G-protein coupling of μ -opioid receptors (OP₃): elevated basal signalling activity

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To determine μ -opioid receptor (OP₃) signalling activity, guanosine 5'-[γ -³⁵S]thiotriphosphate (GTP[³⁵S]) binding to G-proteins was measured in the membranes of human embryonic kidney cells (HEK-293) transfected with μ -opioid receptor (HEK- μ). GTP[³⁵S] binding to HEK- μ membranes was significantly elevated compared with HEK-293 control membranes (without OP₃), and this was abolished by pertussis-toxin pretreatment. The irreversible antagonist β -chlornaltrexamine (β -CNA) dose-dependently decreased elevated basal G-protein coupling of HEK- μ to control levels in cells devoid of OP₃. This characterizes β -CNA as an inverse OP₃ agonist. Immunoprecipitation of solubilized G-proteins with G₁₃ α antisera demonstrated that basal GTP[³⁵S] binding to G₁₃ α was also substantially elevated in

HEK- μ membranes over the control, whereas G₁₃ α protein levels were unchanged. Basal GTP[³⁵S] binding to G₁₁ α /G₁₂ α and G₀ α was also increased twofold in HEK- μ membranes over the control. Morphine further increased coupling to each of these G α proteins with similar potency, but not to G_q/G₁₁ α or G_s α . These results indicate that the wild-type OP₃ can couple constitutively to endogenously expressed G₁₃ α , G₁₁ α /G₁₂ α and G₀ α subunits of G-proteins in HEK-293 cells.

Key words: β -chlornaltrexamine, G-protein α -subunits, immunoprecipitation, morphine, guanosine 5'-[γ -³⁵S]thiotriphosphate binding.

INTRODUCTION

Opioid receptors (μ , δ and κ ; MOR, DOR and KOR; or, more recently, OP₃, OP₁ and OP₂) are members of the superfamily of G-protein-coupled receptors (e.g. [1–4]). Opioid-receptor activation leads to inhibition of adenylate cyclase [5,6], stimulation of potassium-channel conductance [7,8] and inhibition of calcium-channel conductance [9], mediated by pertussis-toxin (PTX)-sensitive G-proteins [10,11]. Receptor activation causes exchange of GDP for GTP on the α -subunit of heterotrimeric G-proteins; G α -GTP can then dissociate from $\beta\gamma$ subunits and both G α -GTP and G $\beta\gamma$ subunits can modulate effector activity [12,13]. An intrinsic GTPase activity of G α then hydrolyses GTP to GDP, and G α -GDP and G $\beta\gamma$ reassociate. Activation of G-proteins can be measured using radiolabelled, poorly hydrolysed analogues of GTP {5'-[γ -³⁵S]thiotriphosphate (GTP[³⁵S]) and 5'-[α -³²P]triphosphate ([α -³²P]GTP) azidoanilide} [14–17]. These analogues remain bound to G α subunits and their accumulation can be measured.

Many G-protein-coupled receptors appear to activate G-proteins spontaneously (in the absence of agonist) [18–20]. Constitutive activity of wild-type β -adrenergic [20], 5-hydroxytryptamine ('serotonin') [21], bradykinin [22], δ -opioid receptor [23] and muscarinic [24] receptors has been reported. Constitutive activation of μ -opioid receptor (OP₃) following chronic treatment with opiates has been hypothesized to account for part of the regulatory mechanism underlying narcotic tolerance and dependence [25,26], but clear evidence of the existence of constitutive activity of OP₃ receptors has not been forthcoming. The present study addresses the hypothesis that the OP₃ indeed displays basal signalling activity in the absence of agonist. This was measured with the use of a GTP[³⁵S] binding assay, using cell

membranes expressing the OP₃. Moreover, GTP[³⁵S] binding was determined to individual G α proteins immunoprecipitated with G α -selective antisera [27]. Here we demonstrate enhanced basal activation of the OP₃ to specific endogenously expressed G α proteins.

