Opioid Modulation of the Binding of Guanosine-5’-O-(3-[35S]thio)triphosphate to NG108–15 Cell Membranes: Characterization of Agonist and Inverse Agonist Effects

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ABSTRACT

The ability of the delta agonist DPDPE ([D-Pen²,D-Pen⁵]enkephalin) to stimulate binding of the GTP analog guanosine-5’-O-(3-[35S]thio)triphosphate ([35S]GTPγS) to pertussis toxin-sensitive G proteins has been characterized in membranes from NG108–15 mouse neuroblastoma X rat glioma cells. The presence of GDP, or its hydrolysis-resistant analog GDP[S], and Mg²⁺ ions was essential to observe agonist-mediated stimulation of [35S]GTPγS binding, although the guanine dinucleotides alone had complex inhibitory and stimulatory effects on [35S]GTPγS binding. The relative ability of the delta agonists benzylidenenaltrexone and naltriben to inhibit DPDPE-stimulated [35S]GTPγS binding suggested the opioid receptor involved was of the delta-2 subtype. Ligand binding assays demonstrated biphasic binding of these antagonists to this single receptor type. [35S]GTPγS binding was also stimulated by [D-Ser²,Leu⁵,Thr⁶]enkephalin > deltorphin II = DPDPE = etorphine > levallorphan = diprenorphine = nalorphine = naltrindole. The delta agonists benzylidenenaltrexone, TIPP (Tyr-Tic-Phe-Phe) and naltriben had no effect, but ICI 174864 (N,N-diallyl-Tyr-Aib-Phe-Leu-OH) acted as an inverse agonist and inhibited [35S]GTPγS binding. Pertussis toxin pretreatment blocked agonist stimulation of [35S]GTPγS binding and also reduced basal binding, thus confirming the presence of constitutively active delta receptors. Replacement of Na⁺ in the assay buffer with K⁺ afforded an increased level of basal [35S]GTPγS binding and an apparent increase in both the inverse agonist activity of ICI 174864 and the agonist activity of the partial agonist diprenorphine relative to the full agonist [D-Ser²,Leu⁵,Thr⁶]enkephalin. The stimulation of [35S]GTPγS binding to NG108–15 cell membranes allows a functional measurement of delta opioid activity that can provide systems of differing relative efficacy.

There is considerable interest in the development of delta opioid-selective, nonpeptide compounds as analgesic agents that may show an improved side effect profile over morphine and other currently available mu agonist therapies (Porreca et al., 1995). To expedite this process, a full understanding of the action of delta opioid agonists at the cellular level is essential, particularly because compounds active at this receptor may show agonist, antagonist and inverse agonist properties (Costa and Herz, 1989). Opioid receptors are members of the family of seven-transmembrane domain receptors and couple to inhibitory G proteins, such that opioid agonists stimulate the exchange of bound GDP for GTP, which can be measured as an increase in the binding of the stable GTP analog [35S]GTPγS to cell membranes (Sim et al., 1995; Traynor and Nahorski, 1995). Such opioid agonist-mediated stimulation of [35S]GTPγS binding has an absolute requirement for GDP (Traynor and Nahorski, 1995). In addition, μM levels of GDP are needed for optimum agonist-stimulation of [35S]GTPγS binding at several receptor systems (Gierschik et al., 1991; Lazareno et al., 1993; Lorenzen et al., 1993; Offermanns et al., 1994). However, the level of GDP required varies across systems. Thus, to observe agonist responses at m1 and m3 receptors expressed in CHO or human embryonic kidney cells, only low levels (0.1 μM) of GDP are required, whereas for m2- and m4-mediated increases in the same cells, 10-fold higher levels of GDP are needed (Lazareno et al., 1993; Offermanns et al., 1994). In contrast, the alpha-2 adrenergic-mediated stimulation of [35S]GTPγS binding in PC-12 cells requires no GDP (Tian et al., 1994).

