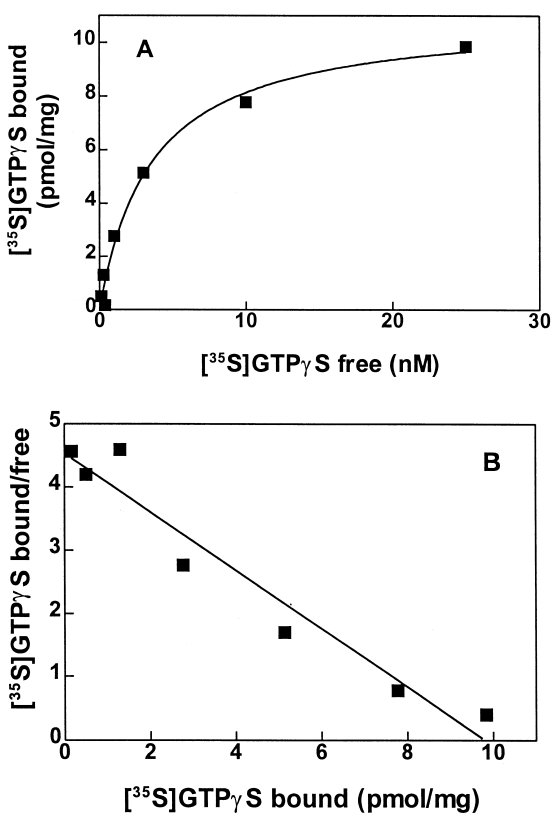

Fig. 5. Saturation analysis of DAMGO-stimulated [35S]GTPγS binding to membranes of C6μ cells. Saturation binding of [^{35}S]GTPγS was performed in the absence or presence of 10 μM DAMGO. Assays were carried out for 120 min at 25°C in the presence of 30 μM GDP with 15 μg membrane protein as described in Section 2. Unstimulated [^{35}S]GTPγS binding was subtracted from the DAMGO-stimulated value at each point. Shown is the saturation curve (A) and Scatchard plot (B) from a representative assay carried out three times.

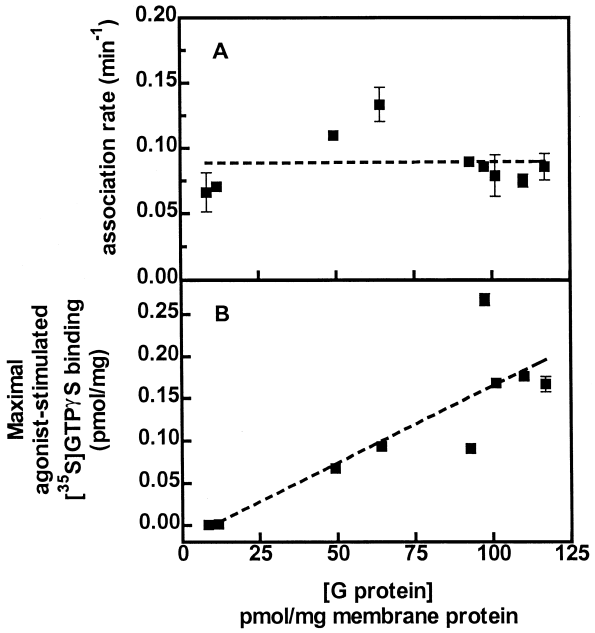

Fig. 6. Agonist-stimulated [^{35}S]GTPγS binding in membranes prepared from control and pertussis-toxin treated C6μ cells. Cells were treated 4–20 h with 2–20 ng/ml of pertussis toxin followed by a preparation of membranes. [^{35}S]GTPγS binding was measured in the presence of 1 μM DAMGO as described in Section 2. (A) Relationship between the rate of [^{35}S]GTPγS binding and the level of G protein. The average rate was 0.088 ± 0.007 min^{-1}. There was no correlation between the level of G protein and the rate of [^{35}S]GTPγS binding ($r^2 = 0.0004$) and the slope of the line was not significantly different from zero. (B) Relationship between the maximal [^{35}S]GTPγS bound and the level of G protein. Data points represent the mean ± range from 2 experiments in membranes from control and treated cells. To assess the level of functional G protein in membranes, cholate extracts of control and treated membranes were prepared, and following pertussis toxin-catalyzed [^{32}P]ADP-ribose incorporation, the samples were run on 12% SDS-PAGE. Radioactive bands migrating the same as ADP-ribosylated bovine brain G_o /G_i were cut from the gel and counted.

