British Journal of Pharmacology (2006) 147, 671–680
www.nature.com/bjp

Differential activation of G-proteins by μ-opioid receptor agonists

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1 We investigated the ability of the activated μ-opioid receptor (MOR) to differentiate between myristoylated $G_{\alpha i1}$ and $G_{\alpha oA}$ type G_α proteins, and the maximal activity of a range of synthetic and endogenous agonists to activate each G_α protein.

2 Membranes from HEK293 cells stably expressing transfected MOR were chaotrope extracted to denature endogenous G-proteins and reconstituted with specific purified G-proteins. The G_α subunits were generated in bacteria and were demonstrated to be recognised equivalently to bovine brain purified G_α protein by CB_1 cannabinoid receptors. The ability of agonists to catalyse the MOR-dependent GDP/[^{35}S]GTPγS exchange was then compared for $G_{\alpha i1}$ and $G_{\alpha oA}$.

3 Activation of MOR by DAMGO produced a high-affinity saturable interaction for $G_{\alpha oA}$ ($K_m = 20 \pm 1$ nM) but a low-affinity interaction with $G_{\alpha i1}$ ($K_m = 116 \pm 12$ nM). DAMGO, met-enkephalin and leucine-enkephalin displayed maximal G_α activation among the agonists evaluated. Endomorphins 1 and 2, methadone and β-endorphin activated both G_α to more than 75% of the maximal response, whereas fentanyl partially activated both G-proteins.

4 Buprenorphine and morphine demonstrated a statistically significant difference between the maximal activities between $G_{\alpha i1}$ and $G_{\alpha oA}$. Interestingly, DAMGO, morphine, endomorphins 1 and 2, displayed significant differences in the potencies for the activation of the two G_α. Differences in maximal activity and potency, for $G_{\alpha i1}$ *versus* $G_{\alpha oA}$, are both indicative of agonist selective activation of G-proteins in response to MOR activation.

5 These findings may provide a starting point for the design of drugs that demonstrate greater selectivity between these two G-proteins and therefore produce a more limited range of effects.
British Journal of Pharmacology (2006) **147,** 671–680. doi:10.1038/sj.bjp.0706661;
published online 16 January 2006

Keywords: Opioid; G-protein; MOR; agonist selectivity of G-proteins; inverse agonist; analgesia

Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulphonyl fluoride; β-CNA, β-chlornaltrexamine; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; DEAE sephacel, diethylaminoethyl sephacel; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FBS, fetal bovine serum; G418, geneticin; G-protein, guanine nucleotide binding protein; GDP, guanosine diphosphate; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; HEK293 cells, human embryonic kidney cells; HU210, (6aR-*trans*-3-(1,1-dimethylheptyl)-6*a*,7,10,10*a*-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[*b*,*d*]pyran-9-methanol; MOPS, 4-morpholinepropanesulphonic acid; MOR, μ-opioid receptor; ORL1, opioid receptor-like 1 receptor; P2, post nuclear fraction; PBS, phosphate-buffered saline; PTX, pertussis toxin; [^{35}S]GTPγS, [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate; Sf9, *Spodoptera frugiperda* cells; SR141716, (6aR-*trans*-3-(1,1-dimethylheptyl)-6*a*,7,10,10*a*-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo [*b*,*d*]pyran-9-methanol; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Tris/HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride

Introduction

The opioid receptors belong to the G-protein-coupled receptor (GPCR) superfamily. There are three different opioid receptor subtypes – μ, κ and δ and the structurally homologous ORL1 (Dooley & Houghten, 2000), which has also been suggested to bind certain opioid ligands (Lutfy *et al*., 2003). The μ-opioid receptor (MOR) is primarily involved in the regulation of pain (Przewlocki & Przewlocka, 2001). Like many other GPCRs, MOR couples to multiple G-protein α subunits, including all members of the $G_{\alpha i/o}$ family (Childers, 1991), including the pertussis toxin insensitive $G_{\alpha z}$ (Wong *et al*., 1992), and $G_{\alpha 16}$ (Offermanns & Simon, 1995). Activation of $G_{\alpha i/o}$ subunits by MOR has been shown to decrease forskolin-induced intracellular levels of cAMP, inhibit calcium channels and activate inwardly rectifying potassium (K_{ir}) channels (North *et al*., 1987; Moises *et al*., 1994). In addition to the clinically useful analgesic effect, activation of MOR also leads to various other effects, such as vomiting, nausea, hypothermia, constipation, respiratory suppression and death. It has been hypothesised that the different effects of the drugs acting through MOR might be mediated by the activation of different G-proteins (Sanchez-Blazquez *et al*., 2001).

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K_{ir} channels, which are activated by opioid ligands through $G_{\alpha i/o}$ subunits, have been suggested to be the primary pathway involved in the production of analgesia by opioids and other analgesic drugs (Mark & Herlitze, 2000). It has been shown that the ATP-sensitive potassium (K_{ATP}) channels, members of the K_{ir} family, are differentially modulated by distinct $G_{\alpha i/o}$ subunits. In a study by Sanchez *et al*. (1998) $G_{\alpha i1}$ increased channel activity to a greater extent than $G_{\alpha i2}$. Therefore, we hypothesise that preferential activation of different G_{α} subunits by certain opioid ligands may lead to a more selective response, devoid of adverse effects and maximising analgesia.

The ability of certain drugs to preferentially activate one intracellular pathway over another has been termed 'stimulus trafficking'. Kenakin first proposed the concept of stimulus trafficking after observing a change in potency order of agonists in activating different intracellular pathways in numerous studies (for review, see Kenakin, 2003). Preferential activation of one signalling cascade over another has previously been shown to occur for cannabinoid receptors (CB_1) (Glass & Northup, 1999), serotonin receptors (Berg *et al*., 2001) and adrenergic receptors (Eason *et al*., 1994; Kukkonen *et al*., 2001). The opioid receptors are good candidates for the investigation of agonist-specific receptor conformations because only four different receptors have been cloned with the potential to bind opioid ligands, yet there are at least 10 different endogenous opioid agonists (Bodnar & Klein, 2004). A possible explanation for the need of surplus endogenous ligands is for differential activation of the receptors.

In vivo studies have suggested that G_{α} coupling to MOR is agonist-selective (Sanchez-Blazquez *et al*., 1995; 2001). These studies showed that some opioid drugs produced different levels of analgesia in $G_{\alpha i1}$ or $G_{\alpha o1}$ knockdown mice. Analgesia produced by endomorphins 1 and 2, and methadone was dependent on $G_{\alpha i1}$. Methadone also displayed reduced analgesia in $G_{\alpha o1}$ knockdown mice (Sanchez-Blazquez *et al*., 2001). However, analgesia can be influenced by many other receptors, such as cannabinoid and adrenergic receptors. As both these classes of receptors are coupled to G-proteins, including $G_{\alpha i}$ and $G_{\alpha o}$, these analgesic pathways may not function normally in G_{α} knockdown mice (Meng *et al*., 1998; Bie *et al*., 2003). In order to further investigate this, we have utilised $G_{\alpha i1}$ and $G_{\alpha oA}$ in this study, as representative G-proteins of the $G_{\alpha i}$ and $G_{\alpha o}$ subfamilies.