EXPERIMENTAL

Materials

GTP[³⁵S] and the G-protein antisera [G₁₁ α /G₁₂ α (AS/7), G₁₃ α (EC/2), G₀ α (GC/2), G_q α /G₁₁ α (QL) and G_s α (RM/1)] were from New England Nuclear (Boston, MA, U.S.A.). Protein G-Sepharose was from Pharmacia Biotech (Sweden). Normal rabbit serum and Nonidet P40 (NP40) were from Calbiochem (La Jolla, CA, U.S.A.). Morphine sulphate was from the National Institute on Drug Abuse, NIH, Bethesda, MD, U.S.A. β -Chlornaltrexamine dihydrochloride (β -CNA) was from Research Biochemicals International (Natick, MA, U.S.A.). SDS/PAGE and immunoblotting reagents were from Bio-Rad. (Hercules, CA, U.S.A.). All other reagents were from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Cell culture and membrane preparation

Human embryonic kidney (HEK) 293 cells transfected either with cDNA encoding recombinant rat OP₃ (HEK- μ) [28] (expressing $\approx$ 4 pmol of [³H]diprenorphine sites/mg of membrane protein) or with pRC vector alone (without OP₃ cDNA, HEK-vec) were grown to confluence in T-175 cm³ tissue-culture flasks containing 30 ml of media (a 1:1 mix of Dulbecco's modified Eagle's medium and Ham's F-12 medium, supplemented with

Abbreviations used: OP₃, μ -opioid receptor; HEK- μ , human embryonic kidney cells (HEK 293) transfected with μ -opioid receptor; HEK-vec, HEK 293 cells transfected with vector alone (without μ -opioid-receptor cDNA); β -CNA, β -chlornaltrexamine; PTX, pertussis toxin; GTP[³⁵S], guanosine 5'-[γ -³⁵S]thiotriphosphate; NP40, Nonidet P40; DAMGO, [D-Ala², MePhe⁴, Gly(ol)⁵]enkephalin.

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10 % (v/v) foetal-bovine serum and 200 $\mu\text{g}/\text{ml}$ G418). Cells were washed three times with PBS, homogenized in 30 ml of buffer containing 10 mM Hepes, pH 7.4, and 10 mM EDTA, and then centrifuged at 30000 g for 10 min at 4 °C. The pellets were resuspended in 1 ml of 10 mM Hepes (pH 7.4)/0.1 mM EDTA, divided into portions and stored at -80 °C.

GTP[³⁵S] binding assay

The procedure for measuring GTP[³⁵S] binding to cell membrane homogenates (total GTP[³⁵S] binding) was described previously [27]. Aliquots of frozen cell membranes were diluted in assay buffer (10 mM Hepes/10 mM MgCl_2 /100 mM NaCl, pH 7.4) to yield final protein concentrations of 10–35 $\mu\text{g}/50 \mu\text{l}$. Membranes were then incubated with GTP[³⁵S] (0.4 nM for total G-protein binding, 1 nM for individual $\text{G}\alpha$ -subunit binding) at 30 °C for the indicated time periods. In experiments measuring total GTP[³⁵S] binding, the membrane pellets obtained after centrifugation were washed twice with 0.5 ml of assay buffer and radioactivity measured by liquid-scintillation counting. GTP[³⁵S] binding to individual $\text{G}\alpha$ subunits was determined as described in [27]. Briefly, after washing twice with assay buffer, membranes were solubilized and precleared with 100 μl of buffer containing 50 mM Tris/HCl, pH 7.4, 1 % NP40, 100 mM NaCl, 1 mM EDTA, 0.1 % SDS and 15 μl of normal rabbit serum at 4 °C for 2 h, then spun at 13000 g for 10 min at 4 °C. The supernatants were transferred to a set of new tubes and after addition of 40 μl of 20 % slurry Protein G beads and 15 μl of anti- $\text{G}\alpha$ antiserum (1:20 dilution), incubated at 4 °C for another 12 h. Then the beads were spun down and washed twice with PBS at 4 °C and radioactivity measured by scintillation counting. For inverse-agonist experiments, membranes (10 μg) were incubated with different concentrations of β -CNA in 50 mM Hepes (pH 7.4)/100 mM NaCl/10 mM EDTA/5 mM MgCl_2 /1 mM DTT/10 μM GDP/0.1 % BSA at 30 °C for 20 min, then the reactions were stopped by filtration. The filters were washed twice with 50 mM Tris/HCl (pH 7.4)/50 mM NaCl, and radioactivities were counted.