3.2. Effect of G protein density

The maximal level of G-protein that could be stimulated by agonist was measured in C6μ cells expressing 2.6 ± 0.3 pmol/mg protein ($n = 4$), as determined with [^{3}H]naloxone. C6μ cells were incubated in the presence or absence of a maximally stimulating concentration of DAMGO (10 μM) and the level of G proteins activated measured by the homologous displacement of [^{35}S]GTPγS by unlabeled GTPγS. Analysis of the difference between the displacement curves in the absence and presence of DAMGO, which was best fit to a single binding site using GraphPad Prism, afforded a K_d for GTPγS binding of 2.8 ± 0.2 nM and a B_{max} of 10.92 ± 0.21 pmol/mg protein. (Fig. 5). Thus, 4.2 mol G protein can be activated per 1 mol μ-opioid receptor in the C6μ cells. To examine the role of G protein concentration in agonist-stimulated [^{35}S]GTPγS binding association rates [^{35}S]GTPγS binding was measured in membranes from control cells and C6μ cells pretreated with pertussis toxin to reduce functional G protein. Over a 10-fold range of inhibitory G protein concentration, the [^{35}S]GTPγS association rate was independent of membrane G protein content (Fig. 6). However, maximal agonist-stimulated [^{35}S]GTPγS binding did correlate with decreasing membrane G protein content (Fig. 6).

3.3. G protein activation by μ OR and δ OR

As shown earlier, the partial agonist nalbuphine induced [^{35}S]GTPγS binding at a threefold slower rate than DAMGO (Fig. 2B). In contrast, there was no significant difference between the association rate of [^{35}S]GTPγS binding stimulated by the full agonist DSLET and the partial agonist SIOM (Fig. 2B). In addition, even with the full agonist the [^{35}S]GTPγS association rate induced by δ-opioid receptor occupation was 35% slower than that induced by occupancy of the μ-opioid receptor (Fig. 7). To rule out artifacts due to the different agonists used (DAMGO and DSLET) the rate of G protein activation by the same agonist acting at both receptors was evaluated. [Met^5]enkephalin and [Leu^5]enkephalin are full agonists compared with DAMGO and DSLET at the μ- and δ-opioid

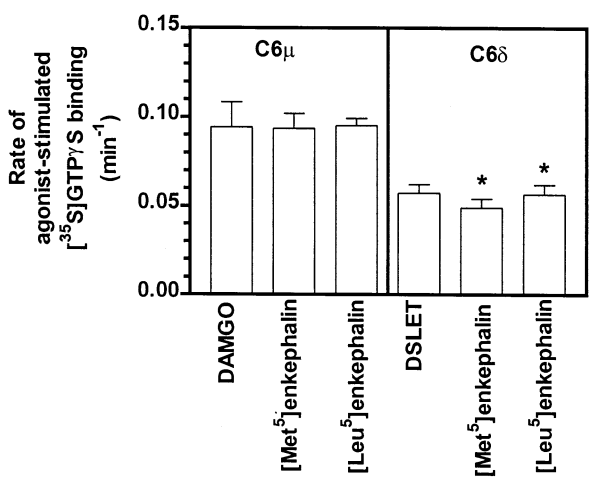

Fig. 7. Stimulation of [^{35}S]GTPγS binding by full agonists in membranes prepared from C6μ and C6δ cells. The rates of agonist-stimulated [^{35}S]GTPγS binding were determined using 3 μM DAMGO, 1 μM [Met^5]enkephalin, 1 μM [Leu^5]enkephalin, and 10 μM DSLET in membranes prepared from C6μ and C6δ cells as described in Materials and Methods. Shown is the average and S.E.M for either two ([Met^5]enkephalin and [Leu^5]enkephalin), four (DSLET) or five (DAMGO) experiments. The corresponding halftimes of association are 7.4 ± 1 and 12.2 ± 1 min. for DAMGO and DSLET, respectively. An asterisk (*) indicates significantly different rates in C6μ and C6δ membranes for the same agonist ($P < 0.05$ in a two-tailed, unpaired *t*-test). These experiments were performed in membranes containing 19.6 pmol μ-opioid receptors/mg membrane protein and 4.5 pmol δ-opioid receptors/mg membrane protein. The maximal agonist-stimulated [^{35}S]GTPγS binding in these membranes was 0.147 ± 0.012 pmol/mg membrane protein ($n = 5$) and 0.207 ± 0.015 pmol/mg membrane protein ($n = 4$) for C6μ and C6δ membranes, respectively.