To determine proximal events in MOR signal transduction, we used an *in situ* reconstitution technique to study agonist-dependent MOR activation of different pathways at the G-protein level. This approach enables the precise characterisation of the coupling properties of the receptors to individual G-protein subtypes and has previously been utilised to investigate 5-HT_{2c} receptors coupling to $G_{\alpha q}$ (Hartman & Northup, 1996), gastrin-releasing peptide receptor coupling to $G_{\alpha q}$ (Hellmich *et al*., 1997) and bombesin receptor coupling to various G_{α} subunits (Jian *et al*., 1999) and to demonstrate agonist selective G-protein activation of cannabinoid CB_1 receptors (Glass & Northup, 1999). These studies utilised G-proteins isolated and purified from native tissues, such as bovine brain or squid retina to obtain G-proteins with the appropriate post-translational modifications for proper functional interaction with receptor. However, it is quite arduous to obtain clearly homogeneous samples of the structurally similar G-proteins from native membrane sources. In this study, we have utilised homogeneously myristoylated recombinant $G_{\alpha i1}$ and $G_{\alpha oA}$ subunits generated through bacterial expression and $\beta_1\gamma_2$ subunits produced in Sf9 insect cells. We demonstrate that these homogeneous gene products act comparably to those proteins isolated from bovine brain. The $\beta_1\gamma_2$ dimer was used throughout our experiments, as it is the main $\beta\gamma$ dimer expressed in the brain (Clapham & Neer, 1993). In our experiments, *in situ* reconstitution of MOR receptors with purified G-protein subunits revealed G-protein-selective agonist activation with select opioid ligands. We have also demonstrated that the basal and agonist-driven G-protein activation of certain opioid ligands through MOR is Mg^{2+} dependent.

Methods

CB_1 receptor expression and quantification

Membranes from Sf9 cells infected with baculoviruses encoding the human CB_1 receptor were prepared and washed with urea as previously described (Glass & Northup, 1999). The final pellet was suspended in solution A (10 mM MOPS, 1 mM EGTA and 10 μM AEBSF, pH 7.5) with 200 mM sucrose, and aliquots were snap frozen and stored at -80°C. Binding site abundance was determined by saturation binding assay using 0.1–30 nM [^{3}H]SR141716A (CB_1 antagonist) as previously described (Glass & Northup, 1999).

MOR transfection

HEK293 cells were transfected with pcDNA3 containing hMOR gene utilising Lipofectamine 2000 following manufacturer's instructions. Cells were selected under 400 μg ml^{-1} G418 selection pressure for at least a week. Clonal isolation was then performed by limiting dilution. The expression levels of a range of clones were screened by whole-cell receptor binding assays as described below, and the clone expressing the highest level of receptors was utilised for all subsequent studies. High receptor expression was required to obtain an observable signal in our assays.

Whole-cell binding assay to select HEK293 cell clones expressing MOR

Following clonal isolation of transfected cells, clones were screened for receptor expression by whole-cell binding assay utilising [^{3}H]diprenorphine. Cells were plated in a 24-well plate at 2×10^5 cells per well and allowed to grow overnight, before exposure to 5 nM [^{3}H]diprenorphine in Krebs buffer (5.6 mM glucose, 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM HEPES, pH 7.4, 1 mM ascorbic acid) at 30°C for 1 h. Nonspecific binding was determined by the addition of 2.5 μM naloxone. The reaction mix was removed and cells were gently washed with ice-cold Krebs buffer. Then, 250 μl of 0.1 M NaOH was added per well and the plate was agitated for 20 min. From the resulting cell lysate, 200 μl was transferred to another plate, scintillation fluid was added and radioactivity was counted in a Wallac Trilux Microbeta counter.

Membrane preparation and chaotrope extraction

HEK293 cells expressing MOR were grown to 80% confluence. Media was removed and the cells were harvested into solution A. The cells were lysed using a dounce homogeniser, with 40 strokes. P2 membrane fractions were collected by repeated centrifugation. Nuclei and cell debri were removed by centrifugation at $1600 \times g$ for 10 min at 4°C. The supernatant was centrifuged at $40{,}000 \times g$ for 30 min at 4°C to collect the P2 membrane fraction. Protein content of the P2 was determined using a Lowry protein assay, performed according to manufacturer's protocol (Bio-Rad, Auckland, New Zealand).

To denature endogenous G-proteins, membranes were diluted to 0.75 mg ml^{-1} in 7 M urea in solution B (50 mM Tris/HCl, pH 7.5; 5 mM $MgCl_2$; 1 mM EDTA, 10 μM AEBSF) and incubated on ice for 30 min before four-fold dilution in solution B. The pellet was sedimented at $125{,}000 \times g$ for 1 h at 4°C, then washed in solution B, and repelleted before resuspension and stored at −80°C in solution A with 200 mM sucrose.

Saturation-binding assay to quantify receptor number

Receptor number in 40 μg of P2 membranes was determined by saturation-binding assay with [^{3}H]diprenorphine (0.625–10 nM). Nonspecific binding was determined by the addition of 5 μM naloxone as a displacer. The binding reaction was performed for 1 h at 30°C in solution C (10 mM MOPS pH 7.5, 1 mM $MgCl_2$ and 100 mM NaCl). The reaction was then filtered through GF-B filter paper, washed 3 times with ice-cold solution C. MeltiLex was then melted onto the dried filter and trapped radioactivity was quantified by scintillation counting in a Wallac Trilux Microbeta counter.

Purification of G_α subunits

Myristoylated recombinant $G_{\alpha i1}$ and $G_{\alpha oA}$ were produced in *Escherichia coli*, expressing G_α and *N*-myristoyltransferase, following previously published procedures (Mumby & Linder, 1994) with modifications as described below. Bacterial pellets expressing myristoylated G_α were collected and resuspended in TED buffer (50 mM Tris/HCl pH 8, 1 mM EDTA, 1 mM DTT, 100 μM AEBSF). Lysozyme was added to final concentration of 0.2 mg ml^{-1} and the mixture was incubated on ice for 30 min. Subsequently, $MgSO_4$ was added to a final concentration of 5 mM and 50,000 U l^{-1} of DnaseI was added, this was then incubated on ice for 30 min. The lysate was centrifuged in Beckman JA14 at $19{,}000 \times g$ for 1 h at 4°C. The supernatant was collected and combined with 200 ml DEAE-A25 Sephadex that has been equilibrated in TED buffer. This was incubated on ice for 30 min with stirring. The resin was collected on a Whatman No. 4 filter in a Buchner funnel and washed with 200 ml TED. The flow through was loaded onto 400 ml DEAE-Sephacel column and this was eluted overnight with 2 l gradient from 0 to 300 mM NaCl, collecting 700 drop fractions. G_α subunit concentration was assessed by [^{35}S]GTP$_\gamma$S binding (Northup *et al*., 1982) and by pertussis toxin-catalysed ADP-ribosylation (Fawzi *et al*., 1991). The progress of [^{35}S]GTP$_\gamma$S binding or ADP-ribosylation was monitored over a time course until the reactions were complete, usually between 90 and 120 min. The positive fractions were pooled and adjusted to 1.2 M $(NH_4)_2SO_4$, 100 μM AEBSF and 25 μM GDP were added to stabilise G_α. The G-protein mixture was incubated for 10 min and centrifuged at $10{,}000\,g$ for 10 min to remove precipitated protein. The supernatant was applied to an equilibrated 200 ml Phenyl Sepharose column and eluted with a 1 l descending gradient of 1.2–0 M $(NH_4)_2SO_4$ in TED buffer containing 25 μM GDP, collecting 15 ml fractions. TED buffer was applied (250 ml) with 25 μM GDP. Fractions collected were analysed for the presence of G_α using pertussis toxin-catalysed ADP-ribosylation assay. Fractions from the second peak of activity, containing the myristoylated G_α, were collected and diluted to 20 mM $(NH_4)_2SO_4$ and applied to a 100 ml fast Q column and equilibrated with TED buffer containing 10 μM GDP. The final purified protein was eluted with a 500 ml gradient of NaCl (0–300 mM) and 6 ml fractions were collected, snap frozen and stored at −80°C.