ABBREVIATIONS: [D-Ala²,Glu⁴]deltorphin II, [Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH₂]; DPDPE, [D-Pen²,D-Pen⁵]enkephalin; DSLET, [D-Ser²,Leu⁵]enkephalin-Thr⁶; BNTX, 7-benzylidenenaltrexone; NTB, naltriben; TIPP, Tyr-Tic-Phe-Phe (Tic = tetrahydroisoquinoline-3-carboxylic acid); GTPγS, guanosine-5’-O-(3-thiotriphosphate); DAMGO, [D-Ala²,MePhe⁴,Gly⁵]enkephalin; CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified Eagle’s medium.
The mu opioid receptors in SH-SY5Y cells couple to Gα and Gβγ proteins, including Gα12, Gα13, and Gα11, but have a preference for Gα11 (Laugwitz et al., 1993). In contrast, delta opioid receptors in NG108–15 cells couple especially to Gα12/13 (McKenzie and Milligan, 1990). However, when expressed in CHO cells, both mu and delta receptors couple to similar populations of Gα protein subtypes (Chakrabarti et al., 1995). Such differences or similarities in the coupling of opioid receptors to G protein subtypes may define the specific requirements for GDP and certain ions to optimize agonist-stimulated [35S]GTPγS response and contribute to the properties of the receptors.

We report on the characterization of delta opioid modulation of [35S]GTPγS binding response in NG108–15 cells. A complex relationship exists between the concentration of GDP and its effect on [35S]GTPγS binding, but opioid agonist-mediated stimulation of this response follows a simple relationship, increasing with increasing GDP. Changes in [35S]GTPγS binding provide a functional method for investigating delta opioid activity and allow for study of the action of agonists and inverse agonists, as exemplified by ICI 174864. A preliminary account of some of the results has been presented (Szekeres and Traynor, 1995).

Materials and Methods

Chemicals and drugs. [3H]Diprenorphine (1.11 TBq/nmol) was purchased from Amersham (Aylesbury, UK) and [35S]GTPγS (46.1 TBq/nmol) was purchased from Dupont New England Nuclear Research Products (Boston, MA). DAMGO, DPDPE, DSLET, pertussis toxin, and all other chemicals were of analytical grade and purchased from Sigma Chemical (St. Louis, MO). [d-Ala2,6-Glu5]Deltorphin II (Tyr-d-Ala-Phe-Val-Glu-Val-Gly-NH2) was obtained from Peninsula (St. Helens, UK), and BNTX, NTB and ICI 174864 [N,N-diallyl-Tyr-Aib-Phe-Leu-OH (Aib = α-aminoisobutyric acid)] were purchased from RBI (St. Albans, UK). The following compounds were kindly donated as indicated: CI977 [5R-(5a,7a,8β)-N-methyl-N-[7-(1-pyrroldinyl)-1-oxaspiro[4,5]dec-8-yl]-4-benzofuranacetamide-HCl], Parke-Davis, Cambridge, UK), etorphine and diprenorphine (Reckitt & Colman, Hull, UK); nalorphine and levallorphan (Zeneca Pharmaceuticals, Alderley Park, UK) and TIPP (Tyr-Tic-Phe-Phe; Tic = tetrahydroisoquinoline-3-carboxylic acid) (Dr. S. J. Paterson, St. Thomas’ Hospital, London, UK). Eosincent scintillation fluid was purchased from National Diagnostics. DMEM (without sodium pyruvate; with 4500 mg/l glucose), DMEM/Nutrient Mix F-12, fetal calf serum, and 4 mM MgCl2 (buffer A) and homogenized using a tissue tesser (twice at 5 sec, 30,000 rpm). The resultant crude membrane fraction was collected by centrifugation (50,000 × g, 15 min), washed in buffer A and recentrifuged as before. The pellet was finally resuspended in buffer A to give a protein concentration of ~0.25 mg/ml (Lowry et al., 1951). All procedures were performed at 0° to 4°C. In experiments to determine opioid binding, buffer A was replaced with 50 mM Tris-HCl, pH 7.4, in all procedures.

Opioid binding assays. Binding was performed in Tris-HCl buffer to afford parameters for high-affinity binding of the ligands (Rodriguez et al., 1992) as follows: membrane protein (~250 μg) was incubated in Tris-HCl, pH 7.4, with [3H]Diprenorphine (0.04–10 nM) or [3H]DPDPE (0.04–20 nM) in a final volume of 1 ml. After allowing the system to reach equilibrium for 1 hr at 25°C (Cotton et al., 1985; Ho et al., 1997), the mixture was rapidly vacuum-filtered through GF/B filters to separate bound from free ligand, and the filters were rinsed three times in 3 ml of ice-cold buffer (Tris-HCl, pH 7.4). Radioactivity retained on the filters was determined by liquid scintillation counting. Nonspecific binding was defined as the binding remaining in the presence of 10 μM naloxone. Specific binding was typically >80% of total binding at the radioligand Kd.

