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RESEARCH PAPER

Binding of GTPγ[^{35}S] is regulated by GDP and receptor activation. Studies with the nociceptin/orphanin FQ receptor

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Background and purpose: We have examined the effects of ligand efficacy and receptor density on the binding of guanosine 5′-[γ-thio]triphosphate (GTPγS) and GDP to the nociceptin/orphanin FQ (N/OFQ) peptide receptor (NOP)-coupled G-proteins.
Experimental approach: In GTPγ[^{35}S] binding experiments, using stable (CHO_{hNOP}) and inducible ($CHO_{INDhNOP}$) recombinant human and rat NOP we have measured: (i) ligand-specific GDP requirements; (ii) the effects of receptor density on guanine nucleotide affinity/capacity; and (iii) the effect of ligand efficacy on GTPγS association kinetics.
Key results: GTPγS competition curves were shallow and modelled by high- and low-affinity components that were relatively consistent between cell types and tissue preparations. In the presence of 1 μM N/OFQ a high-affinity GDP binding site was also present, but the fraction of total binding was reduced. In an efficacy-dependent manner, the partial agonists [F/G]N/OFQ(1-13)NH_2 ([Phe^{1}ψ(CH_2-NH)Gly^2]-nociceptin(1-13)NH_2) and naloxone benzoylhydrazone both reduced the fraction of high-affinity sites for GDP (relative to basal). While the pIC_{50} for high-affinity GDP binding site did not decrease in the presence of 1 μM N/OFQ, N/OFQ produced a significant reduction in pIC_{50} for the low-affinity site. Agonist-mediated decrease in affinity for GDP binding was efficacy-dependent. GDP displayed three affinities: high, conserved in the presence and absence of ligand; intermediate, present as a low fraction under basal conditions; low (efficacy-dependent), present during receptor activation representing the majority of binding.
Conclusions and implications: The affinity of GTPγ[^{35}S] was regulated by GDP and receptor activation caused increased binding of GTPγ[^{35}S] through a reduction in GDP affinity.
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Keywords: nociceptin/orphanin FQ; opioids; guanine nucleotides; binding

Abbreviations: CHO, Chinese hamster ovary; CHO_{hNOP}, CHO cells stably expressing the human NOP receptor; $CHO_{INDhNOP}$, CHO cells stably expressing the ecdysone-inducible expression system for the human NOP; dpm, disintegrations per minute; [F/G]N/OFQ(1-13)NH_2, [Phe^{1}ψ(CH_2-NH)Gly^2]-nociceptin(1-13)NH_2; GTPγS, guanosine 5′-[γ-thio]triphosphate; K_{obs}, observed association rate; N/OFQ, nociceptin/orphanin FQ; NalBzOH, naloxone benzoylhydrazone; NOP, nociceptin/orphanin FQ opioid peptide receptor

Introduction

As a member of the opioid receptor family, the nociceptin/orphanin FQ (N/OFQ) opioid peptide receptor (NOP; receptor nomenclature follows Alexander *et al.*, 2008) has been shown to produce a variety of biological responses including modulation of pain, depression, anxiety, food intake, cognition and cardiovascular function (Lambert, 2008).

To date a variety of NOP ligands varying in efficacy from full agonists to partial agonists to antagonists of peptide and non-peptide nature have been identified (Calo *et al.*, 2000a,b; Zaveri *et al.*, 2005; Chiou *et al.*, 2007; Lambert, 2008). We have previously suggested that to correctly define the efficacy of these ligands, testing in multiple systems and measurements at different end points in the signal cascade is required (McDonald *et al.*, 2003). We have made an extensive study of the peptide ligand [F/G]N/OFQ(1-13)NH_2 ([Phe^{1}ψ(CH_2-NH)Gly^2]-nociceptin(1-13)NH_2) and can produce the full range of agonist, partial agonist and antagonist behaviour depending on NOP density, assay and end point (McDonald *et al.*, 2003).

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The response measured for a ligand will be influenced by numerous factors including cell surface receptor density (receptor reserve), G-protein density (coupling reserve), cell type [different isoforms of effector(s)] and the response measured. Indeed ligand efficacy can differ regarding the ability to affect different intracellular responses in a single cell line (Cordeaux *et al.*, 2000).

The kinetics for binding of guanosine 5′-[γ-thio]triphosphate (GTPγS) to oligomeric G_i and G_o has been shown to be three to four orders of magnitude slower than that expected for a diffusion-controlled reaction and resolved as a complex biomolecular interaction where an inhibitor, GDP, must first dissociate in order that GTPγS can bind (Ferguson *et al.*, 1986). Studies with cannabinoid receptors have shown that the affinity of GTPγS is unaffected by receptor activation whereas the affinity of GDP is negatively regulated (decreased) (Breivogel *et al.*, 1998). Thus it is believed that the formation of a ternary complex reduces GDP affinity, leading to increased association of GTP.

The use of non-hydrolysable, radiolabelled analogues of GTP, such as GTPγ[^{35}S] allow receptor activation to be measured at the earliest point in a signal transduction cascade, that is, GDP for GTP exchange. In order that ligand-stimulated binding of GTPγ[^{35}S] can be measured, micromolar concentrations of GDP are required to reduce basal binding of GTPγ[^{35}S] (Traynor and Nahorski, 1995; Albrecht *et al.*, 1998). Maximal binding of GTPγ[^{35}S], as stimulated by different ligands can be used to determine relative differences in ligand efficacy. It has been shown with the hNOP receptor that ligands of different efficacy can vary in their requirement for GDP (Berger *et al.*, 2000a; Bigoni *et al.*, 2002; McDonald *et al.*, 2003). Differences in agonist GDP requirements, that is, the concentration of GDP to give a maximal response, have been shown for the $G_{\alpha i/0}$-coupled cannabinoid receptor (Breivogel *et al.*, 1998).