Purification of $G_{\beta 1\gamma 2}$ proteins

Heterodimeric $\beta_1\gamma_2$ were isolated from Sf9 cells coinfected with baculoviruses encoding these subunits. P2 membranes were prepared as described above and extracted with 1% cholate and the $\beta_1\gamma_2$ was purified essentially as described by Wildman *et al*. (1993). Briefly, the cholate extract was chromatographed over a 50 ml column of DEAE Sephacel eluted with a gradient of 0–250 mM NaCl in solution D (20 mM Tris pH 8.0, 1 mM EDTA, 1 mM DTT) and 1% cholate. The $G_{\beta 1\gamma 2}$ protein pool was collected, concentrated to about 4 ml with an Amicon stirred filtration cell and applied to a 200 ml column of Ultrogel ACA 34 sizing gel. The subunits were eluted from the column with solution D with 100 mM NaCl and 1% cholate. The subunits were then placed into a storage solution (10 mM MOPS pH 7.5, 1 mM $MgCl_2$, 100 mM NaCl with 8 mM CHAPS) by chromatography on a Sephadex G50 column, snap frozen and stored at −80°C.

In situ *reconstitution of cannabinoid CB_1 receptors with G-protein subunits*

Receptor catalysed GDP/[^{35}S]GTP$_\gamma$S exchange was determined as previously described (Glass & Northup, 1999) by incubation of ~4 nM of CB_1 receptor (8–12 μg membrane protein), with varying concentrations of G_α subunits in the presence of a saturating concentration of $\beta_1\gamma_2$ (100 nM) (Glass & Northup, 1999). As [^{35}S]GTP$_\gamma$S binding proceeded linearly beyond 10 min this time was used to estimate rates in all experiments (Glass & Northup, 1999). The assays were carried out at 30°C in a final reaction volume of 50 μl containing 10 mM MOPS pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 0.5% (w/v) BSA, 2.5 μM GDP and [^{35}S]GTP$_\gamma$S (0.4–0.8 nM to $2–5 \times 10^5$ c.p.m.). G-protein-binding activity was measured with urea-washed membrane reconstituted with purified G_α and $G_{\beta\gamma}$ proteins in the presence and absence of saturating levels of HU210 (1 μM). Reactions were terminated by the addition of 2.5 ml of solution E (20 mM Tris/HCl pH 8, 100 mM NaCl, 11 mM $MgCl_2$), and filtered over nitrocellulose membranes on a Millipore vacuum manifold. Filters were washed 4 times with 2.5 ml of solution E and then dried, before addition of Starscint scintillation fluid and counting in a Wallac Trilux. All experiments were carried out in siliconised test tubes, to prevent adsorption of HU210 to the tubes.

In situ *reconstitution of MOR with G-protein subunits*

MOR catalysed GDP/[^{35}S]GTP$_\gamma$S exchange was determined by incubation of MOR containing membranes (~4 nM, 10 μg protein per reaction) with varying concentrations of G_α subunits in the presence of saturating $\beta_1\gamma_2$ (200 nM). The assays were carried out as described above with a slightly modified reaction mixture containing 50 mM Tris/HCl pH 7.5, 3 mM $MgCl_2$, 0.2 mM EDTA with all other reagents as specified above. Reactions were terminated and the filters scintillation counted as described above. Agonist concentration–response experiments were performed to compare the opioid receptor activation of $G_{\alpha i1}$ or $G_{\alpha oA}$, using several different endogenous, synthetic or plant-derived agonists compared to the highly efficacious synthetic opioid DAMGO. All experiments were performed using K_m levels of either $G_{\alpha i1}$ or $G_{\alpha oA}$, under conditions described above.

Magnesium dependence of β*-CNA, naloxone, buprenorphine and DAMGO*

The *in situ* reconstitution assay was used to determine the influence of Mg^{2+} on the basal activity of MOR and to test the effect of Mg^{2+} on the maximal activity of opioid drugs (β-CNA, naloxone, buprenorphine and DAMGO) at 1 μM at MOR. Urea-washed membranes (4 nM, 10 μg per reaction) were incubated with 20 nM $G_{\alpha oA}$ or 116 nM $G_{\alpha i1}$ and 200 nM $\beta_1\gamma_2$ with 0, 1, 3 and 10 mM additional Mg^{2+}, in a reaction mix of 10 mM MOPS pH 7.5, 1 mM EDTA, 100 mM NaCl, 0.5% (w/v) BSA, 4 μM GDP and ~0.5 nM [^{35}S]GTP$_\gamma$S. The reaction was carried out as described above.

Data analysis

The [^{3}H]diprenorphine saturation binding experiments were conducted in triplicate and were repeated at least 3 times. Nonspecific binding was subtracted from total binding. Nonlinear regression analysis for a single site Michaelis–Menton interaction was fitted to the resulting data with GraphPad Prism (GraphPad Software, San Diego, CA, U.S.A.; version 3.02), and using the B_{max} from this curve, the receptor number was calculated using the specific activity of [^{3}H]diprenorphine.

G_α protein saturation experiments were performed at least 3 times for both $G_{\alpha i1}$ and $G_{\alpha oA}$ in duplicate for both CB_1 and MOR. K_m values for the agonist catalysed GDP/[^{35}S]GTP$_\gamma$S exchange were calculated using nonlinear regression analysis for a single site Michaelis–Menton interaction with GraphPad Prism (GraphPad Software, CA, U.S.A.; version 3.02).

Agonist saturation analyses were determined for both $G_{\alpha oA}$ and $G_{\alpha i1}$. Experiments were performed at approximate K_m values (20 nM $G_{\alpha oA}$ and 116 nM $G_{\alpha i1}$). All experiments were repeated at least 3 times with duplicate determinations of each condition. Seven to nine different concentrations of each agonist were used and the resulting curve was used to determine the EC_{50} values and the maximal G_α activation for each agonist, given as a percentage of the maximal G_α activation produced at saturating concentrations of DAMGO (10 μM), a highly efficacious MOR agonist (Traynor *et al*., 2002), using the GraphPad Prism (version 3.02) sigmoidal dose–response curve analysis. Statistical analysis, comparing results for $G_{\alpha i1}$ *versus* $G_{\alpha oA}$, was performed on all data using GraphPad Prism, version 3.02, *t*-test analysis.