[35S]GTPγS binding assay. Unless otherwise stated, cell membranes (~250 μg of protein) were incubated in buffer A containing [35S]GTPγS (100 μM) and GDP (100 μM) for 1 hr at 30°C in a total volume of 1 ml and in the presence of varying concentrations of opioids. Samples were then rapidly vacuum-filtered through GF/B filters and washed three times with 3 ml of ice-cold buffer A. Bound radioactivity was quantified by liquid scintillation counting. Nonspecific binding was defined using 10 μM unlabeled GTPγS. Specific binding was typically 90% to 95% of total binding. To examine the stability of [35S]GTPγS at the end of the incubation assay, 100 μl samples of the incubation mixture were taken, centrifuged and applied to cellulose-PEI thin-layer liquid chromatography plates that were developed using a solvent system of 0.5 M K2HPO4 containing 0.01 M β-mercaptoethanol. After drying, the plates were cut into 1-cm strips and counted for radioactivity as above. The purity of the [35S]GTPγS used in the assays, as determined by thin-layer liquid chromatography, was >90%.

Data analysis. Binding data were analyzed with the program LIGAND (Munson and Rodbard, 1980) to provide estimates for the total receptor number (Bmax), binding affinity (Kd or Ki) and slope of the binding isotherms. The data was fitted to both one- and two-site models. To determine whether the two-site model produced a statistically better fit of the data than the one-site model, the variance ratio test (F test) facility of LIGAND was used, where P < .05 was considered significant. The EC50 for stimulation of [35S]GTPγS binding obtained at various drug concentrations was determined from nonlinear curve fitting of the data fitted, using GraphPAD Prism (GraphPAD, San Diego, CA). Apparent antagonist affinity constants (Kd) were calculated from [35S]GTPγS binding assay concentration-effect curves in the absence or presence of a single concentration of antagonist using the equation Kd = [antagonist] × [DR]−1, where DR = (EC50 in the presence of antagonist)/EC50 in the absence of antagonist) (Kosterlitz and Watt, 1968). Statistical comparisons between groups of data were made by Student’s t test, where P < .05 was considered significant.

Results

Characterization of basal and agonist-stimulated [35S]GTPγS binding. The NG108–15 cells used in this study expressed a homogeneous delta opioid receptor population. This was demonstrated by the ability of the selective delta opioid agonist DPDPE (Ki = 1.64 ± 0.07 nM; Hill coefficient, 0.95 ± 0.06; n = 3) but not the mu and kappa opioids DAMGO and CI977 (Ki > 1000 nM) to displace binding of the nonselective antagonist [3H]diprenorphine. In

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addition, the number of receptors labeled by [3H]diprenorphine (559 ± 61 fmol/mg/protein, $K_D = 0.32 ± 0.01$ nM, $n = 3$) was the same as the number labeled by [3H]DPDPE (501 ± 94 fmol/mg/protein, $K_D = 1.68 ± 0.51$ nM). The CHO cells used expressed delta receptors at a density of 404 ± 23 fmol/mg of protein ($n = 3$) determined with [3H]diprenorphine ($K_D = 0.2 ± 0.01$ nM).

Preliminary data (not shown) indicated that DPDPE (1 μM) maximally stimulated the binding of the labeled GTP analog [35S]GTPγS to membranes from NG108–15 cells. Optimum stimulation of [35S]GTPγS binding was achieved at 30°C in the presence of 100 μM GDP. Agonist-stimulated binding of the labeled nucleotide was linear up to ~150 μg of membrane protein, reaching maximal effect at 250 μg of protein, and increased linearly with time up to 90 min at 30°C, at which time a 2.2-fold stimulation over basal binding was observed. Higher temperatures resulted in a decreased effectiveness of the agonist response (1.6-fold stimulation at 37°C), whereas at 20°C, a 2.5-fold stimulation was observed but over 180 min.