SDS/PAGE and Western-blot analysis

Cell membranes (5–40 μg) were separated by SDS/PAGE (10 % polyacrylamide gel) as described by Laemmli [29]. Separated proteins were electrophoretically transferred to PVDF membrane (0.2 μm) [30]. The membrane was blocked in 5 % non-fat dried milk in TBST [Tris-buffered saline (20 mM Tris/HCl (pH 7.6)/137 mM NaCl)/0.05 % Tween-20] for 2 h at room temperature with agitation, followed by 2 h incubation in the presence of the primary antibody $\text{G}_{13}\alpha$ antisera (1:1000 dilution). After washing in TBST, a secondary antibody [goat anti-rabbit IgG conjugated to alkaline phosphatase (1:2000 dilution)] was added for 2 h at room temperature, followed by development with AP Development Buffer (Bio-Rad).

Data analysis

Results expressed as means $\pm$ S.E.M. for at least three experiments, each performed in duplicate. Curve fits of dose-response curves were analysed, and EC_{50} values were obtained using KaleidaGraph. Statistical analysis was performed by Student's t test or ANOVA. Western blots were scanned and analysed using Adobe Photoshop and NIH-image.

RESULTS

GTP[³⁵S] binding to HEK-vec and HEK- μ membranes

Total GTP[³⁵S] binding was determined in HEK-vec and HEK- μ membrane preparations. Expression of OP_3 in HEK-cell membranes resulted in a significant increase (20–25 %) in basal levels of GTP[³⁵S] binding compared with HEK-vec membranes (Figure 1), in both the absence and presence of 1 μM GDP, after a 5 min incubation at 30 °C. Addition of a maximally effective concentration of the full agonist morphine (10 μM) to HEK- μ membranes resulted in a 85 % increase in GTP[³⁵S] binding compared with HEK-vec basal activity, in both the absence and presence of GDP (EC_{50} values for morphine were 1.7 nM in the absence of GDP and 7.4 nM in the presence of GDP respectively; results not shown). Thus about 25 % of the overall response attributable to OP_3 appears to be accounted for by basal receptor activity in the absence of agonist. In contrast, morphine had no significant effect on GTP[³⁵S] binding to HEK-vec membranes (results not shown). PTX pretreatment (100 ng/ml for 16 h) of HEK-vec cells had no significant effect on basal GTP[³⁵S] binding under the assay conditions. The lack of a demonstrable effect of PTX on basal GTP[³⁵S] binding in non-transfected cell membranes is probably a result of the rather small amount of membrane protein per sample incubation (12 μg of protein in this experiment) when compared with previous studies. In contrast, PTX pretreatment abolished the enhanced basal GTP[³⁵S] binding in HEK- μ membranes, in both the absence and presence of GDP. Moreover, PTX treatment suppressed morphine-stimulated GTP[³⁵S] binding (> 90 %) (results not shown), demonstrating that G_i/G_o type- α subunits were indeed inactivated. These results indicate the presence of substantial basal signalling activity of OP_3 to PTX-sensitive G-proteins. Basal activity was detectable regardless of the presence of GDP

Figure 1 Effect of PTX and GDP on basal and agonist-mediated GTP[³⁵S] binding to membranes of HEK-vec and HEK- μ cells

GTP[³⁵S] ('[³⁵S]GTP γ S' in this and subsequent Figures) binding experiments were conducted as described in the Experimental section either with no drug added or in the presence of 10 μM morphine, in membranes (12 $\mu\text{g}/\text{tube}$) of HEK-vec and HEK- μ cells. Experiments were terminated after a 5 min incubation at 30 °C. Basal activity was also determined in membranes after PTX pretreatment of the intact cells (100 ng/ml for 16 h). Similar measurements were determined both in the absence and presence of GDP (1 μM). Data are expressed as a percentage (mean $\pm$ S.E.M.) of the response in HEK-vec membranes. The 100 % value represents approx. 4000–6000 d.p.m.; statistical significance versus HEK-vec: * P < 0.05; ** P < 0.01; ANOVA; n = 4–2.