In Mg^{2+} dependence experiments, the ratios of the basal activity were determined by dividing the basal activity at each Mg^{2+} concentration by the basal activity in the absence of added Mg^{2+}. Signal ratios were determined by dividing the agonist-specific signal at each Mg^{2+} concentration by the basal activity at the same concentration. To analyse differences between the two G_α subunits and between different Mg^{2+} concentrations, statistical analysis was performed on all data using GraphPad Prism, version 3.02 two-way ANOVA. To analyse differences between two groups of values, *t*-test analysis was used (GraphPad Prism, version 3.02).

Materials

DAMGO was purchased from Tocris, Australian Laboratory Services NZ Ltd, Auckland, New Zealand. Endomorphin 1, endomorphin 2, leucine-enkephalin, met-enkephalin, β-endorphin, morphine, methadone, fentanyl, buprenorphine, naloxone and β-CNA were purchased from Sigma, Sydney, Australia. [^{3}H]Diprenorphine, [^{35}S]GTP$_\gamma$S, MeltiLex A (Wallac) and Starscint were purchased from Perkin-Elmer, Melbourne, Australia. Lipofectamine 2000, G418, GDP, DMEM, FBS and trypsin-EDTA were purchased from Invitrogen, Auckland, New Zealand. [^{3}H]SR141716A was purchased from Amersham Biosciences, Auckland, New Zealand. DEAE-Sephacel, Phenyl Sepharose, Fast Q and Sephadex G50 columns were purchased from Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A. Ultrogel ACA 34 sizing gel was received from IBF Biotechnics, Villeneuve-la-Garenne, France. Lowry assay reagents were purchased from Bio-Rad, Auckland, New Zealand. HEK293 cells were purchased from ATCC (Manassas, VA, U.S.A.) and Sf9 cells were received from Invitrogen, Rockville, MD, U.S.A. A pcDNA3 expression plasmid encoding for human MOR was a generous gift from Professor L. Devi (Mount Sinai School of Medicine, NY, U.S.A.).

Results

Validation of recombinant myristoylated G-proteins

This study has utilised $G_{\alpha i1}$ and $G_{\alpha oA}$ generated by recombinant expression in *E. coli* to evaluate receptor activation of specific G_α proteins. In order to ensure that the recombinant G-proteins behaved similarly to the previously utilised brain G-proteins, we first assessed the affinity of their interaction with cannabinoid CB_1 receptors. All reactions were carried out in the presence of saturating $\beta_1\gamma_2$ (100 nM) (Glass & Northup, 1999) to determine the contribution of G_α subunit to the extent of activation. Cannabinoid CB_1 receptor (~4 nM) was incubated with varying concentrations of G_α in the presence or absence of the high-affinity cannabinoid agonist HU210. As previously demonstrated (Glass & Northup, 1999), activated CB_1 receptors catalysed GDP/[^{35}S]GTP$_\gamma$S exchange for both $G_{\alpha i1}$ (Figure 1a) and $G_{\alpha oA}$ (Figure 1b) with high affinity. The affinity of interaction of CB_1 receptors with $G_{\alpha i1}$ was identical to that previously reported for a $G_{\alpha i}$ trimer pool from bovine brain (Figure 1a; $K_m = 26 \pm 2$, $n = 3$ *versus* 28 ± 2 nM; Glass & Northup, 1999). In contrast, recombinant $G_{\alpha oA}$ displayed a

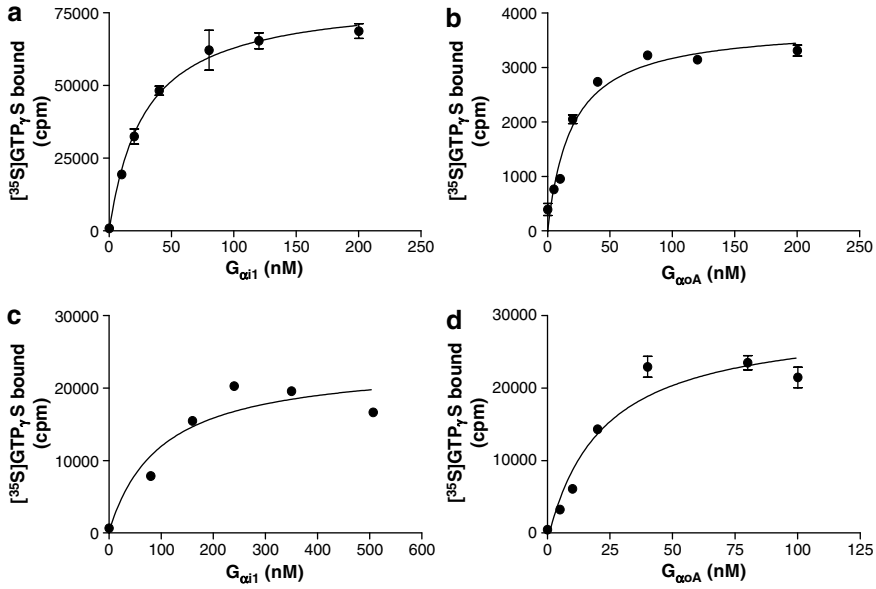

Figure 1 Reconstitution of CB_1 (a, b) and MOR (c, d) receptors with $G_{\alpha i1}$ (a, c) and $G_{\alpha oA}$ (b, d). Urea-extracted CB_1 or MOR membranes were assessed for agonist stimulated [^{35}S]GTP$_\gamma$S binding at the indicated concentrations of G_α protein in the presence of 100 nM $\beta_1\gamma_2$ or 200 nM $\beta_1\gamma_2$, respectively, with either 1 μM HU210 (a, b) or 1 μM DAMGO (c, d). Specific agonist-stimulated [^{35}S]GTP$_\gamma$S binding was calculated by subtracting binding in the presence of agonist from binding in the absence of agonist at each G_α concentration. The data presented are the averages and errors of duplicate values from a representative experiment. The experiments were performed 3 times.

2.3-fold higher affinity for CB_1 receptor than that observed for bovine brain $G_{\alpha o}$ ($K_m = 29 \pm 4$, $n = 3$ *versus* 81 ± 9 previously; Glass & Northup, 1999) (Figure 1b). Agonist stimulation at K_m $G_{\alpha i1}$ and $G_{\alpha oA}$ concentrations resulted in an approximately three-fold increase above basal activity.