A further complexity to confound measurements of ligand efficacy is that the stoichiometry between activated receptor and G-protein can vary greatly. Indeed when highly expressed in Chinese hamster ovary (CHO) cells, the Mu opioid receptor receptor activates ~20-fold less G-proteins per receptor compared with MOP receptors at a relatively lower expression in SK-N-SH cells (Selley *et al.*, 1998). While this may simply be an issue of coupling efficiency between the two cells tested, it is important to determine if the same receptor in a given cell line could alter its ability to couple to G-protein(s) via changes in cell surface receptor number.

The aim of this study was to examine how the binding of GTPγS and GDP to NOP-coupled G-proteins is affected by ligands of differing efficacy. Specifically we have examined: (i) ligand-specific GDP requirements; (ii) the effects of hNOP receptor density on guanine nucleotide affinity/capacity; and (iii) the effect of ligand efficacy on GTPγS association kinetics. As a full agonist, a high-efficacy and a low-efficacy partial agonist we have used N/OFQ, [F/G]N/OFQ(1-13)NH_2 and naloxone benzoylhydrazone (NalBzOH) respectively.

Methods

Tissue culture and rat brain preparations

Chinese hamster ovary cells stably expressing the human NOP receptor (CHO_{hNOP}) and the ecdysone-inducible expression system for the human NOP ($CHO_{INDhNOP}$) were cultured in DMEM/F12 (1:1) (5% fetal calf serum) and HAMS/F12 (10% fetal calf serum), respectively, both supplemented with penicillin (100 IU·mL^{-1}), streptomycin (100 μg·mL^{-1}) and fungizone (2.5 μg·mL^{-1}) at 37°C with 5% CO_2 humidified air. Geneticin (G418) was used to maintain expression of the NOP receptor plasmid in CHO_{hNOP} and $CHO_{INDhNOP}$ cells at 0.2 μg·mL^{-1} and 1 μg·mL^{-1} respectively. In addition zeocin was further included in $CHO_{INDhNOP}$ stock media at 250 μg·mL^{-1} for selection of the plasmid encoding the ecdysone-inducible expression system. Cells were subcultured twice weekly and used for experimentation when confluent. $CHO_{INDhNOP}$ cells were induced to express the human NOP receptor by addition of fresh media supplemented with ponasterone A (5 and 10 μM), 20 h prior to experimentation; cultures were confluent at the time of harvesting.

All animal care and experimental procedures complied with Home Office regulations. The cerebral cortex from four female Wistar rats (200–250 g; bred in house) were used. Animals were killed by stunning and cervical dislocation (schedule 1).

Membrane preparation

Rat cortical tissue, CHO_{hNOP} and $CHO_{INDhNOP}$ cells were suspended in homogenization buffer consisting of 50 mM Tris-HCl and 0.2 mM EGTA, pH 7.4. After homogenization, membrane fragments were collected by centrifugation at 20 375× *g* for 10 min at 4°C. Membrane fragments were resuspended in fresh homogenization buffer and the procedure repeated twice more. This extensive washing was to remove any endogenous guanine nucleotides. Finally, membrane fragments were suspended in the required volume of assay buffer, consisting of 50 mM Tris-HCl, 100 mM NaCl, 1 mM $MgCl_2$ and 0.2 mM EGTA, pH 7.4. Protein concentration of membrane fragments was determined using the method described by Lowry *et al.* (1951).

GTPγ[35S] assays

All assays were performed using membrane fragments containing 20 μg of protein from either CHO_{hNOP}, $CHO_{INDhNOP}$ or rat cerebral cortex. Samples were incubated in 0.5 mL volumes of assay buffer (as above) further supplemented with bovine serum albumin (0.1%), bacitracin (150 μM), peptidase inhibitors (amastatin, bestatin, phosphoramidon and captopril, all at 10 μM) and GTPγ[^{35}S] (~70 pM). For isotope dilution (total saturation) assays membranes were incubated for 4 h at 30°C with gentle shaking, unlabelled GTPγS was included over the range 1 pM–10 μM, and GDP was absent.

In agonist-stimulated GTPγ[^{35}S] binding assays, agonists were included at a fixed 1 μM concentration (10 μM for the low-efficacy agonist NalBzOH), GTPγS was included over the range 10 pM–1 μM, and 5 μM GDP was added. GDP binding parameters were determined by assessing the ability of a range of GDP concentrations (10 pM–1 mM) to displace a fixed GTPγ[^{35}S] concentration (~70 pM). This was carried out in the presence and absence of different ligands, included at 1 μM (10 μM for the low-efficacy NalBzOH). These assays were incubated for 1 h at 30°C with gentle shaking.

For time course assays membranes were incubated with GDP at 1 μM or 0.1 μM along with N/OFQ (1 μM) or NalBzOH

(10 μM). The time course was conducted over 4 h at 30°C with gentle shaking. Membranes were added last and the time course performed essentially in reverse in order to harvest at a single end point.

In all cases non-specific binding was defined in the presence of 10 μM GTPγS, and reactions were terminated by vacuum filtration through Whatman G/F B filters. The filters with the membranes were kept in scintillation fluid for 8 h at room temperature prior to counting.