Figure 2 Dose–response curve of β -CNA effect on basal GTP[35 S] binding to HEK- μ membranes

Different concentrations of β -CNA were incubated with HEK- μ membranes at 30 °C for 20 min followed by filtration as described in the Experimental section. Results are means $\pm$ S.E.M., $n = 3$. Statistical significance versus basal: * $P < 0.05$, ** $P < 0.01$; ANOVA; 10 μ M β -CNA has no effect on HEK-vec membrane (●).

(Figure 1), which tends to suppress weak basal G-protein-coupled-receptor signalling.

β -CNA decreases basal GTP[35 S] binding of HEK- μ

Under the assay conditions described here, the irreversible OP_3 antagonist β -CNA dose-dependently reduced basal GTP[35 S] binding of HEK- μ , but not HEK-vec (Figure 2). The EC_{50} value for β -CNA is 52 ± 1.8 nM. β -CNA was the most effective inverse agonist among a panel of known opioid antagonists tested (D. Wang and W. Sadee, unpublished work). This result confirms the presence of basal G-protein coupling of OP_3 . It remains to be resolved whether β -CNA acts as a non-covalent competitive or an irreversible ligand, or both, over the 20 min incubation period.

Basal GTP[35 S] binding after immunoprecipitation with $G_{i3}\alpha$ antisera in HEK- μ compared with HEK-vec membranes

We have previously shown that OP_3 -mediated GTP[35 S] binding following immunoprecipitation with specific G-protein antisera is most pronounced using $G_{i3}\alpha$ antisera, in HEK- μ cell membranes [27]. This antiserum was therefore used to determine basal and agonist-stimulated GTP[35 S] binding following immunoprecipitation. Comparisons of maximal agonist (morphine)-stimulated GTP[35 S] binding between total GTP[35 S] binding to HEK- μ membranes and GTP[35 S] binding following solubilization and immunoprecipitation with $G_{i3}\alpha$ antisera (1:200 dilution), showed that approx. 6% of the total pool of labelled G-proteins were immunoprecipitated as GTP[35 S] binding to $G_{i3}\alpha$. A larger percentage recovery could be achieved with increasing concentrations of antisera. However, the concentration of antisera used was minimized to reduce the expense of the experiments while still providing a good signal-to-noise ratio and level of reproducibility. Under these assay conditions, recovery levels were nearly identical between different experiments and therefore suitable for quantitative analysis. Further, immunoprecipitation of $G_{i3}\alpha$ reduced the background level of GTP[35 S] binding compared with whole membranes, thereby enhancing the signal-to-noise ratio of the measurements.

In the absence of opioid receptor ligands, HEK- μ membranes produced a $142 \pm 25\%$ (three independent experiments) greater GTP[35 S] binding compared with HEK-vec membranes, after

Figure 3 Basal GTP[35 S] binding to HEK-vec and HEK- μ membranes after immunoprecipitation with $G_{i3}\alpha$ antisera

Comparisons of the basal GTP[35 S] binding to $G_{i3}\alpha$ proteins were conducted in membranes from HEK-vec and HEK- μ (30 μ g/tube) before and after pretreatment with PTX (100 ng/ml for 16 h), after a 30 min time period, without added GDP. Data are expressed as a percentage (mean $\pm$ S.E.M., $n = 3$) of the HEK-vec basal response in the absence of PTX pretreatment (1355 ± 35 d.p.m.). Statistical significance versus HEK-vec control: ** $P < 0.01$, ANOVA.

immunoprecipitation with $G_{i3}\alpha$ antisera, following a 30 min incubation (Figure 3). PTX pretreatment of HEK- μ cells decreased basal GTP[35 S] binding to a level indistinguishable from that observed with PTX-pretreated HEK-vec membranes ($P > 0.05$) (Figure 3). This result indicates that the enhanced GTP[35 S] binding activity of HEK- μ cells results largely from basal μ -opioid receptor signalling. A small decrease in GTP[35 S] binding caused by PTX pretreatment of HEK-vec membranes further suggests the presence of detectable basal $G_{i3}\alpha$ activity unrelated to OP_3 .