Reconstitution of opioid receptor signal transduction

HEK293 cells were transfected with a plasmid construct expressing MOR and clonally isolated. Individual clones were screened for receptor expression by whole-cell binding assay using [^{3}H]diprenorphine. Membranes from the selected clone contained a receptor number of 21 ± 3 pmol mg^{-1} of membrane protein, determined by saturation binding assay. The remainder of the harvested membranes were urea-washed, as described in Methods. [^{3}H]Diprenorphine binding was equivalent in membranes before and following urea washing, indicating that the receptors are not denatured by the chaotrope extraction (data not shown). No opioid regulation of the rate of [^{35}S]GTP$_\gamma$S binding was observed in the absence of expressed MOR (data not shown). The opioid receptor containing membranes displayed a low rate of GDP/[^{35}S]GTP$_\gamma$S exchange for $G_{\alpha i1}$ and $G_{\alpha oA}$ in the absence of agonist, which was related to G_α protein concentration in a linear fashion (data not shown). The addition of DAMGO significantly increased the rate of GDP/[^{35}S]GTP$_\gamma$S exchange for both G_α. When the contribution of the endogenous membrane [^{35}S]GTP$_\gamma$S binding and basal G-protein activity were removed from the total binding signal to assess the specific agonist-stimulated GDP/[^{35}S]GTP$_\gamma$S exchange rates, the data were best fit by a single site saturation isotherm (Figure 1c and d). From the nonlinear regression, a five-fold higher apparent affinity of MOR for $G_{\alpha oA}$ than for $G_{\alpha i1}$, with K_m of 20 ± 1 nM, compared to 116 ± 12 nM could be determined. Agonist stimulation at K_m $G_{\alpha i1}$ and $G_{\alpha oA}$ concentrations resulted in an approximately three-fold increase above basal activity.

Agonist concentration–response curves

To address whether agonist-selective G-protein regulation occurs, we performed a set of agonist concentration–response experiments. These experiments compared the ligand saturation of the opioid receptor activation of $G_{\alpha i1}$ or $G_{\alpha oA}$, with several different endogenous, synthetic or plant-derived agonists compared to the highly efficacious synthetic opioid DAMGO (Traynor *et al*., 2002). All experiments were performed using K_m levels of either $G_{\alpha i1}$ or $G_{\alpha oA}$.

All endogenous opioid ligands displayed high maximal activity in the activation of both G-protein α subunits. Endomorphins 1 and 2 and β-endorphin activated both $G_{\alpha i1}$ and $G_{\alpha oA}$ to more than 75% of the maximal response produced by DAMGO (Figure 2 and Table 1). Both met-enkephalin and leucine-enkephalin maximally activated both $G_{\alpha i1}$ and $G_{\alpha oA}$ (Table 1). Plant-derived and synthetic opioid ligands generally produced lower maximal activities in the activation of $G_{\alpha i1}$ and $G_{\alpha oA}$ than the endogenous opioids. Morphine caused a partial (66 ± 4%) activation of $G_{\alpha i1}$ (Figure 2a), but activated $G_{\alpha oA}$ to a greater extent (88 ± 2% of maximal; Figure 2b). Fentanyl displayed high potency in activating both $G_{\alpha i1}$ and $G_{\alpha oA}$ and it activated both G_α subunits partially (Table 1). Buprenorphine partially activated both G_α subunits, but for $G_{\alpha i1}$ produced a very low maximal activity, 29 ± 4% of the maximal response (Figure 2a) compared to 48 ± 2% for $G_{\alpha oA}$ (Figure 2b). Methadone activated both $G_{\alpha i1}$ and $G_{\alpha oA}$ to more than 75% of the maximal response (Table 1).

Interestingly, morphine and buprenorphine showed a statistically significant difference between the extents of

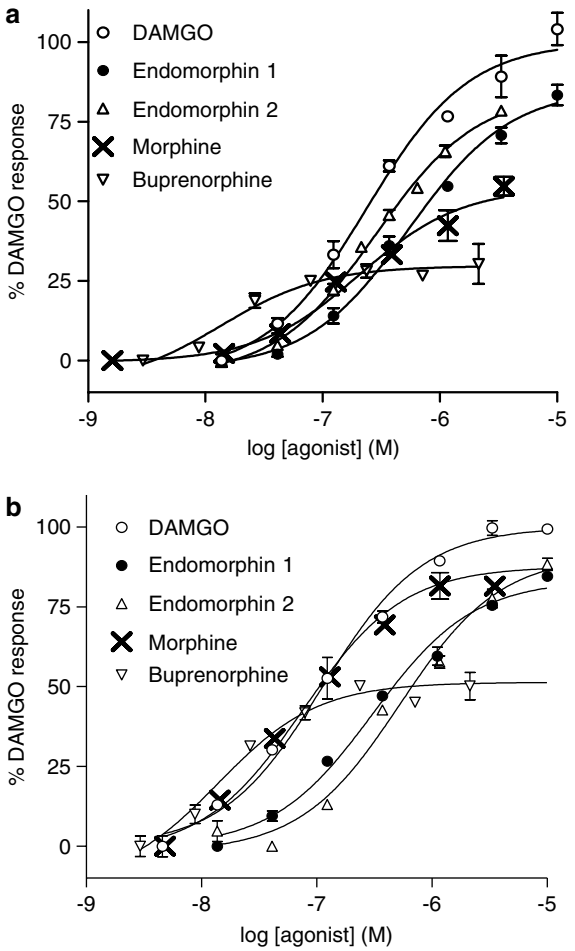

Figure 2 Agonist saturation-binding analysis for MOR agonist stimulated [^{35}S]GTP$_\gamma$S binding to purified G-proteins. Urea-washed MOR membranes were incubated at 116 nM $G_{\alpha i1}$ (a) and 20 nM $G_{\alpha oA}$ (b) with 200 nM added $\beta_1\gamma_2$ and the indicated concentrations of endomorphin 1, endomorphin 2, morphine, buprenorphine and DAMGO. The data presented are the averages and errors of duplicate values from a representative experiment, given as a percentage of the maximal DAMGO response (10 μM). The experiments were performed at least 3 times.

activation of the two G_α subunits as shown in Figure 2. For both morphine and buprenorphine, maximal activation of $G_{\alpha oA}$ was greater than that of $G_{\alpha i1}$. The potencies of certain opioid agonists for the activation of $G_{\alpha i1}$ compared to $G_{\alpha oA}$ also differed. The EC_{50} values of DAMGO, endomorphin 1, endomorphin 2 and morphine were significantly different for $G_{\alpha i1}$ *versus* $G_{\alpha oA}$ (Table 1). DAMGO, morphine and endomorphin 1 displayed higher potency in activating $G_{\alpha oA}$ than $G_{\alpha i1}$, whereas endomorphin 2 activated $G_{\alpha i1}$ with greater potency compared to $G_{\alpha oA}$.

Effect of magnesium on the basal activity of MOR and on the maximal activity of opioid ligands

The activation of $G_{\alpha i1}$ and $G_{\alpha oA}$ through MOR increased in the absence of an agonist (basal activity), with increasing concentrations of Mg^{2+} (Figure 3). This increase was statistically significant ($P<0.0001$), at each Mg^{2+} concentration, when compared to activation in the absence of additional

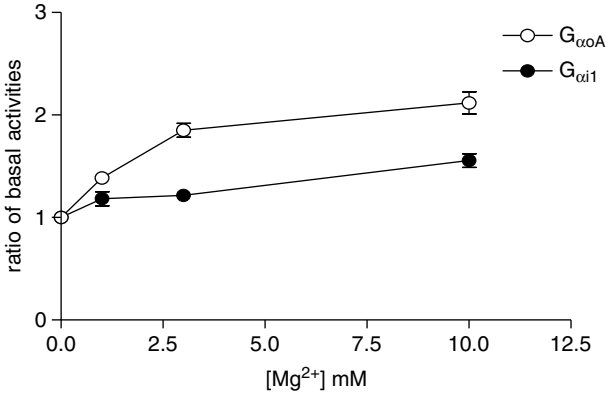

Figure 3 Magnesium dependence of basal activity through MOR. [^{35}S]GTP$_\gamma$S binding, in the presence of different magnesium concentrations, was measured with 20 nM $G_{\alpha oA}$ or 116 nM $G_{\alpha i1}$ and 200 nM $\beta_1\gamma_2$. The data are represented as ratios, calculated by dividing basal activity at each Mg^{2+} concentration by basal activity in the absence of additional Mg^{2+}.