Statistical analysis

Raw disintegrations per minute (dpm) data were plotted to sigmoid curves of variable slope and where slope factors were less than unity data were further fitted to an equation,

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom})\left[\frac{\text{Fraction 1}}{1+10^{\lambda-\text{LogEC}_{50\text{-}1}}} + \frac{1-\text{Fraction\_1}}{1+10^{X-\text{LogEC}_{50\text{-}2}}}\right],$$

'where Y is binding and X is the logarithm of the concentration of the unlabeled ligand. FRACTION_1 is the fraction of receptors having affinity described by $\log EC_{50\text{-}1}$. The remainder of receptors have an affinity described by $\log EC_{50\text{-}2}$ that assumed competition for two binding sites (GraphPad Prism V3-help menu)'. When used, raw dpm binding affinity data are presented as pIC_{50} ($\log_{10}$ of the concentration required to inhibit 50% of the binding of GTPγ[^{35}S]). These data were derived using GraphPad Prism V3. Pseudo-isotope dilution (G-protein saturation) assays (4 h) were also analysed according to Scatchard assuming the presence of two sites; this was performed using KELL (from Biosoft) and data are presented as GTPγS affinity K_D (nM) and B_{max} (pmol·mg^{-1}). Data are presented as mean ± standard error of the mean (SEM) for a minimum of three individual experiments. Where appropriate net GTPγ[^{35}S] binding is calculated by subtracting the basal curve from the 'stimulated' curve. ANOVA and *t*-tests with *post hoc* correction, as appropriate, were used to determine statistical differences with $P < 0.05$ considered significant.

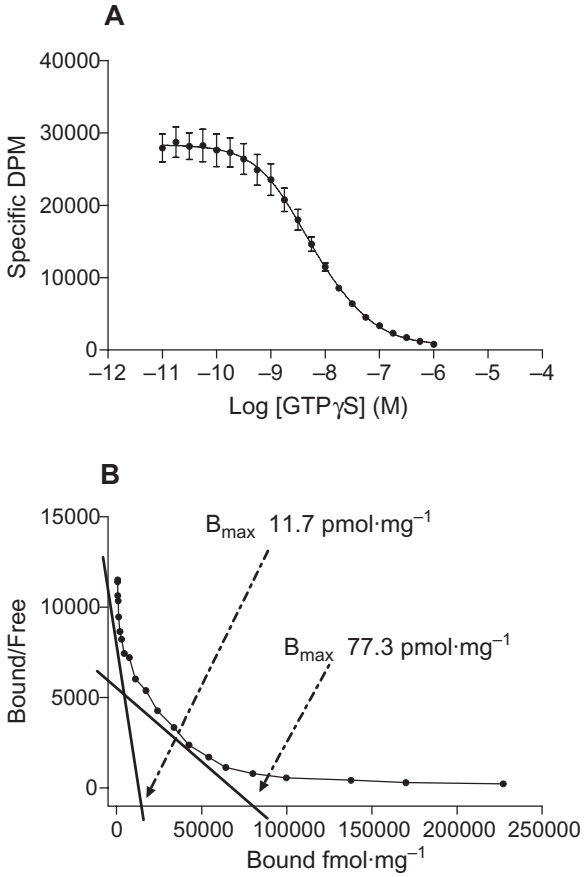

Figure 1 GTPγS isotope dilution of GTPγ[^{35}S] in CHO_{hNOP} membranes. (A) Data plotted as raw disintegrations per minute (means ± SEM, $n = 3$) using a semi-log plot fit to a sigmoid curve of variable slope. (B) The same data analysed using a two-site Scatchard analysis. CHO_{hNOP}, Chinese hamster ovary cells stably expressing the human nociceptin/orphanin FQ opioid peptide receptor; GTPγS, guanosine 5′-[γ-thio]triphosphate.

Results

GTPγ[^{35}S] isotope dilution binding

GTPγS/GTPγ[^{35}S] isotope dilution curves were shallow (slope ~0.8) (Figure 1 and Table 1) and could be analysed with a two-site model. In general there was a high-affinity component with pIC_{50} between 8.58 and 9.05 and a lower-affinity component with pIC_{50} between 7.42 and 7.70 (Table 1). In general these high- and low-affinity sites were consistent between cell types and tissue preparations.

Scatchard analysis yielded curvilinear plots that were best fitted by a two-site model (Figure 1) with K_D for the high-affinity site between 1.44 and 5.98 nM (equivalent pK_D of 8.84–8.22) and between 95 and 326 nM (equivalent pK_D of 7.02–6.48) for the low-affinity site. The capacity of the high-affinity site was relatively low, about 6–12 pmol·mg^{-1} protein, compared with the low-affinity site that had a B_{max} ~10-fold greater of about 60–80 pmol·mg^{-1} (Table 1).