DPDPE (1 μM) stimulation of [35S]GTPγS binding to crude membranes from NG108–15 cells was critically dependent on the presence of GDP. The role of this nucleotide was complex because both basal and delta opioid-stimulated [35S]GTPγS binding showed a triphasic response to GDP (fig. 1a). GDP concentrations over the range of 0.1 to 3 μM inhibited [35S]GTPγS binding, but at >3 μM GDP, [35S]GTPγS binding was increased, followed by a reversal at higher concentrations (>30 μM) of GDP. With concentrations of GDP of ≥3 μM, differences were seen in the effects on the binding of [35S]GTPγS in the presence and absence of DPDPE (1 μM), resulting in a separation of basal and agonist-stimulated binding and thus a measurable agonist response. At 100 μM GDP, a stimulation of [35S]GTPγS binding over basal representing an agonist-mediated increase in [35S]GTPγS of 55.7 ± 2.2 (n = 3) fmol/mg of protein was obtained (fig. 1a, inset). The poorly hydrolyzable guanine nucleotide analog GDPβS mimicked the effect of GDP in all aspects, including the ability to facilitate agonist-mediated [35S]GTPγS binding (fig. 1b). However, neither of the dinucleotides ADP and UDP nor the mononucleotide GMP could substitute for GDP in affording delta agonist-mediated stimulation of [35S]GTPγS binding. Moreover, none of these nucleotides inhibited basal [35S]GTPγS binding, but all three produced a concentration-dependent increase in [35S]GTPγS binding in the absence of delta opioid agonist, resulting in a >300% increase in binding over basal values (fig. 2). Preliminary metabolism studies suggested that the presence of unlabeled nucleotides stabilized the [35S]GTPγS against degradation. After 60 min at 30°C, only 25 ± 5% of the added radioactivity was recovered as [35S]GTPγS. However, in the presence of 100 μM GDP or ADP, recovery increased to 49 ± 4% and 74 ± 7%, respectively ($n = 3$).

DPDPE (1 μM) also stimulated the binding of [35S]GTPγS to membranes from CHO cells expressing the recombinant mouse delta receptor. In contrast to the response in NG108–15 cells, the level of GDP needed to achieve a separation of basal and agonist-stimulated binding was much lower, with an optimum concentration at 3 μM, and the effects of GDP on [35S]GTPγS binding were only inhibitory (fig. 3).

The presence of Na⁺ ions was not necessary to observe
agonist-stimulated [35S]GTPγS binding, but this cation inhibited control binding preferentially over DPDPE-stimulated binding, resulting in an optimal window for stimulation over the concentration range of 10 to 100 mM Na⁺ (fig. 4). In comparison, agonist-stimulated binding of [35S]GTPγS cells had an absolute requirement for Mg⁺⁺, although this ion had a biphasic action on both control and DPDPE-stimulated [35S]GTPγS binding (fig. 5). Optimum agonist-stimulated binding [35S]GTPγS was observed at 4 mM Mg⁺⁺.

Conditions used in all subsequent experiments were 80 to 150 μg of membrane protein in the presence of 100 μM GDP, 100 mM NaCl and 4 mM MgCl₂ at 30°C for 1 hr.

Effects of agonists and antagonists on [35S]GTPγS binding. DPDPE caused a concentration-dependent increase in the binding of [35S]GTPγS over control, affording an
lowered basal binding of $[^{35}S]GTP\gamma S$ in these cells by 56.0 ± 2.2%, to 18.7 ± 0.8 fmol/mg protein from a basal level of 41.2 ± 1.2 fmol/mg protein (P < .01; n = 3).

The stimulatory effect of DPDPE was antagonized by naloxone (300 nM), which shifted the concentration-effect curve to the right by ~13-fold to give an apparent $K_a$ value for naloxone of 19.6 ± 3.6 nM, which is indicative of delta opioid receptor involvement (Leslie, 1987). The putative delta-1 opioid receptor antagonist BNTX (Portoghese et al., 1992) and putative delta-2 opioid receptor antagonist NTB (Sofuoglu et al., 1991) shifted the dose-effect curve of DPDPE to the left in a parallel fashion, allowing calculation of apparent $K_a$ values of 1.5 ± 0.1 and 0.078 ± 0.01 nM, respectively (fig. 6).

DPDPE is a putative delta-1 agonist (Mattia et al., 1992). The putative delta-2 agonists [d-Ala²,Glu⁴]Deltorphin II and DSLET (Mattia et al., 1992) also stimulated the binding of $[^{35}S]GTP\gamma S$ to membranes from NG108–15 cells. [d-Ala²,Glu⁴]Deltorphin II afforded a similar maximal response to DPDPE (102.6 ± 1.1% of the DPDPE response), but DSLET afforded a higher maximum, representing 119.4 ± 2.7% of the DPDPE response (P < .05) (fig. 8). The EC₅₀ values for these compounds were 19.2 ± 5.8 and 5.3 ± 0.6 nM, respectively (data not shown). Etorphine had a similar maximum to DPDPE, whereas levallorphan, diprenorphine and nalorphine were all weaker, partial agonists. Of the delta opioid antagonists, naltrindole showed a small degree of agonist activity, whereas TIPP, BNTX and NTB did not significantly alter the basal level of $[^{35}S]GTP\gamma S$ binding (fig. 8). In contrast, ICI 174864 inhibited $[^{35}S]GTP\gamma S$ binding by 17.0 ± 6.3%. Replacement of the Na⁺ ions in the assay medium with K⁺ ions resulted in an increased level of basal $[^{35}S]GTP\gamma S$ binding and a consequently much greater inhibition of $[^{35}S]GTP\gamma S$ binding by ICI 174864 such that an EC₅₀ value of 77.2 ± 27 nM could be determined (fig. 9). This inhibitory effect of ICI 174864 was blocked when the assay was per-