Western-blot analysis of the content of $G_{i3}\alpha$ in membranes from HEK-vec and HEK- μ

The $G_{i3}\alpha$ expression level was determined by quantitative Western-blot analysis. The $G_{i3}\alpha$ antiserum used revealed the presence of a single immunoreactive band consistent with $G_{i3}\alpha$ protein. The results show that HEK- μ and HEK-vec membranes contain the same level of $G_{i3}\alpha$ (results not shown). Therefore, the difference in GTP[35 S] binding between HEK- μ and HEK-vec is not due to different levels of $G_{i3}\alpha$ in these two cell clones, but rather appears to result from an enhanced activation of $G_{i3}\alpha$ proteins in HEK cell membranes expressing OP_3 .

Comparisons of basal GTP[35 S] binding activity between HEK- μ and HEK-vec after immunoprecipitation with various G-protein antisera

Basal GTP[35 S] binding between HEK- μ and HEK-vec was compared after immunoprecipitation with the selective G-protein antisera (Figure 4). Basal activity in HEK- μ membranes was approx. 2-fold greater than in HEK-vec after immunoprecipitation with $G_{i1}\alpha$ / $G_{i2}\alpha$, $G_{o\alpha}$ and $G_{i3}\alpha$ antisera. Basal activity was not significantly different between these two cell clones after immunoprecipitation with $G_s\alpha$ antisera, whereas immunoprecipitation with $G_q\alpha$ / $G_{11}\alpha$ antisera resulted in a reduction (by 36%) in basal GTP[35 S] binding in HEK- μ compared with HEK-vec membranes. To test the selectivity of the $G_s\alpha$ and $G_q\alpha$ / $G_{11}\alpha$ antisera, we determined their ability to detect activation of these $G\alpha$ subunits by their cognate receptors. Agonist stimulation (1 mM carbachol and 1 μ M α -melanocyte-stimu-

Figure 4 Comparisons of basal GTP[³⁵S] binding between HEK-vec and HEK-μ cell membranes after immunoprecipitation with various G-protein antisera

Experiments were carried out in the presence of equal protein concentrations of either HEK-vec or HEK-μ cell membranes for 30 min. Samples were immunoprecipitated with different G-protein antisera (1:200 dilution), and basal GTP[³⁵S] binding was compared. Data are expressed as the means ± S.E.M. for three experiments each performed in duplicate. Statistical significance versus HEK-vec: **P* < 0.05, Student's *t* test.

Figure 5 Morphine- and naloxone-stimulated GTP[³⁵S] binding to specific G-proteins in HEK-μ membranes

GTP[³⁵S] binding experiments were conducted in HEK-μ membranes in the absence or presence of morphine (10 μM) or naloxone (10 μM) for 1 min. Samples were then solubilized and immunoprecipitated with various G-protein antisera (1:200 final dilution). Data are means ± S.E.M. for three experiments each performed in duplicate. Statistical significances versus basal: **P* < 0.05, ***P* < 0.01, ANOVA.

lating hormone) expressing m1 muscarinic receptors (HEK-m1, expressing 2 pmol of receptor/mg of protein) or melanocortin receptors (HEK-mc4) increased GTP[³⁵S] binding more than tenfold above basal activity, measured with G_qα/G₁₁α and G_sα antisera respectively (results not shown). Basal signalling was minimal or undetectable. This demonstrated that coupling to G_qα/G₁₁α and G_sα can be detected sensitively under these assay conditions. Muscarinic wild-type receptor also activated G₁₃α upon carbachol stimulation (results not shown); however, in contrast with OP₃, no basal signalling activity of G₁₃α (102 ± 5% versus vector membranes) was detectable. Our analysis may not be indicative of the absolute levels of Gα coupling by OP₃ among individual Gα and G_oα subunits, since we have not determined overall recovery for each separately.