Table 1 Agonist–concentration response experiments

	$G_{\alpha i1}$		$G_{\alpha oA}$	
	% Maximal activation	*EC_{50}* (nM)	*% Maximal activation*	*EC_{50}* (nM)
DAMGO[a]	100	243±20	100	132±19***
Endomorphin 1[a]	86±3	653±113	85±5	327±44*
Endomorphin 2[a]	89±3	252±24	91±4	421±55*
β-Endorphin	78±1	365±100	84±3	298±80
Met-enkephalin	103±5	504±108	96±1	278±17
Leucine-enkephalin	101±5	1011±241	99±3	842±190
Morphine[a,b]	66±4	213±39	88±2**	89±15*
Fentanyl	69±4	119±19	72±3	67±38
Methadone	88±1	185±60	83±4	64±24
Buprenorphine[b]	29±4	21±4	48±2**	15±2

Maximal activation of $G_{\alpha i1}$ and $G_{\alpha oA}$ by each drug is given as a percentage of maximal DAMGO-induced activation. Also shown are the EC_{50} values for the activation of $G_{\alpha i1}$ and $G_{\alpha oA}$ by each drug.
*$P<0.05$, **$P<0.01$ and ***$P<0.001$ by Student's *t*-test.
Morphine produced a statistically significant difference for both the % maximal activation and EC_{50} values. Data are mean±s.e.m.
[a]Drugs that showed a statistically significant difference between the EC_{50} values for the two G_α.
[b]Drugs that showed a statistically significant difference between % maximal activation for $G_{\alpha i1}$ versus $G_{\alpha oA}$.

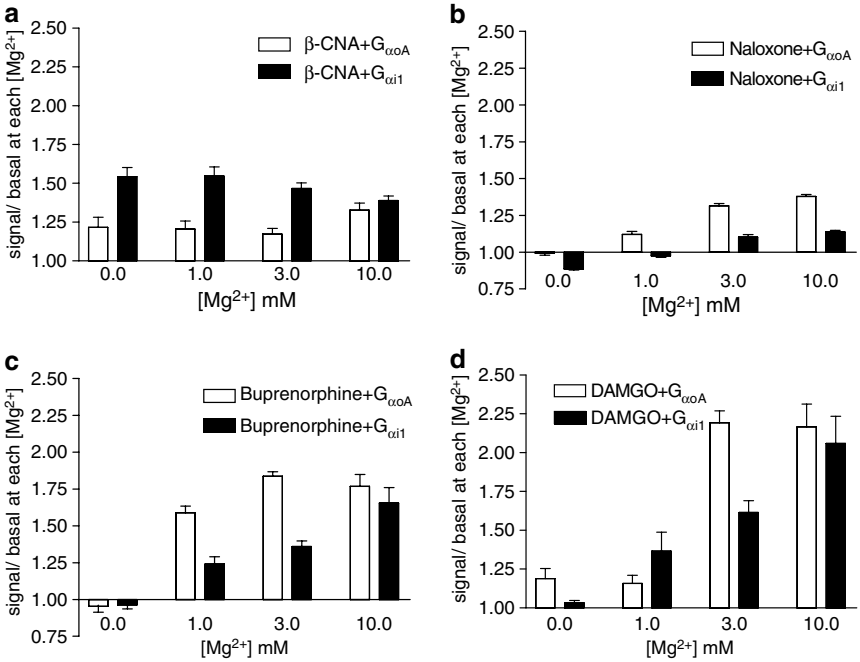

Figure 4 Magnesium dependence of β-CNA (a), naloxone (b), buprenorphine (c) and DAMGO (d). Magnesium dependence of the efficacy of these four drugs through MOR was tested by measuring [^{35}S]GTP$_\gamma$S binding in the presence of 0, 1, 3 or 10 mM added Mg^{2+}. G-proteins were used at 20 nM $G_{\alpha oA}$ or 116 nM $G_{\alpha i1}$ and 200 nM $\beta_1\gamma_2$. The data are represented as ratios, calculated by dividing ligand-mediated signal by the signal produced in the absence of ligand at each Mg^{2+} concentration.

Mg^{2+}, using two-way ANOVA analysis. For $G_{\alpha oA}$, the basal activity was 2.1 times higher with the addition of 10 mM Mg^{2+}. When $G_{\alpha i1}$ was used, the increase in Mg^{2+} concentration had a lesser effect than for $G_{\alpha oA}$ (Figure 3). For this G-protein the basal activity was 1.6 times higher at 10 mM added Mg^{2+} than in the absence of additional Mg^{2+} (Figure 3). The difference in Mg^{2+} sensitivity between the two G-proteins was statistically significant ($P<0.0001$).

Four different drugs were investigated in this study to determine their sensitivity to Mg^{2+} in their ability to activate either $G_{\alpha i1}$ or $G_{\alpha oA}$. β-CNA was reported to behave as an inverse agonist and naloxone as an antagonist in previous studies (Liu & Prather, 2001; Wang *et al*., 2001). DAMGO and buprenorphine were chosen to investigate Mg^{2+} dependence of agonists (full and partial, respectively). Two-way ANOVA analysis was used to compare the effect of Mg^{2+} on the signalling ability of each drug, and between the two G-proteins. In contrast to previous reports, in this assay β-CNA did not display inverse agonist properties at any Mg^{2+} concentrations. For both G-protein α subunits, β-CNA behaved as an agonist at all Mg^{2+} concentrations (Figure 4a). With no added Mg^{2+}, β-CNA produced only a small activation of $G_{\alpha oA}$, which was not significantly different from the basal activity at the same Mg^{2+} concentration, as determined by a *t*-test analysis. β-CNA behaved as an agonist in the absence of added Mg^{2+} in its activation of $G_{\alpha i1}$. Statistical analysis using two-way ANOVA revealed that the effect of increasing Mg^{2+} concentration was not statistically significant for the activation of either G_α subunit by β-CNA. However, the difference between the two G-proteins was significant and $G_{\alpha i1}$ was activated to a greater extent than $G_{\alpha oA}$ ($P<0.0001$). Naloxone did not activate $G_{\alpha oA}$ in the absence of additional Mg^{2+} and displayed a low level of activity at higher Mg^{2+} concentrations (Figure 4b). The only evidence of inverse agonism at MOR was observed for naloxone and $G_{\alpha i1}$; naloxone decreased G-protein bound [^{35}S]GTP$_\gamma$S in the absence of added Mg^{2+} (Figure 4b). In contrast, with the addition of 1 mM Mg^{2+}, naloxone-induced activation of $G_{\alpha i1}$ was not significantly different from basal activity. At higher Mg^{2+} concentrations, naloxone acted as a weak agonist in its activation of $G_{\alpha i1}$ (Figure 4b). The increase in the extent of MOR activation by naloxone with increasing Mg^{2+} was statistically significant ($P<0.0001$), as was the difference between $G_{\alpha i1}$ and $G_{\alpha oA}$ and with this drug $G_{\alpha oA}$ was activated more efficiently at all Mg^{2+} concentrations. Buprenorphine-induced activation was not significantly different from basal activity in the absence of added Mg^{2+}. Buprenorphine behaved as a weak agonist at all Mg^{2+} concentrations, for both $G_{\alpha i1}$ and $G_{\alpha oA}$ (Figure 4c). DAMGO-induced G_α activation was equal to basal activity in the absence of added Mg^{2+} and behaved as an agonist for both $G_{\alpha i1}$ and $G_{\alpha oA}$ at all higher Mg^{2+} concentrations (Figure 4d). For both buprenorphine and DAMGO, the increase in MOR signalling with increasing Mg^{2+} concentration and the difference between the extent of activation of $G_{\alpha i1}$ and $G_{\alpha oA}$ were statistically significant ($P<0.0001$). Buprenorphine activated $G_{\alpha oA}$ more efficiently than $G_{\alpha i1}$ at all tested Mg^{2+} concentrations. DAMGO also preferentially activated $G_{\alpha oA}$ at most Mg^{2+} concentrations but interestingly, at 1 mM additional Mg^{2+}, DAMGO preferentially activated $G_{\alpha i1}$ (Figure 4d).