Table 1
Enkephalin efficacy at the μ OR and δ OR[a]

Ligand	μ OR		δ OR	
	EC_{50} (nM)	Efficacy (%)[b]	EC_{50} (nM)	Efficacy (%)[c]
[Leu^5]enkephalin	122 ± 12	101.5 ± 1.5	100 ± 3	94 ± 1
[Met^5]enkephalin	40 ± 8	105 ± 3	66 ± 2.5	100 ± 1

[a]Agonist stimulated [^{35}S]GTPγS binding was evaluated in duplicate at seven agonist concentrations in a 30 min assay in C6μ and C6δ membranes as described in Section 2. The EC_{50} and efficacy values from two independent experiments were averaged and the mean and range are reported.
[b]Relative to maximally efficacious DAMGO (3 μM).
[c]Relative to maximally efficacious DSLET (10 μM).

receptor respectively (Table 1). With these peptides μ-opioid receptor occupancy stimulated [^{35}S]GTPγS binding at a significantly faster rate than δ-opioid receptor occupancy. In contrast, the maximal agonist-stimulated [^{35}S]GTPγS binding was higher in the membranes from the C6δ cells (0.207 ± 0.015 pmol/mg membrane protein, $n = 4$) than the C6μ cells (0.147 ± 0.012 pmol/mg membrane protein, $n = 5$).

4. Discussion

In C6 cells G protein activation by opioid agonists can be measured by the stimulation of binding of [^{35}S]GTPγS (Traynor and Nahorski, 1995; Emmerson et al., 1996). A reduction in μ-opioid receptor number in C6μ cells by β-funaltrexamine treatment did not significantly alter the maximal level of [^{35}S]GTPγS binding stimulated by the μ-opioid receptor agonist DAMGO, nor did variation of receptor number from 5 to 20 pmol/mg protein alter the time taken to attain maximal [^{35}S]GTPγS binding. Similar findings were obtained at the δ-opioid receptor expressed in C6 cells even though fewer receptors were involved (1.4 to 4.3 pmol/mg). The fact that the level of [^{35}S]GTPγS binding is the same across different receptor numbers would support a collision coupling model whereby the activated opioid receptor acts as a catalyst which is able to reach all G protein molecules and cause guanine nucleotide exchange. However, this model also requires a decrease in rate of G protein activation as lower number of receptors require more time to interact with all the available G protein. This was not seen and so does not support a collision-coupling model. A possible explanation for this inconsistency is that there is a large excess of receptors in the C6μ and C6δ cells used in this study. Even when this number is decreased by four- to fivefold there are sufficient receptors remaining to activate all available G proteins, i.e. there is a large receptor reserve in these cells. However, this is not true for the partial agonist nalbuphine, where maximal G protein activation decreases following the reduction of receptor number, yet there is no change in rate. To confirm this, prolonged treatment with β-chlornaltrexamine to decrease receptor number to approximately 300 fmol/mg protein did cause a reduction in the maximal level of G protein stimulated by DAMGO. Similarly, in SH-SY5Y cell membranes a reduction in receptor number also caused an equivalent drop in the level of [^{35}S]GTPγS binding stimulated suggesting a close relationship between receptors and G protein, but also indicating a lack of receptor reserve in this system. Moreover, these reductions in maximal effect were not accompanied by any change in the time taken for DAMGO to activate the remaining G proteins.

Reduction in the level of functional inhibitory G protein in C6μ cells by treatment with pertussis toxin led, as expected, to a decrease in the level of [^{35}S]GTPγS labeling induced by agonist action. However, the rate at which the remaining G proteins were labeled by [^{35}S]GTPγS was not reduced.

Taken together these findings support a model in which there is some organization of receptors and G-protein, rather than a random collision-coupling model. Analysis of μ-opioid receptors and G protein levels stimulated following μ-opioid receptor occupation would suggest an average ''compartment'' in C6μ cells consists of a ratio of one receptor to four G proteins. Previous experiments giving a 1: 2.4 ratio of μ-opioid receptor to G protein in SH-SY5Y cells suggests a similar organization (Traynor and Nahorski, 1995).