Figure 6 Dose-response curves for morphine-stimulated GTP[³⁵S] binding in HEK-μ membranes after immunoprecipitation with various G-protein antisera

GTP[³⁵S] binding dose-response curves to morphine after 1 min incubation were determined in HEK-μ membranes. Samples were then immunoprecipitated with either G₁₁α/G₁₂α, G_oα or G₁₃α antisera (1:200 dilution). Data are represented as means ± S.E.M. for three experiments each performed in duplicate (see also Table 1).

Morphine- and naloxone-stimulated GTP[³⁵S] binding in HEK-μ membranes after immunoprecipitation with various G-protein antisera

Using the initial rates of GTP[³⁵S] binding in the first 1 min of incubation, morphine produced a robust response of 3.9-fold, 2.5-fold and 3.7-fold above basal GTP[³⁵S] binding to G-proteins immunoprecipitated with G₁₁α/G₁₂α, G_oα and G₁₃α antisera respectively (see also [27]; Figure 5). As described above, no stimulation was detectable with G_qα/G₁₁α and G_sα antisera after 1 min (Figure 5) or 90 min incubation (results not shown), even though coupling to these subunits can be sensitively measured in our assay. Although naloxone is considered an antagonist, it also produced a small stimulation of GTP[³⁵S] binding under the assay condition used (1.4-fold, 1.6-fold and 1.9-fold above basal for G₁₁α/G₁₂α, G_oα and G₁₃α respectively) (Figure 5).

Neither morphine nor naloxone induced detectable GTP[³⁵S] binding above basal levels in HEK-vec after immunoprecipitation with G₁₃α antisera. PTX pretreatment of cells (100 ng/ml for 16 h) abolished both morphine- and naloxone-stimulated GTP[³⁵S] binding at G₁₁α/G₁₂α, G_oα and G₁₃α (results not shown).

Dose-response curves for morphine and naloxone at specific G-proteins in HEK-μ membranes

Dose-response curves were obtained at optimal time periods of incubation (highest signal-to-noise ratio) for morphine (1 min) and naloxone (30 min) in HEK-μ membranes. In this experiment, morphine produced a dose-dependent increase in GTP[³⁵S] binding above basal levels after immunoprecipitation with G₁₁α/G₁₂α, G_oα and G₁₃α antisera, reaching a maximal increase above basal of 3.1-, 3.0- and 5.2-fold respectively (Figure 6 and Table 1). In each case, the EC₅₀ for morphine ranged from 0.4 to 0.7 nM, with no significant difference between each of the G-protein subtypes studied.

Naloxone also produced a small dose-dependent increase in GTP[³⁵S] binding above basal levels after immunoprecipitation with G₁₁α/G₁₂α, G_oα and G₁₃α antisera, reaching a maximal increase above basal of 1.3-, 1.6- and 1.8-fold respectively (Table 1; data obtained with no GDP added). The EC₅₀ for naloxone

Table 1 Basal, morphine (after 1 min incubation) and naloxone (after 30 min stimulation)-stimulated GTP[³⁵S] binding to HEK- μ membranes after immunoprecipitation with G protein antiseraData represent means $\pm$ S.E.M. for three experiments each performed in duplicate.

	Stimulation (d.p.m.)		
	Basal	Maximal	EC ₅₀ (log M)
Morphine (1 min)			
G ₁₁ α /G ₁₂ α	134 $\pm$ 5	410 $\pm$ 29	-9.15 $\pm$ 0.11
G _o α	147 $\pm$ 12	442 $\pm$ 47	-9.25 $\pm$ 0.16
G ₁₃ α	525 $\pm$ 17	2708 $\pm$ 368	-9.33 $\pm$ 0.03
Naloxone (30 min)			
G ₁₁ α /G ₁₂ α	448 $\pm$ 25	598 $\pm$ 53	-9.27 $\pm$ 0.41
G _o α	407 $\pm$ 13	640 $\pm$ 42	-9.14 $\pm$ 0.37
G ₁₃ α	3042 $\pm$ 70	5329 $\pm$ 176	-9.33 $\pm$ 0.04

was similar to that of morphine, ranging from 0.4 to 0.7 nM, and no significant differences were noted for EC₅₀ values at the individual G α subunits.