Discussion

Opioid drugs produce a variety of pharmacological effects, such as analgesia, nausea and respiratory arrest. It is feasible

that these different effects are produced through activation of distinct intracellular pathways. The aim of this study was to investigate how agonists differentially regulate G-protein coupling to MOR. We investigated the ability of a range of endogenous and synthetic opioid ligands to activate $G_{\alpha i1}$ *versus* $G_{\alpha oA}$, using *in situ* reconstitution model, which directly studies the activation of G-proteins by the receptor. This method has previously been utilised to investigate $G_{\alpha i}$ and $G_{\alpha o}$ coupling of CB_1 and CB_2 receptors (Glass & Northup, 1999) and $G_{\alpha q}$ coupling of 5-HT_{2c} receptors (Hartman & Northup, 1996), and has demonstrated stimulus trafficking for CB_1 (Glass & Northup, 1999). In this study, chaotrope extraction of the HEK-MOR membranes did not alter the receptor affinity for diprenorphine in membrane binding assays. Furthermore, a previous study has demonstrated the ability to restore high-affinity opioid binding by the addition of G-proteins to urea-washed HEK-MOR membranes (Lim & Neubig, 2001), indicating that MOR remain undenatured and fully functional following this treatment.

Initial experiments were conducted to determine the relative affinity of the receptor for each G_α subunit. These studies utilised the highly efficacious agonist DAMGO (Traynor *et al*., 2002), to activate the receptor at increasing concentrations of either $G_{\alpha i1}$ or $G_{\alpha oA}$ in the presence of saturating concentrations of $\beta_1\gamma_2$. $G_{\alpha oA}$ demonstrated a significantly higher apparent affinity for activated MOR than did $G_{\alpha i1}$ (20 ± 1 nM compared to 116 ± 12 nM). These results are comparable to a previously published study (Laugwitz *et al*., 1993), where MOR displayed a two-fold preferential coupling to $G_{\alpha oA}$ in comparison to $G_{\alpha i1}$. In this study, differential coupling of G-protein α subunits to MOR was assessed by measuring the levels of radioactivity ([α-^{32}P]GTP) incorporated in immunoprecipitated G-protein α subunits after addition of 1 μM DAMGO. It might be argued that as naturally occurring G_α express two post-translational modifications, myristoylation and palmitoylation (Mumby *et al*., 1990; Linder *et al*., 1993), our purified G_α might function differently from bovine-brain derived G_α because they lack palmitoylation. In our study, we have shown that activated CB_1 receptors catalysed the GDP/[^{35}S]GTP$_\gamma$S exchange for both $G_{\alpha oA}$ and $G_{\alpha i1}$ with high affinity. For $G_{\alpha i1}$, the apparent affinity was identical to the apparent affinity produced by bovine brain derived $G_{\alpha i}$ (Glass & Northup, 1999). The relative affinity for $G_{\alpha oA}$ produced in *E. coli* was 2.3 times higher than for $G_{\alpha o}$ purified from bovine brain (Glass & Northup, 1999). One of the functions of palmitoylation is to promote membrane association (Smotrys & Linder, 2004), which may be a possible reason for this difference. Supporting our results is a study by Cao & Youguo (2005) in which they observed a three-fold higher affinity of nonpalmitoylated $G_{\alpha o}$ for [^{35}S]GTP$_\gamma$S compared to palmitoylated $G_{\alpha o}$. Also, bovine brain-derived $G_{\alpha o}$ is a combination of $G_{\alpha o1}$ and $G_{\alpha o2}$, which offers another possible explanation for the difference in apparent affinities.

We then investigated the ability of a range of synthetic, plant-derived and endogenous opioid ligands to activate each G_α protein. Interestingly, four of the drugs tested – DAMGO, endomorphins 1 and 2 and morphine – displayed significant differences in their potencies for the activation of the two G_α proteins. We found that two of the 10 agonists tested, buprenorphine and morphine, produced different maximal activities for the activation of one G_α over another, with $G_{\alpha oA}$ being activated to a greater extent in both cases. For buprenorphine there was a small but statistically significant difference in the maximal activity for $G_{\alpha i1}$ *versus* $G_{\alpha oA}$. Previously, buprenorphine has been suggested to act as a partial agonist at MOR, consistent with our findings (Wesson, 2004). In our investigation, morphine activated $G_{\alpha oA}$ to more than 75% of the maximal response, but it activated $G_{\alpha i1}$ only partially. This weak activation of $G_{\alpha i}$ likely underlies partial agonism of morphine towards inhibition of the cAMP pathway, which occurs *via* $G_{\alpha i/o}$ (Massotte *et al*., 2002; Gharagozlou *et al*., 2003). These differences in maximal activity and potency between $G_{\alpha i1}$ and $G_{\alpha oA}$ are both indicative of stimulus trafficking of G-proteins in MOR activation (Kenakin, 2003).

Our finding that four drugs, DAMGO, endomorphins 1 and 2, and morphine produced significant differences in potencies for $G_{\alpha i1}$ *versus* $G_{\alpha oA}$ suggests that the cellular response elicited upon agonist binding can be modulated by the concentration of the agonist, leading to a more selective response. Endomorphin 1 preferentially recruited $G_{\alpha oA}$, whereas endomorphin 2 displayed a lower EC_{50} for $G_{\alpha i1}$. Therefore, 350 nM endomorphin 1 will result in significant activation of $G_{\alpha oA}$ pathway but only minimal $G_{\alpha i1}$ pathway activation. Both endomorphins 1 and 2 are four amino-acid peptides differing in one amino-acid residue (tryptophan for endomorphin 1 *versus* phenylalanine for endomorphin 2) (Zadina *et al*., 1997). This amino-acid residue may be directly involved in binding to the MOR, perhaps changing the overall structure of the receptor significantly thus changing the extent of activation of MOR-$G_{\alpha i1}$ *versus* MOR-$G_{\alpha oA}$. Differences between potencies produced by the two endomorphins have also been reported in a study by Storr *et al*. (2002), comparing electrically induced twitch contractions on smooth muscle to the peristaltic reflex. In this study, endomorphin 1 produced comparable IC_{50} for both of these effects; however, endomorphin 2 was less potent in the twitch contraction experiments and more potent in the reflex model than endomorphin 1 (Storr *et al*., 2002).