EC₅₀ value of 31.2 ± 3.1 nM (n = 12) and reaching maximal stimulation at 1 μM of the opioid (figs. 6 and 7). This stimulation was completely blocked after incubation of the cells with pertussis toxin (100 ng/ml) for 24 hr before preparation of the membranes (fig. 7), confirming the involvement of G proteins of the $G_\gamma/G_\delta$ class. Pertussis toxin treatment also

Fig. 6. Stimulation of $[^{35}S]GTP\gamma S$ (100 pM) binding to membranes of NG108–15 cells by DPDPE alone (■) or in the presence of 300 nM naloxone (○), 10 nM BNTX (▲) or 0.2 nM NTB (▲). Assays were performed as described in Materials and Methods for 60 min at 30°C, and values represent mean ± S.E.M. from three separate determinations performed in duplicate. Binding of $[^{35}S]GTP\gamma S$ was 39.1 ± 5.3 fmol/mg protein in the absence of DPDPE and 107.9 ± 9.2 fmol/mg of protein in the presence of DPDPE (10 μM).

Fig. 7. DPDPE-mediated stimulation of $[^{35}S]GTP\gamma S$ (100 pM) binding to NG108–15 membranes prepared from control (●) and pertussis toxin (100 ng/ml, 24 hr)-treated (▲) cells. Assays were performed as described in Materials and Methods for 60 min at 30°C. Points represent mean ± S.E.M. from three separate determinations performed in duplicate. All pertussis toxin-treated values were significantly (P < .01) lower than the basal value.

Fig. 8. Relative activity of various ligands to stimulate $[^{35}S]GTP\gamma S$ (100 pM) binding to NG108–15 cell membranes. Concentrations used were DSLET, [d-Ala²,Glu⁴]Deltorphin II (DELT II) and DPDPE (3 μM); etorphine, levallorphan, diprenorphine, nalorphine, naltrindole, NTB and ICI 174864 (10 μM); BNTX (1 μM); and TIPP (5 μM). Points represent mean ± S.E.M. from at least three separate determinations performed in duplicate and are normalized to the maximal effect produced by DSLET that represented 37.6 ± 2.9 fmol $[^{35}S]GTP\gamma S$ bound/mg of protein. Assays were performed as described in Materials and Methods for 60 min at 30°C. †Significantly greater than the DPDPE response (P < .05). *Significantly below basal value (P < .05).
formed in the presence of naloxone (fig. 9). Replacement of Na\(^+\) ions with K\(^+\) ions also resulted in an increased efficacy of the partial agonist diprenorphine relative to the full agonist DPDPE (fig. 10).

To further characterize the delta opioid receptor in NG108–15 membranes, displacement of [\(^3\)H]diprenorphine (0.50 nM) binding by BNTX and NTB was studied (fig. 11). Surprisingly, both antagonists afforded shallow displacement curves, with Hill coefficients of 0.45 ± 0.02 and 0.51 ± 0.05 for BNTX and NTB, respectively. Such shallow slopes suggest the presence of receptor heterogeneity, and computer modeling of the data using the program LIGAND (Munson and Rodbard, 1980) revealed that a two-site model gave a significantly better fit of the data than a one-site model (P < .01). As expected from the relative ability of the two antagonists to shift the [\(^3\)S]GTP\(_\gamma\)S binding curve for DPDPE, the affinity of NTB was higher at both sites than that of BNTX (table 1). In contrast, displacement of [\(^3\)H]diprenorphine by the delta agonist DPDPE was monophasic with a Hill coefficient of 0.95 ± 0.06 and an affinity (K\(_i\)) of 1.64 ± 0.07 nM (fig. 11).