There is evidence to support the idea of a compartmentalization of opioid receptors and specific G protein. In SH-SY5Y cells μ-opioid receptors preferentially couple to $G\alpha i_3$ while δ-opioid receptors preferentially activate $G\alpha i_1$ (Laugwitz et al., 1993), suggesting some specificity of action or compartmentalization. Perhaps more convincingly in NG108-15 cells there is no evidence for the sharing of pertussis toxin-sensitive G-proteins between δ-opioid receptors and α_2-adrenoceptors, even though both receptors couple to the same subtype (Gi_2) of Gi (Graeser and Neubig, 1993). However, in SH-SY5Y cells δ-opioid and μ-opioid receptors show synergistic actions with muscarinic cholinergic receptors at the level of phospholipase C (Connor and Henderson, 1996) and in rat olfactory bulb opioid receptors act synergistically with receptors for vasoactive intestinal peptide or corticotrophin releasing hormone at the level of adenylyl cyclase (Olianas and Onali, 1993). These findings suggest that any compartmentalization is limited to receptors and G protein.

An intriguing finding of this study was the fact that the association rate for [^{35}S]GTPγS binding in the presence of a full μ-opioid receptor agonist was greater than that stimulated by a full δ-opioid receptor agonist, even when the same agonist was used. Although the actual numbers of receptors differed in the C6μ and C6δ cells this was not responsible for the difference as there was no relationship between the association rate for [^{35}S]GTPγS binding and

receptor number. Perhaps related to this is the finding that the partial δ-opioid receptor agonist SIOM, which gives 23% of the level of [^{35}S]GTPγS binding stimulated by full agonist DSLET (similar to levels reported by Clark et al. (1997)) caused stimulation of [^{35}S]GTPγS binding at an association rate not significantly different from a full agonist. In contrast, the partial μ-opioid receptor agonist nalbuphine, which gives 31% of the maximal [^{35}S]GTPγS response seen with the full agonist DAMGO (similar to levels reported by Emmerson et al. (1996)), led to a much slower increase in the binding of [^{35}S]GTPγS than DAMGO (Clark et al., submitted). The reasons for these differences are not clear, but may suggest that agonist occupancy of μ-opioid and δ-opioid receptors activates different types of G protein. Indeed this would also explain why δ-opioid receptor occupancy in the C6δ cells caused a much greater level of [^{35}S]GTPγS binding than μ-opioid receptor occupancy in the C6μ cells. Although studies with opioid receptors transfected in CHO (Chinese Hamster Ovary) cells suggest μ-opioid and δ-opioid receptor agonists stimulate similar G proteins (Prather et al., 1994; Chakrabarti et al., 1995), work in SH-SY5Y cells has suggested that agonist-occupied μ- and δ-receptors have different abilities to activate pertussis-sensitive G-proteins (Laugwitz et al., 1993). The μ-opioid receptor agonist DAMGO stimulates incorporation of the photoaffinity label [α-^{32}P]GTP-azidoanilide into $G\alpha i_3$ much better than into $G\alpha i_1$ while the opposite is true for agonists at the δ-opioid receptor, though it is not clear how this relates to C6 cells where the predominant pertussis toxin-sensitive G-protein appears to be $G\alpha i_2$. This hypothesis would also require SIOM to be a full agonist at a particular sub-population of G proteins. This hypothesis is supported by the ability of SIOM to stimulate [^{35}S]GTPγS binding at a rate similar to the full agonist DSLET, although it could be that the assay is not sensitive enough to detect small differences. Certainly in the mouse vas deferens, where δ-opioid receptor occupancy leads to an inhibition of electrically-induced contractions, SIOM is a full agonist in contrast to its partial agonist property in the C6δ cell (Portoghese et al., 1993).

In conclusion the findings do not lend support to a random collision-coupling model for G protein activation by agonist-occupied μ-opioid or δ-opioid receptors but rather support a compartmentalization model (Neubig, 1994, 1998) in which receptors and G protein are in some way associated, with an excess of G protein over receptors.

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References

Breivogel, C.S., Selley, D.E., Childers, S.R., 1998. Cannabinoid receptor agonist efficacy for stimulating [^{35}S]GTPγS binding to rat cerebellar membranes correlates with agonist-induced decreases in GDP affinity. J. Biol. Chem. 273, 16865–16873.

Chakrabarti, S., Prather, P.L., Yu, L., Law, P.-Y., Loh, H.H., 1995. Expression of the μ-opioid receptor in CHO cells: ability of μ-opioid ligands to promote α-azidoanilido[^{32}P]GTP labeling of multiple G protein α subunits. J. Neurochem. 64, 2534–2543.

Clark, M.J., Emmerson, P.J., Mansour, A.M., Akil, H., Woods, J.H., Portoghese, P.S., Remmers, A.E., Medzihradsky, F., 1997. Opioid efficacy in a C6 glioma cell line stably expressing the delta opioid receptor. J. Pharmacol. Exp. Ther. 283, 501–510.