Effects of receptor density on GTPγ[^{35}S] binding

N/OFQ-stimulated GTPγ[^{35}S] binding was measured in $CHO_{INDhNOP}$ cells induced to express pseudo-physiological and supra-physiological NOP densities by pretreatment with ponasterone A. We have previously shown that treatment of these cells with 5 and 10 μM ponasterone A yielded B_{max} values of 191 and 1101 fmol of [*leucyl*-^{3}H]N/OFQ bound per milligram of protein respectively (McDonald *et al.*, 2003). Under basal conditions GTPγS binding affinity was lower than that seen in isotope dilution experiments but was consistent for the two receptor densities, pIC_{50} of 7.27 ± 0.11 at 5 μM ponasterone A and 6.87 ± 0.17 at 10 μM ponasterone A (these values were not significantly different $P > 0.05$). This lower GTPγS binding affinity is attributed to the presence of 5 μM GDP used in these experiments (Table 2). There was an increase in maximal N/OFQ-stimulated (1 μM) GTPγS binding as a consequence of increased receptor density but this failed to reach statistical significance (2455–4147 dpm, $P < 0.05$). The stoichiometry between receptor density and G-protein recruitment (specific at 5 μM GDP) was different for the two induction concentrations: 44.2 fmol·mg^{-1} protein of GTPγS was bound at a NOP density of 191 fmol·mg^{-1} protein (5 μM ponasterone A) giving a ratio of 0.23; 74.7 fmol·mg^{-1} protein of GTPγS was bound at a NOP density of 1101 fmol·mg^{-1} protein (10 μM ponasterone A) giving a ratio of 0.07.

Table 1 Displacement of GTPγ[^{35}S] by increasing concentrations of GTPγS in membranes prepared from CHO_{hNOP}, $CHO_{INDhNOP}$ non-induced and $CHO_{INDhNOP}$ induced with 10 μM ponasterone A in the absence and presence of 1 μM N/OFQ and rat cerebral cortex homogenate

Membrane	*dpm sigmoid plot*				*Scatchard analysis*				
	1-site analysis		*2-site analysis*		*High* K_D (pK_D)	B_{max} *(high)*	*Low* K_D (pK_D)	B_{max} *(low)*	*% High*
	pIC_{50} *(nM)*	*Slope*	*High* pIC_{50} *(nM)*	*Low* pIC_{50} *(nM)*					
CHO_{hNOP}	8.24 ± 0.01 (5.82 ± 0.15)	0.81 ± 0.07	8.58 ± 0.13 (2.88 ± 0.89)	7.42 ± 0.30 (56.5 ± 31.1)	2.87 ± 0.27 (8.54 ± 0.04)	11.7 ± 2.60	226 ± 79 (6.67 ± 0.14)	77.3 ± 4.6	12.98 ± 2.39
$CHO_{INDhNOP}$ (non-induced)	8.26 ± 0.05 (5.63 ± 0.73)	0.79 ± 0.01	8.80 ± 0.14 (1.93 ± 0.55)	7.74 ± 0.16 (23.1 ± 6.80)	3.09 ± 0.18 (8.52 ± 0.03)	10.6 ± 0.46	326 ± 159 (6.60 ± 0.21)	62.2 ± 11.1	15.34 ± 1.95
$CHO_{INDhNOP}$ (10 μM induced)	8.20 ± 0.04 (6.36 ± 0.60)	0.73 ± 0.07	8.67 ± 0.11 (2.29 ± 0.57)	7.48 ± 0.06 (34.0 ± 4.54)	3.10 ± 0.14 (8.51 ± 0.02)	9.41 ± 0.98	163 ± 28.3 (6.79 ± 0.07)	78.3 ± 17.1	12.21 ± 4.03
$CHO_{INDhNOP}$ (10 μM induced) +1 μM N/OFQ	8.32 ± 0.04 (4.77 ± 0.27)	0.61 ± 0.03	9.05 ± 0.07 (0.92 ± 0.13)	7.70 ± 0.02 (20.1 ± 0.95)	2.23 ± 0.23 (8.66 ± 0.04)	6.43 ± 0.75	140 ± 24.1 (6.87 ± 0.09)	79.2 ± 6.9	7.63 ± 1.09
Rat cerebral cortex	8.41 ± 0.01 (3.94 ± 0.05)	0.84 ± 0.02	8.62 ± 0.06 (2.48 ± 0.30)	7.46 ± 0.14 (39.26 ± 9.13)	1.44 ± 0.04 (8.84 ± 0.01)	10.1 ± 0.91	95.1 ± 10.4 (7.03 ± 0.05)	61.4 ± 6.41	14.15 ± 0.19

Data are mean ± SEM for $n \geq 4$ experiments. B_{max} is pmol·mg^{-1} protein.
Statistical analysis (when significant by ANOVA, Bonferroni multiple comparison test was used) revealed small differences in the one-site pIC_{50} and slope, two-site low-affinity site and Scatchard high affinity K_D. These differences were most often against rat and numerically less than 2.5-fold maximum. These were considered functionally unimportant.
CHO, Chinese hamster ovary; CHO_{hNOP}, CHO cells stably expressing the human NOP receptor; $CHO_{INDhNOP}$, CHO cells stably expressing the ecdysone-inducible expression system for the human NOP; dpm, disintegrations per minute; GTPγS, guanosine 5′-[γ-thio]triphosphate; N/OFQ, nociceptin/orphanin FQ; NOP, nociceptin/orphanin FQ opioid peptide receptor.

Agonist-stimulated GTPγ[^{35}S] binding

The effects of N/OFQ (1 μM), [F/G]N/OFQ(1-13)NH_2 (1 μM) and NalBzOH (10 μM) on the binding of GTPγ[^{35}S] to membranes prepared from CHO_{hNOP} cells stably expressing the human NOP receptor were measured (Table 3). In order that agonist-stimulated GTPγ[^{35}S] binding could be measured, a low concentration of GDP (5 μM) was used. All ligands stimulated the binding of GTPγ[^{35}S] to a single nanomolar affinity site, although the affinity was affected by ligand efficacy (Table 3). Relative to the other ligands, NalBzOH displayed a decreased ability to promote GTPγ[^{35}S] binding and the affinity of GTPγS was lower but there was no difference for any of the derived parameters for N/OFQ and [F/G]N/OFQ(1-13)NH_2 (Table 3). A possible explanation for this discrepancy, regarding differences in relative efficacy, with earlier results (Berger *et al.*, 2000b; McDonald *et al.*, 2003), could be the lower concentration of GDP used here, that is, 5 μM.