Discussion

The NG108–15 cell membranes used in this study were shown to express the same number of delta opioid receptors measured by either the delta agonist [\(^3\)H]DPDPE or the delta antagonist [\(^3\)H]diprenorphine. Because this cell line expresses only opioid receptors of the delta type, this demonstrates that in Tris buffer the receptors are all (within the limits of measurement) in a conformational state recognized with high affinity by both agonists and antagonists. According to the ternary complex model of receptor/G protein interaction (Birnbaumer et al., 1990), this would indicate that the receptors are in a form tightly coupled to G protein.

To demonstrate delta opioid agonist stimulation of [\(^3\)S]GTP\(_\gamma\)S binding in membranes from NG108–15 and CHO cells, the presence of GDP, or its hydrolysis resistant analog GDP\(_\beta\)S, was necessary. This dependence on GDP has also been observed in studies on the stimulation of [\(^3\)S]GTP\(_\gamma\)S binding by mu opioid agonists in SH-SY5Y cell membranes (Traynor and Nahorski, 1995) and in C6 cells expressing the cloned \(\mu\) receptor (Emmerson et al., 1996), adenosine A\(_1\) agonists in bovine brain (Lorenzen et al., 1993), muscarinic agonists in porcine cardiac membranes and CHO cells (Hill et al., 1989; Lazareno et al., 1993) and chemotactic peptide fMet-Leu-Phe in membranes from HL-60 cells (Gierschik et al., 1991). GDP is thought to bind to empty guanine nucleotide binding sites on G proteins and hence reduce the basal level of [\(^3\)S]GTP\(_\gamma\)S binding (Wieland et al., 1992), such that agonist-induced exchange of GDP for [\(^3\)S]GTP\(_\gamma\)S can be observed. However, GDP and GDP\(_\beta\)S affected both basal and agonist-stimulated [\(^3\)S]GTP\(_\gamma\)S binding in the NG108–15 cell membranes in a complex manner. Thus, GDP and GDP\(_\beta\)S reduced binding of [\(^3\)S]GTP\(_\gamma\)S at concentrations below 3 \(\mu\)M, but above this concentration an increase in [\(^3\)S]GTP\(_\gamma\)S binding was observed, and this reverted to an inhibition at higher concentrations.

The delta opioid-mediated stimulation of [\(^3\)S]GTP\(_\gamma\)S binding was not observed if GDP or GDP\(_\beta\)S was replaced by GMP, ADP or UDP. These latter nucleotides did not inhibit basal [\(^3\)S]GTP\(_\gamma\)S binding but did stimulate the binding of [\(^3\)S]GTP\(_\gamma\)S in the absence of opioid agonist. Indeed, for ADP, UDP and GMP, the level of stimulation was far in excess of that seen with GDP or GDP\(_\beta\)S. A number of explanations are...
possible for this finding. The nucleotides may be stimulating the binding of [35S]GTPγS via a G protein-coupled “nucleotide” receptor, although no nucleotide receptor with such a profile is known. However, a preliminary metabolism study suggests the presence of the second unlabeled nucleotide protects [35S]GTPγS from breakdown, probably by various nucleotidases and phosphatases present in the membrane preparation (Wieland et al., 1992), thereby increasing the level of [35S]GTPγS available to bind to G protein. This is supported by the finding that a reduced level of agonist stimulated [35S]GTPγS binding was seen at 37°C, whereas stimulation was increased at 20°C. The decrease in binding at higher concentrations of GDP or GDPβS (>30 μM) presumably results from a competition between the guanine dinucleotides and [35S]GTPγS for the nucleotide binding site on the G protein(s). As a consequence, this effect is not seen with ADP, UDP or GMP due to the specificity of the nucleotide binding site on the G protein (Rodbell et al., 1971, 1980).

These complex effects of GDP on [35S]GTPγS binding in NG108–15 cell membranes are not a property of the delta opioid receptor system because a similar relationship is not seen in membranes from CHO cells expressing the mouse delta receptor. Thus, the effect is cell, not receptor, specific and is independent of the ability of the agonist bound form of the delta receptor to stimulate [35S]GTPγS binding. The difference in GDP requirements for expression of delta agonism at the same receptor expressed in different cells highlights considerations that must be examined when looking at receptor-mediated responses outside endogenous systems (Kenakin, 1996).