Time course of GTP[³⁵S] binding

Time-course studies were performed for GTP[³⁵S] incorporation into membranes of HEK-vec and HEK- μ cells. Shown in Figure 7 are time courses for basal OP₃ activity (above HEK-vec control) and morphine (10 μ M)- and naloxone (10 μ M)-stimulated GTP[³⁵S] binding. Experiments including GDP (10 μ M) gave qualitatively similar time courses, except for a greatly attenuated naloxone response (results not shown). Basal OP₃ stimulation of total GTP[³⁵S] incorporation into membranes was significantly elevated over the control in all but one time point in this experiment (Figure 7A). Morphine induced a rapid increase in total GTP[³⁵S] binding to HEK- μ membranes, which peaked at 15 min and then declined somewhat. In contrast, naloxone stimulated a steady increase over the basal level, with no apparent decline at longer incubation times. As a result, naloxone stimulated more GTP[³⁵S] binding than morphine over longer incubation periods (Figure 7A). Similar results were obtained following solubilization of the membranes and immunoprecipitation with G₁₃ α antiserum (Figure 7B). Basal activity and

naloxone-induced activity were more prominent because of reduced background GTP[³⁵S] binding after immunoprecipitation. Morphine again caused a rapid initial surge in GTP[³⁵S] binding, followed by a slow decline (Figure 7B).

The necessity of using early time points to distinguish between agonist and basal binding of guanine nucleotides has been observed previously. [D-Ala²,MePhe⁴,Gly(ol)⁵]enkephalin (DAMGO)-stimulated elevation of [α -³²P]GTP azidoanilide into G₁₃ α in SH-SY5Y cell membranes was maximally elevated from basal levels after 6 min [31]. Similarly, dopamine-induced binding of [α -³²P]GTP in rat brain membranes could only be observed at early incubations (3 min, 30 °C) [32].

The gradual loss of GTP[³⁵S] binding to G₁₃ α upon morphine incubations exceeding 5–15 min suggests that additional processes occur, such as morphine-induced desensitization of the OP₃, GTP[³⁵S] dissociation from G₁₃ α , or G₁₃ α dissociation from the membrane during incubation with morphine. To investigate whether activated G-proteins could be detected in the supernatant rather than the membrane pellet, supernatants were immunoprecipitated with G₁₃ α antisera. No increase in GTP[³⁵S] binding was observed in the supernatant after 1 min compared with basal conditions. However, an increase in both morphine- and naloxone-induced GTP[³⁵S] binding to G₁₃ α above basal was detected after 30 min (966 and 662 d.p.m. respectively, $n = 2$), suggesting that some of the activated G-proteins may leave the membrane environment upon extended stimulation with agonist. However, this result only partially accounts for the loss of GTP[³⁵S] binding after the initial morphine-induced peak. Clearly, an additional unknown process appears to occur with morphine, but not as a result of basal and naloxone-induced activity.

Incubation of HEK- μ membranes with morphine (1 μ M) and naloxone (10 μ M) together resulted in a time course similar to that of naloxone alone (results not shown). Therefore naloxone can inhibit the initial large morphine-induced GTP[³⁵S] binding at 1 min.

Similar time-course profiles were observed in HEK- μ membranes using G₁₁ α /G₁₂ α antisera and G_o α antisera (results not shown), although basal coupling and drug treatments produced lower levels of GTP[³⁵S] binding than those observed after immunoprecipitation with G₁₃ α antisera (see Figure 4). In each case, morphine-induced GTP[³⁵S] binding was maximal after

Figure 7 Time course of GTP[³⁵S] binding in HEK- μ membranes and following solubilization and immunoprecipitation with G₁₃ α antisera

GTP[³⁵S] binding experiments were conducted either with no drug added, in the presence of morphine (10 μ M) or in the presence of naloxone (10 μ M) for the times indicated. Total GTP[³⁵S] binding to HEK- μ membranes (A) and GTP[³⁵S] binding following solubilization and immunoprecipitation with G₁₃ α antisera (1:200 final dilution) in HEK- μ membranes (B), were determined. Data are represented as means $\pm$ S.E.M. for three experiments each performed in duplicate. Statistical significance versus HEK-vec basal: * $P < 0.05$, ** $P < 0.01$, ANOVA.