An *in vivo* study, in which the expression of various G_α subunits was reduced by administration of oligodeoxynucleotides directed at their mRNA, has suggested that G_α knockdown mice displayed differential decreases in the functionality of distinct opioid drugs, depending on the G_α subunits that were knocked down (Sanchez-Blazquez *et al*., 2001). In this study, analgesia produced in G_α knockdown mice after the administration of endomorphins 1 and 2 and methadone was dependent on $G_{\alpha i1}$, but DAMGO, morphine and buprenorphine-induced analgesia was $G_{\alpha i1}$ independent (Sanchez-Blazquez *et al*., 2001). Only methadone-induced analgesia was reduced in $G_{\alpha o1}$ knockdown mice. These results are mainly inconsistent with the findings of our study, where all of these six drugs produced an equal or higher maximal activation of $G_{\alpha oA}$ than of $G_{\alpha i1}$ and five out of the six drugs (endomorphin 2 excluded) produced a higher potency for $G_{\alpha oA}$ than for $G_{\alpha i1}$. A possible explanation for this discrepancy is that the ratios of different G_α subunits are not consistent throughout different pain pathways. Therefore, if certain opioid drugs preferentially act on one of these pathways over another, they may be affected differently depending on the G_α subunits knocked down.

Specific cellular effects of different opioid ligands have also been reported in the development of desensitisation and internalisation. Unlike most other opioid agonists, morphine-activated MOR avoids phosphorylation by G-protein-coupled

receptor kinase and the subsequent desensitisation produced by arrestin binding (Whistler & von Zastrow, 1998). Morphine also does not induce internalisation of MOR (Keith *et al*., 1998), unlike endogenous opioid ligands and methadone (Trapaidze *et al*., 2000). This further shows that different opioid agonists produce distinct intracellular effects upon receptor binding, and these effects are also indicative of agonist-specific receptor conformations.

The maximal activities of most opioid drugs for the activation of $G_{\alpha i1}$ *versus* $G_{\alpha oA}$ did not differ in our study. Met-enkephalin and leucine-enkephalin were full agonists for both $G_{\alpha i1}$ and $G_{\alpha oA}$. Endomorphins 1 and 2 and β-endorphin activated both G_{α} subunits to more than 75% of the maximal response, as did methadone. Contrary to our findings, methadone has previously been reported to be a full agonist with efficacy equal to that of DAMGO (Selley *et al*., 1998). However, in the study by Selley *et al*. (1998), MOR-expressing CHO membranes were used, which may contain G-protein subunits other than $G_{\alpha i/o}$, such as $G_{\alpha z}$, possibly accounting for these differences. Fentanyl displayed high potency for the activation of both G-proteins, which is in accordance with previous data on this drug (Lee & Lee, 2003). It was a partial agonist for both $G_{\alpha i1}$ and $G_{\alpha oA}$, which is consistent with a finding by Selley *et al*. (1997), where fentanyl was a partial agonist in a [^{35}S]GTP$_{\gamma}$S binding assay in the rat thalamus, in comparison to DAMGO.

Our next aim was to investigate the influence of Mg^{2+} on the extent of MOR basal activation *via* $G_{\alpha i1}$ and $G_{\alpha oA}$ and the resulting effect on the maximal activity of four opioid ligands through MOR. Magnesium has previously been shown to be essential for high-affinity interaction of GTP$_{\gamma}$S with G_{α} (Gilman, 1987; Zelent *et al*., 2001). However, intracellular Mg^{2+} concentrations are not constant, but rather have been shown to fluctuate. For example, Murphy *et al*. (1989) have shown that the intracellular [Mg^{2+}] in cultured embryonic chicken heart cells, can increase from its basal level of 0.48 to 1.6 mM, depending on the intracellular [Ca^{2+}]. We found that the basal activity of MOR increased with increasing Mg^{2+} for both $G_{\alpha i1}$ and $G_{\alpha oA}$ and this effect was more pronounced for $G_{\alpha oA}$ than for $G_{\alpha i1}$, which may suggest that Mg^{2+} stabilises the $G_{\alpha oA}$-[^{35}S]GTP$_{\gamma}$S complex to a greater extent than $G_{\alpha i1}$-[^{35}S]GTP$_{\gamma}$S. The effect of Mg^{2+} on the extent of activation of G_{α} also differed for the four drugs and we found that with increasing maximal activity of a drug, Mg^{2+} sensitivity also increased. We observed a large influence of Mg^{2+} on $G_{\alpha i1}$ activation with DAMGO and buprenorphine, yet none with β-CNA. For naloxone, buprenorphine and DAMGO, Mg^{2+} had a much greater effect when using $G_{\alpha oA}$ compared to β-CNA. Several studies have suggested that β-CNA displayed inverse agonism through MOR (Burford *et al*., 2000; Wang *et al*., 2001). Wang *et al*. (2001) observed inverse agonism of β-CNA at 0 and 1 mM Mg^{2+} (peaking at 1 mM). In our system β-CNA behaved as a neutral antagonist (i.e. producing neither an increase nor decrease in basal activity) or a weak agonist at all Mg^{2+} concentrations, for both $G_{\alpha i1}$ and $G_{\alpha oA}$, which may partly be due to the low level of MOR basal activity in our system. The only evidence of inverse agonism was observed with naloxone acting through the $G_{\alpha i1}$ pathway in the absence of added Mg^{2+}. Buprenorphine and DAMGO were agonists at all Mg^{2+} concentrations; however, in the absence of added Mg^{2+} neither compound produced a significant stimulation of either G-protein activation. Interestingly, at 1 mM added Mg^{2+} DAMGO preferentially activated $G_{\alpha i1}$ over $G_{\alpha oA}$; however, at 3 mM a switch occurred between the two G_{α}, and $G_{\alpha oA}$ was activated to a greater extent than $G_{\alpha i1}$. Although the physiological significance of our findings still requires further investigation, our results suggest that Mg^{2+} concentration may be another factor influencing the selectivity of pathway activation by MOR in *in vitro* studies.

Our study showed that there are statistically significant differences in maximal activities and potencies of certain opioid ligands for the activation of $G_{\alpha i1}$ *versus* $G_{\alpha oA}$. Although further investigation is still required to elucidate the physiological effects resulting from the activation of each intracellular pathway, these findings may provide a starting point for the design or structural screening of drugs that demonstrate greater selectivity for the beneficial pathways activated through MOR, such as analgesia mediated by activation of K_{ir} channels.

This work was funded by a Marsden Fast Start Grant from the Royal Society of New Zealand, a grant from the Auckland Medical Research Foundation and an equipment grant from Lottery Health New Zealand. We thank Dr Christopher Kearn for critical reading of the manuscript.

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(Received August 10, 2005
Revised November 2, 2005
Accepted December 14, 2005
Published online 16 January 2006)