The ionic environment is also a very important parameter in modulating [35S]GTPγS binding. To observe agonist stimulation of [35S]GTPγS binding in NG108–15 cell membranes, Mg2+ ions were required. It is difficult to conclude the mechanism by which Mg2+ is causing its effects because this cation has many actions on G protein events (Birnbaumer et al., 1990). However, the increase in both basal and agonist-stimulated [35S]GTPγS binding may be accounted for by the increased rate of dissociation of GDP (Higashijima et al., 1987) and an increased rate of association of [35S]GTPγS (Bokoch et al., 1984; Sternweis et al., 1981) with a decreased rate of dissociation (Higashijima et al., 1987). In addition, delta opioid binding is likely to be enhanced by Mg2+ (Rodriguez et al., 1992; Standifer et al., 1993), resulting in a differential increase in agonist-stimulated over basal [35S]GTPγS binding. Above 10 mM, Mg2+ had a strong inhibitory effect on both basal and agonist-stimulated [35S]GTPγS binding.

Na+ ions were not essential to produce an agonist-mediated increase in [35S]GTPγS binding. The effect of Na+ ions on [35S]GTPγS binding was to decrease both basal and agonist-stimulated [35S]GTPγS binding. However, in the presence of PDPE (1 μM), the effect of Na+ was shifted, leading to an increased stimulation window at Na+ concentrations of 10 to 100 mM. This inhibitory effect of Na+ on G protein activity has been interpreted as reflecting a modulation of the affinity of “empty” opioid delta receptors for G protein, that is, reducing the activity of constitutively active receptors (Costa et al., 1990, 1992; Lefkowitz et al., 1993). As expected, because delta opioid receptors are known to couple to G proteins of the Gαi/Gαo family (Laugwitz et al., 1993; Offermanns et al., 1991; Prather et al., 1994, 1992), treatment of NG108–15 cells with pertussis toxin (100 ng/ml; 24 hr) completely abolished delta opioid receptor-mediated stimulation of [35S]GTPγS binding. Furthermore, pertussis toxin pretreatment reduced control [35S]GTPγS binding. Because pertussis toxin only uncouples G proteins from receptors and does not alter other properties of G proteins, such as their ability to bind GTP and interact functionally with adenyl cyclase (Katada et al., 1986), this provides evidence for the presence of constitutively active receptors in membrane preparations of NG108–15 cells.

Only a single delta opioid receptor has been cloned from NG108–15 cells (Evans et al., 1992; Kieffer et al., 1992). However, the agonist effect of PDPE was antagonized by the nonselective opioid antagonist naloxone and by the putative delta-1 antagonist BNTX and putative delta-2 antagonist NTB. The higher sensitivity of PDPE to blockade by NTB, rather than BNTX, suggests the homogeneous delta receptor in NG108–15 cells is of the delta-2 subtype, a conclusion also reached by studying the cloned delta receptor from NG108–15 cells expressed in CHO cells (Law et al., 1994). The Kᵩ values reported in the CHO cells for the two antagonists (Law et al., 1994) suggests they have lower affinities for the transfected receptor in CHO cells than for the endogenous receptor in NG108–15 cells. Moreover, the dis-

### Table 1

**Binding affinities of BNTX and NTB at the delta opioid receptor in NG108-15 cell membranes**

Data were obtained from the displacement of specific binding of [3H]diprenorphine (0.5 nM) to membranes from NG108-15 cells by BNTX (≡) and NTB (Δ) and fitted using the LIGAND program (Munson and Rodbard, 1980). The two-site model gave a significantly better fit of the data than a single-site model (P < .01).

<table>
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<tr>
<th>delta Antagonist</th>
<th>Affinity (Kᵩ)</th>
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<tr>
<td></td>
<td>High affinity</td>
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<td></td>
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<tr>
<td>BNTX</td>
<td>0.091 ± 0.027</td>
<td>9.80 ± 2.10</td>
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<td>NTB</td>
<td>0.0102 ± 0.001</td>
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placement of binding of [35S]diprenorphine by BNTX and NTB in delta receptor containing CHO membranes is approximately linear, whereas the displacement of [35S]diprenorphine binding by these compounds in the NG108–15 membranes was biphasic. This biphasic recognition by both BNTX and NTB may depend on the environment, such as the type of associated G proteins, which allows differentiation of the receptor into two apparent binding affinities for these antagonists. However, the agonist DPDPE recognizes only a single site. Because agonists are highly susceptible to the presence of Na+ ions and guanine nucleotides (Childers and Snyder, 1980; Kelly et al., 1982) and therefore to the state of coupling of the receptor, this suggests that the biphasic activity of BNTX and NTB results from a combination of the chemical nature of the compounds themselves and the membrane environment.