GDP binding parameters

The ability of GDP to displace the binding of GTPγ[^{35}S] to CHO_{hNOP} membranes was measured in the absence and presence of 1 μM N/OFQ (Figure 2). In the absence of agonist, GDP displaced the binding of GTPγ[^{35}S] with both high and low affinity, however, the high-affinity site represented the majority (~77%) of the GDP binding (Table 4). In the presence of 1 μM N/OFQ a high-affinity GDP binding site was also present, but this site was a lower fraction (~32%) of the total binding. Decreasing the NOP receptor density to ~200 fmol·mg^{-1} protein, using $CHO_{INDhNOP}$ cells, decreased the ability of N/OFQ to reduce the proportion of high-affinity GDP binding sites, 0.74 (basal) to 0.63 compared with 0.77 (basal) to 0.32 in CHO_{hNOP} cells. N/OFQ still maintained its ability to decrease the affinity of the low-affinity GDP binding site, pIC_{50} of 5.65 (basal) to 4.80, in $CHO_{INDhNOP}$ cells (data not shown).

In CHO_{hNOP} cells, [F/G]N/OFQ(1-13)NH_2 and NalBzOH were tested for their ability to affect the affinity of GDP (Table 4). Both reduced the fraction of high-affinity GDP binding

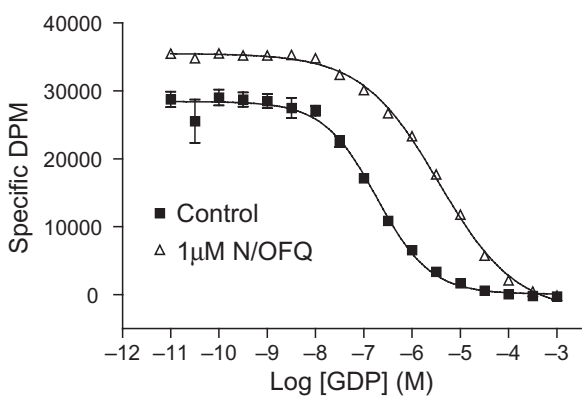

Figure 2 Displacement of GTPγ[^{35}S] binding by increasing concentrations of GDP in the presence and absence of 1 μM N/OFQ, using CHO_{hNOP} membranes. Data are mean ± SEM for $n = 3$ experiments. CHO_{hNOP}, Chinese hamster ovary cells stably expressing the human nociceptin/orphanin FQ opioid peptide receptor; N/OFQ, nociceptin/orphanin FQ.

Table 2 GTPγS binding parameters for $CHO_{INDhNOP}$ cells induced with 5 and 10 μM ponasterone A

[Induction] (Ponasterone A)	*Sigmoid*			*Scatchard*
	Basal (pIC_{50})	*Net (pIC_{50})*	B_{max} *(net dpm)*	K_D *(nM)*
5 μM	7.27 ± 0.11	9.07 ± 0.06	2455 ± 333	1.64 ± 0.24
10 μM	6.87 ± 0.17	9.07 ± 0.07	4147 ± 556	1.06 ± 0.11

Data shown for basal GTPγS binding affinity and net stimulated GTPγS binding affinity are from analysis of raw dpm. B_{max} values were not different ($P = 0.059$) between 5 and 10 μM ponasterone A. K_D values for the net GTPγS binding are taken from the Scatchard analysis. All data are means ± SEM ($n = 3$).
CHO, Chinese hamster ovary; $CHO_{INDhNOP}$, CHO cells stably expressing the ecdysone-inducible expression system for the human NOP; dpm, disintegrations per minute; GTPγS, guanosine 5′-[γ-thio]triphosphate; NOP, nociceptin/orphanin FQ opioid peptide receptor.

Table 3 Basal and agonist-stimulated GTPγ[35S] binding to CHO_{hNOP} membranes in the presence of 5 μM GDP

Agonist	*Sigmoid*			*Scatchard*
	pIC_{50} (nM)	*Slope*	B_{max} *(net dpm)*	K_D *(nM)*
N/OFQ (1 μM)	9.03 ± 0.02 (0.93)	0.91 ± 0.02	19901 ± 1450	0.88 ± 0.03
[F/G] (1 μM)	8.99 ± 0.05 (1.02)	0.85 ± 0.09	15244 ± 1248	1.46 ± 0.03
NalBzOH (10 μM)	8.44 ± 0.06 (4.17)*	0.95 ± 0.12	3358 ± 577*	4.36 ± 0.61*

Data shown are mean ± SEM for $n \geq 3$ experiments.
*$P < 0.05$ compared with N/OFQ and [F/G]N/OFQ(1-13)NH_2 ([F/G]).
CHO, Chinese hamster ovary; CHO_{hNOP}, CHO cells stably expressing the human NOP receptor; dpm, disintegrations per minute; [F/G]N/OFQ(1-13)NH_2, [$Phe^1\psi(CH_2\text{-}NH)Gly^2$]-nociceptin(1-13)$NH_2$; N/OFQ, nociceptin/orphanin FQ; NalBzOH, naloxone benzoylhydrazone; NOP, nociceptin/orphanin FQ opioid peptide receptor.