Connor, M., Henderson, G., 1996. Delta- and mu-opioid receptor mobilization of intracellular calcium in SH-SY5Y human neuroblastoma cells. Br. J. Pharmacol. 117, 333–340.

Emmerson, P.J., Clark, M.J., Mansour, M., Akil, H., Woods, J.H., Medzihradsky, F., 1996. Characterization of opioid agonist efficacy in a C6 Glioma cell line expressing the μ opioid receptor. J. Pharmacol. Exp. Ther. 278, 1121–1127.

Ferguson, K.M., Higashijima, T., Smigel, M.D., Gilman, A.G., 1986. The influence of bound GDP on the kinetics of guanine nucleotide binding to G proteins. J. Biol. Chem. 261, 7393–7399.

Fischel, S.V., Medzihradsky, F., 1981. Scatchard analysis of opiate receptor binding. Mol. Pharmacol. 20, 269–279.

Florio, V.A., Sternweis, P.C., 1989. Mechanisms of muscarinic receptor action on Go in reconstituted phospholipid vesicles. J. Biol. Chem. 264, 3909–3915.

Graeser, D., Neubig, R.R., 1993. Compartmentation of receptors and guanine nucleotide-binding proteins in NG108-15 cells: lack of cross-talk in agonist binding among the alpha 2-adrenergic, muscarinic, and opiate receptors. Mol. Pharmacol. 43, 434–443.

Kasmi, S.M., Misra, R.K., 1987. Comparative pharmacological properties and functional coupling of mu and delta opioid receptor sites in human neuroblastoma SH-SY5Y cells. Mol. Pharmacol. 32, 109–118.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Laugwitz, K.-H., Offermanns, S., Spicher, K., Schultz, G., 1993. Mu and delta opioid receptors differentially couple to G subtypes in membranes of human neuroblastoma SH-SY5Y cells. Neuron 10, 233–242.

Mukhopadhyay, S., Ross, E.M., 1999. Rapid GTP binding and hydrolysis by Gq promoted by receptor and GTPase-activating proteins. Proc. Natl. Acad. Sci. U. S. A. 96, 9539–9544.

Neubig, R.R., 1994. Membrane organization in G-protein mechanisms. FASEB J. 8, 939–946.

Neubig, R.R., 1998. Specificity of receptor–G protein coupling: protein structure and cellular determinants. Semin. Neurosci. 9, 189–197.

Neubig, R.R., Gantzos, R.D., Brasier, R.S., 1985. Agonist and antagonist binding to alpha 2-adrenergic receptors in purified membranes from human platelets. Implications of receptor-inhibitory nucleotide-binding protein stoichiometry. Mol. Pharmacol. 28, 475–486.

Olianas, M.C., Onali, P., 1993. Synergistic interaction of muscarinic and opioid receptors with Gs-linked neurotransmitter receptors to stimulate adenylyl cyclase activity of rat olfactory bulb. J. Neurochem. 61, 2183–2190.

Portoghese, P.S., Moe, S.T., Takemori, A.E., 1993. A selective delta 1 opioid receptor agonist derived from oxymorphone. Evidence for separate recognition sites for delta 1 opioid receptor agonists and antagonists. J. Med. Chem. 36, 2572–2574.

Prather, P.L., McGinn, T.M., Erickson, L.J., Loh, H.H., Law, P.Y., 1994. Ability of δ-opioid receptors to interact with multiple G-proteins is independent of receptor density. J. Biol. Chem. 269, 21293–21302.

Stickle, D., Barber, R., 1993. Analysis of receptor-mediated activation of GTP-binding protein/adenylate cyclase using the encounter coupling model. Mol. Pharmacol. 43, 397–411.

Stickle, D., Barber, R., 1996. Collisions and encounters in simulations of receptor/GTP-binding protein interactions via simple diffusion. Biochim. Biophys. Acta 1310, 242–250.

Tolkovsky, A.M., Levitzki, A., 1978. Mode of coupling between the β-adrenergic receptor and adenylate cyclase in turkey erythrocytes. Biochemistry 17, 3795–3810.

Traynor, J.R., Nahorski, S.R., 1995. Modulation by μ-opioid agonists of guanosine-5′-0-(3-[^{35}S] thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. Mol. Pharmacol. 47, 848–854.

Yu, V.C., Sadee, W., 1988. Efficacy and tolerance of narcotic analgesics at the mu opioid receptor in differentiated human neuroblastoma cells. J. Pharmacol. Exp. Ther. 265, 254–262.