Several opioid agonist alkaloids and peptides were examined for their ability to stimulate [35S]GTPγS binding by using maximally effective concentrations as reported for their activity against adenyl cyclase in these cells (Childers et al., 1993; Law et al., 1983; Roerig et al., 1996). The most efficacious were the peptides, although DSLET was more efficacious than either DPDPE or [d-Ala²,Glu⁴]deltorphin II. Etorphine was equally efficacious with DPDPE and [d-Ala²,Glu⁴]deltorphin II, but other alkaloid derivatives were partial agonists. The relative ability to stimulate [35S]GTPγS binding among these compounds was similar to their ability to inhibit adenyl cyclase. Of the antagonists examined, diprenorphine had some activity, as previously seen at the delta receptor in NG108–15 cells (Law et al., 1983), as did naltrindole, which is reported to have agonist effects at supraspinal delta receptors in the mouse (Stapelfeld et al., 1992). BNTX, NTB and TIPP had no efficacy in this system. In contrast, the antagonist ICI 174864, a delta opioid ligand with negative intrinsic activity (Costa and Herz, 1989), reduced the basal binding of [35S]GTPγS in membranes of NG108–15 cells, confirming the presence of constitutively active receptors of the delta type.

The effect of ICI 174864 in lowering basal [35S]GTPγS was not as pronounced as that of pertussis toxin. This is in contrast with the findings of Mullaney et al. (1996), using the cloned mouse delta receptor expressed in Rat 1 fibroblasts, in which pertussis toxin and ICI 174864 inhibited basal [35S]GTPγS binding to the same extent and suggested ICI 174864 was an inverse agonist with high intrinsic activity. The observed difference could be explained by the fact that in the NG108–15 cells ICI 174864 may not be a full inverse agonist or that other receptors present in NG108–15 cells and also linked to Gαi proteins are active in the agonist-occupied state. Such receptors include muscarinic M₂ receptors (Graesser and Neubig, 1993; Lazareno et al., 1990; Michel et al., 1989), alpha-2B adrenergic (Graesser and Neubig, 1993; McClue and Milligan, 1990) and cannabinoid (Caulfield and Brown, 1992; Mackie et al., 1993).

The inverse agonist effect of ICI 174864 was much more pronounced in buffer in which the Na+ ions had been replaced with K+ ions, an effect previously reported for the inverse agonist activity of ICI 174864 when determined by measures of GTPase activity (Costa and Herz, 1989). Seven-transmembrane domain G protein-linked receptors exist in equilibrium between an inactive (R) and active conformation (R*). In the presence of agonist (A), the R* form is greatly favored and interacts with G protein to form the active ternary complex (AR*G) (Birbaumer et al., 1990). Constitutive activity occurs when receptors exist in the R* state and are able to spontaneously couple to G proteins in the absence of agonist. Ligands with negative intrinsic activity shift the equilibrium in favor of R and thereby lead to a reduction in constitutive activity, manifest in the present experiments as a reduction in basal [35S]GTPγS binding. Thus, despite the fact that Na+ ions and ICI 174864 are acting through different mechanisms, the presence of Na+ will mask the inverse agonist effect of ICI 174864 because both bring about the same result, namely, a reduction in R* with a concomitant rise in R. When Na+ ions in the medium are replaced with K+ ions, the basal binding of [35S]GTPγS is considerably higher, so the inverse agonist effect of ICI 174864 is apparently greater.

The increase in the basal level of binding of [35S]GTPγS afforded by removal of Na+ ions should also have an effect on agonist responses. In the presence of 100 mM Na+, diprenorphine only produced ~50% of the maximal stimulation of [35S]GTPγS binding produced by the full agonist DSLET. However, when Na+ ions were replaced by K+ ions in the reaction buffer, diprenorphine produced 70% of the DSLET response. Thus, because there is a finite number of G proteins that can be labeled by [35S]GTPγS, the relative efficacies of compounds can be varied by alteration of the basal level of coupling through changes in the concentration of Na+ ions.

In conclusion, delta opioid agonists have been shown to stimulate [35S]GTPγS binding to pertussis toxin-sensitive G proteins in membranes from NG108–15 cells in a nalozone-reversible manner. Optimal conditions for this assay have been established to provide a rapid, sensitive and reproducible method to measure the potency and intrinsic activity of G protein activation at an endogenously expressed delta opioid receptor. The relative efficacy of a series of compounds is governed by the level of G protein, which places a ceiling on the maximum effect that can be obtained. Therefore, through judicious manipulation of assay conditions, it should be possible to match the relative delta opioid efficacies in this cell system with those seen in different tissues and thus design screening conditions suitable for the detection of different pharmacological and side effect profiles.

References


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