Table 4 GDP displacement of GTPγ[35S] binding in the absence (control) and presence of different NOP receptor ligands

Ligand	*Slope*	*Fraction (high)*	*High (pIC_{50})*	*Low(pIC_{50})*
Control	0.74 ± 0.01	0.77 ± 0.01	7.04 ± 0.02	5.57 ± 0.03
N/OFQ (1 μM)	0.55 ± 0.03*	0.32 ± 0.01#	6.89 ± 0.08	5.03 ± 0.01*
Control	0.78 ± 0.06	0.77 ± 0.06	7.24 ± 0.03	5.69 ± 0.11
[F/G] (1 μM)	0.54 ± 0.01	0.46 ± 0.08#	7.02 ± 0.16	5.17 ± 0.13*
Control	0.68 ± 0.01	0.74 ± 0.01	7.32 ± 0.08	5.68 ± 0.13
NalBzOH (10 μM)	0.64 ± 0.01	0.50 ± 0.02#$	7.18 ± 0.06	5.75 ± 0.06

Data shown are mean ± SEM from $n = 3$ experiments. The fraction of high-affinity binding sites for control samples were not different from one another ($P > 0.05$), while the fraction of high-affinity binding sites in the presence of agonist were all significantly different to their respective controls (#$P < 0.05$). Efficacy-dependent differences were found between N/OFQ and NalBzOH ($P < 0.05$ relative to N/OFQ fraction (high)). For the low-affinity site, N/OFQ and [F/G]N/OFQ(1-13)NH_2 ([F/G]), but not NalBzOH, modified the pIC_{50} (*$P < 0.05$ compared with respective control value).
[F/G]N/OFQ(1-13)NH_2, [$Phe^1\psi(CH_2\text{-}NH)Gly^2$]-nociceptin(1-13)$NH_2$; N/OFQ, nociceptin/orphanin FQ; NalBzOH, naloxone benzoylhydrazone; NOP, nociceptin/orphanin FQ opioid peptide receptor.

(relative to basal) and this appeared to be efficacy-dependent. While the pIC_{50} for high-affinity GDP binding did not decrease in the presence of 1 μM N/OFQ, the same concentration of N/OFQ produced a statistically significant reduction in the pIC_{50} for the low-affinity fraction. This decrease in GDP affinity was efficacy-dependent, as no change was seen with the low-efficacy partial agonist NalBzOH (10 μM). Hence GDP displayed three distinct binding affinities: (i) a high affinity, conserved in the presence and absence of ligand; (ii) an intermediate affinity, present at a low fraction under basal conditions; and (iii) a low affinity (efficacy-dependent), present during receptor activation representing the majority of binding.

The optimal GDP concentration for maximal net ligand-stimulated GTPγ[35S] binding differed between ligands. These differences were most apparent with NalBzOH. While maximum stimulation of GTPγ[35S] binding was achieved with ~1 μM GDP for N/OFQ, about 10-fold less (~100 nM) was required for NalBzOH (Figure 3). Despite there being differences in the optimal GDP concentration, agonists produced increases in the binding of GTPγ[35S] at all GDP concentrations (Figure 3).

GTPγ[35S] association kinetics

The observed association rate (K_{obs}) for GTPγ[35S] binding increased from 0.017 min^{-1} under basal conditions to 0.045 min^{-1} in the presence of 1 μM N/OFQ (at 1 μM GDP). GTPγ[35S] binding reached a plateau at ~120 min (max = 10 444 dpm) and was stable up to 240 min. In the presence of N/OFQ, GTPγ[35S] binding peaked (max = 24 703 dpm) at ~90 min after which there appeared to be a small, but not statistically significant, decrease in GTPγ[35S] bound (Figure 4). There was no appreciable change in K_{obs} with

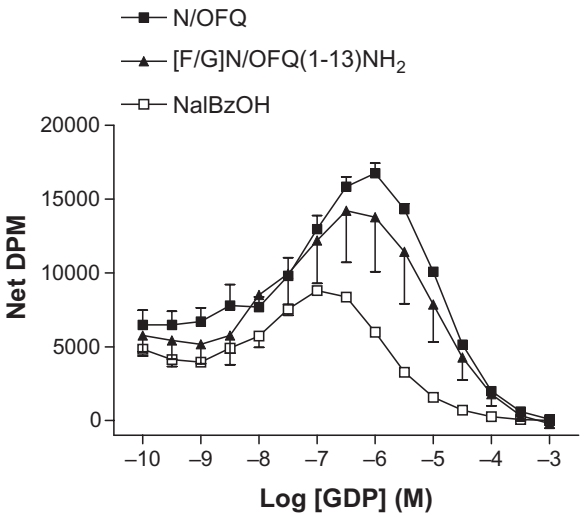

Figure 3 GDP dependence of net agonist-stimulated GTPγ[^{35}S] binding to CHO_{hNOP} membranes. Net binding is calculated as the difference between basal (curve) and agonist stimulated. Data are mean ± SEM for ($n \geq 3$) experiments. CHO_{hNOP}, Chinese hamster ovary cells stably expressing the human nociceptin/orphanin FQ opioid peptide receptor; [F/G]N/OFQ(1-13)NH_2, [Phe^{1}ψ(CH_2-NH)Gly^2]-nociceptin(1-13)NH_2; N/OFQ, nociceptin/orphanin FQ; NalBzOH, naloxone benzoylhydrazone.

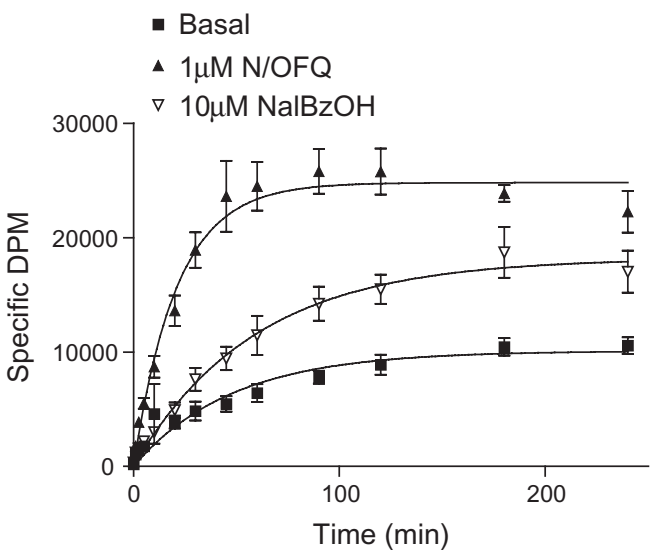

Figure 4 Time course for the binding of GTPγ[^{35}S] to CHO_{hNOP} membranes in the absence and presence of either 1 μM N/OFQ or 10 μM NalBzOH. In this experiment 1 μM GDP was included. Data are mean ± SEM for $n = 3$ experiments. CHO_{hNOP}, Chinese hamster ovary cells stably expressing the human nociceptin/orphanin FQ opioid peptide receptor; N/OFQ, nociceptin/orphanin FQ; NalBzOH, naloxone benzoylhydrazone.

10 μM NalBzOH (0.018 min^{-1} compared with 0.017 min^{-1}) (Figure 4). If the GDP concentration was reduced from 1 to 0.1 μM, the basal rate of association increased to 0.03 min^{-1} and this was further increased to 0.06 min^{-1} in the presence of 1 μM N/OFQ. This N/OFQ-stimulated binding of GTPγ[^{35}S] peaked at ~90 min (data at 0.1 μM GDP not shown).

Discussion

Saturation binding of GTPγ[^{35}S] to a range of cells and tissues can be modelled to two sites, and our results are similar to the report of Albrecht *et al.* (1998), using rat cerebellar membranes. In the presence of different NOP receptor ligands, GTPγS binding was to a single high-affinity site. The affinity of this site was greater than the high-affinity GTPγS binding detected under basal conditions and was conserved with all ligands, except for the partial agonist NalBzOH. The increase in GTPγS affinity promoted by NalBzOH was less than for the other ligands, although greater than basal, suggesting that the ligand increase in GTPγS affinity was related to its efficacy. A lack of difference in stimulated GTPγS affinity in the presence of the other ligands may be due to closer relative intrinsic activities between full and partial agonists at low GDP concentrations (5 μM used in these experiments) (Bigoni *et al.*, 2002). It may also be that the guanine nucleotide binding site activated by these agonists differs from that promoted by the non-liganded receptor, given the threefold difference in GTPγS high-affinity binding seen between CHO_{hNOP} in the absence of N/OFQ (saturation binding) and that in the presence of N/OFQ (agonist binding). Comparison of the GTPγ[^{35}S] saturation binding (GDP omitted) to $CHO_{INDhNOP}$ cells (induced by 10 μM ponasterone A), in the presence and absence of 1 μM N/OFQ, reveals a smaller 1.4-fold difference ($P < 0.05$) in high-affinity binding, perhaps indicative of a different conformation of the guanine nucleotide binding site. These differences in RG-α-GTPγ[^{35}S] and ARG-α-GTPγ[^{35}S] would be in tune with the cubic ternary complex model for G-protein-coupled receptor binding and activation (Kenakin, 2003; Weiss *et al.*, 1996). Therefore three different affinity sites were evident for the binding of GTPγ[^{35}S]: (i) a high-affinity site seen in the presence of agonist (affected by ligand efficacy); (ii) an intermediate-affinity site seen under basal conditions; and (iii) a low-affinity site seen under basal conditions.

At 1 μM GDP, N/OFQ increased both the association rate and maximal binding of GTPγ[^{35}S]. Reducing the GDP concentration 10-fold increased association rates and maximal binding of GTPγ[^{35}S], both in the presence and absence of 1 μM N/OFQ, giving a clear indication that GDP regulated the association of GTPγ[^{35}S]. Therefore, through a reduction in the number of high-affinity binding sites, ligands liberate or reduce the binding of GDP leading to an increase in the binding of GTPγ[^{35}S].

If the maximal capacity for 1 μM N/OFQ-stimulated binding of GTPγS is studied at different receptor densities, it can be seen that the response does not increase linearly. Rather, the stiochometry between receptor and G-protein was greater for the lower expression, that is, at a lower receptor density the amount of G-protein activated per receptor was greater. Similar findings have been reported for the MOP receptor (Selley *et al.*, 1998) whose response per unit receptor (called 'amplification factor' in the article) was 20-fold greater in rat thalamus expressing 0.74 pmol·mg^{-1} protein compared with CHO cells expressing 6.78 pmol·mg^{-1} protein of the recombinant mouse MOP receptor. Further findings were reported between the opioid receptors DOP/KOP, compared with cannabinoid receptors (Sim *et al.*, 1996). Here, despite the observation that in cerebral cortex preparations there is a

10-fold greater cannabinoid receptor density relative to either of the opioid receptors, the maximal stimulation of GTPγ[35S] between the three receptors were remarkably similar. In a situation where there is high receptor expression, the available pool of G-proteins are more likely to adopt the α-GTPγ[35S] bound form reducing the ability of the receptor to interact with multiple G-proteins. When receptor density is lower, there will be less predominance of the α-GTPγ[35S], increasing the chance of multiple receptor G-protein interaction especially given the fixed and relatively short time scale (1 h) of the assays used here. Therefore relative differences in maximal response may be more reliant on the ratio of receptor to G-protein rather than simply to maximal receptor number.

In the absence of ligand, GDP displaced GTPγ[35S] with a relatively high affinity, pIC_{50} of 7.04–7.32 and low affinity pIC_{50} 5.57–5.69 (Table 4). The high-affinity GDP site represented 77% of the total binding. In the presence of ligand (1 μM or 10 μM of different NOP receptor ligands), GDP was seen to bind to a similar high-affinity site to that under basal (pIC_{50} 6.89–7.18) but representing a smaller fraction of the total binding (~32%). Interestingly the decrease in fraction of the high-affinity GDP binding sites was dependent on ligand efficacy, that is, high-efficacy agonists causing a greater reduction (Table 4). Moreover, the affinity of GDP for the lower-affinity binding site was reduced in a manner dependent upon the efficacy of the ligand. NalBzOH caused no change in the lower-affinity GDP binding site but represents the lowest-efficacy partial agonist under investigation (Bigoni *et al.*, 2002; McDonald *et al.*, 2003).

Studies of the GDP dependence of agonist-stimulated specific GTPγ[35S] binding reveals that agonists of different efficacy differed in their optimal requirement for GDP. The low-efficacy agonist NalBzOH showed the most distinct difference in GDP requirement, which is ~10-fold less than for N/OFQ. We have shown previously that a reduction in the GDP concentration can lead to a potentiation, that is, an increase in relative intrinsic activity α, of partial agonists (McDonald *et al.*, 2003). Indeed Berger *et al.* (2000a) has reported similar findings of the differential GDP requirements of partial agonists. This does raise the question regarding which GDP concentration should be used when conducting ligand screens of efficacy (Berger *et al.*, 2000a; McDonald *et al.*, 2003).

It is therefore apparent that there are three affinity sites for the binding of GDP, a conserved high-affinity site, an intermediate-affinity site (in the absence of agonist) and a low-affinity site seen in the presence of high-efficacy agonists. Through rearrangement of the Cheng and Prusoff equation (Cheng and Prusoff, 1973; Breivogel *et al.*, 1998) the effect of GDP on the affinity of GTPγ[35S] at the three hypothesized binding sites can be predicted based on the concentration of GDP present and its affinity for the different binding sites. The pIC_{50} values for GDP determined here were 0.1, 2.5 and 10 μM for the high-, intermediate- and low- (agonist-induced) affinity sites (Table 4) that corresponds to a 51-, 3- and 1.5-fold shift in the affinity of GTPγ[35S] for these respective sites by the addition of 5 μM GDP used here. This predicts the affinity of GTPγ[35S] (assuming a K_D of 2.88 nM) to be 150 nM at the site that shows high affinity for GDP, 8.64 at the intermediate-GDP affinity site and 4.32 nM at the low-GDP affinity site, in the presence of 5 μM GDP. Indeed these values are close to those measured here 115–200 nM (low-affinity basal), 5–7 nM (intermediate-affinity basal) and 0.7–0.85 nM (high single affinity in presence of ligand). Additionally the low- and high-affinity sites for GDP and GTPγ[35S] appear to be reciprocal to one another. Therefore those sites that have high affinity for GDP will have low affinity for GTPγ[35S] and vice versa.

It has been previously suggested that due to the non-hydrolysable nature of GTPγ[35S] that binding is irreversible and hence experiments are performed under non-equilibrium conditions so that data can only be considered apparent. The binding of GTPγ[35S] has been shown to be dissociable from rat cerebellar membranes by the addition of 30 μM 'cold' GTPγS and, consequently, GTPγ[35S] experiments can be performed under equilibrium conditions (Breivogel *et al.*, 1998). In agreement with this study the association kinetics for GTPγ[35S] binding here reached equilibrium after ~90 min. If GTPγ[35S] binding were not dissociable and able to equilibrate then GTPγ[35S] association would continue until all guanine binding sites were saturated. The findings presented here suggest this is not the case and that GTPγ[35S] did reach equilibrium. The basal association of GTPγ[35S] shows the clearest representation of this, as assay conditions are unaffected by ligands of varying efficacy, etc. Also GTPγ[35S] depletion is not a confounding factor in our experiments, as the bound/ free ratio was about 0.1.

While it is clear that the binding of GTPγ[35S] may be performed under equilibrium conditions, data should still be considered apparent when performed with GDP, which will affect GTPγ[35S] binding parameters. Data from saturation experiments performed here may be considered absolute, given the absence of GDP and assays duration (4 h).

In summary, our results showed the affinity of GTPγ[35S] was regulated by GDP and receptor activation caused increased binding of GTPγ[35S] through a reduction in GDP affinity.

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Conflicts of interest

We have no conflicts of interest to declare.

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