1 min. Similar time-course profiles of agonist-induced GTP[³⁵S] binding to HEK- μ membranes after immunoprecipitation with G₁₃ α antisera were observed with lower concentrations of morphine (100 nM) and with the agonists DAMGO and endomorphin I [27].

DISCUSSION

The present study demonstrates that OP₃ expression in HEK cell membranes enhances basal G-protein activation, indicative of constitutive OP₃ activity. Enhanced basal activity was observed both with total GTP[³⁵S] binding to the entire pool of G-proteins in HEK- μ membranes (Figure 1) and after immunoprecipitation with G₁ α antisera, particularly G₁₃ α (Figure 3). Basal OP₃ activity was blocked by PTX pretreatment of HEK- μ cells. This result indicates that basal signalling occurs at PTX-sensitive G-proteins, as observed for agonist-stimulated OP₃ activity. The ability of β -CNA to decrease basal GTP[³⁵S] binding of HEK- μ membrane confirmed the presence of substantial basal OP₃ activity.

Basal activity at G₁₃ α in HEK- δ cell membranes was also detectable under the same assay conditions (results not shown). Basal activity had been reported previously for the δ -opioid receptor (OP₁) [23]. Further, no basal activity was observed in HEK-m1 cell membranes (expressing m1 muscarinic receptors). Therefore, under the assay conditions used, another highly expressed receptor type failed to show any constitutive signalling activity. These comparisons support the view that basal activity detected with OP₃ might be relevant physiologically.

Enhanced basal GTP[³⁵S] binding to G₁₃ α expressed in HEK- μ cell membranes could have been accounted for by an increase in endogenously expressed G₁₃ α levels as a result of OP₃ transfection. However, Western-blot analysis of HEK- μ and HEK-vec membranes showed no significant difference in the levels of G₁₃ α expressed between the two cell clones. This indicates that OP₃ expressed in HEK- μ membranes is capable of constitutively activating endogenous G₁₃ α proteins. Basal activation of G₁₁ α /G₁₂ α and G₀ α was also elevated by approx. 2-fold in HEK- μ compared with HEK-vec membranes. By contrast, basal activation of G₅ α remained unchanged between the two cell clones, and basal activation of G_q α /G₁₁ α was reduced in HEK- μ compared with HEK-vec membranes. Possibly, OP₃ expression could have affected the level of G_q α /G₁₁ α expression, $\beta\gamma$ subunits liberated from G_i/G_o could have scavenged G_q α /G₁₁ α , or OP₃ could have inhibited basal GTP binding (for a discussion on possible inhibitory receptor action, see [33,34]).

Analysis of basal and agonist-dependent G-protein coupling is sensitive to the experimental conditions used. Previous studies investigating GTP[³⁵S] binding without immunoprecipitation have required the presence of excess GDP to dampen basal activity so that agonist-stimulated GTP[³⁵S] binding could be observed above background [14–16]. In the present study we have shown that basal OP₃ activity and agonist-stimulated GTP[³⁵S] binding to HEK- μ membranes are detectable in both the presence and absence of GDP, both in total binding experiments and after immunoprecipitation with G₁₃ α antisera. Therefore basal OP₃ activity was robust under several conditions, even in the presence of GDP, which is known to suppress basal signalling.

The presence of basal receptor activity prompted us to search for inverse OP₃ agonists. The GTP[³⁵S] binding assay described here provides a screen for identifying inverse agonists at OP₃. Inverse agonists have previously been described for OP₁ [24,36]. Further screening of known OP₃ antagonists {naltrexone, diprenorphine, CTAP [H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂(cyclic)], β -funaltrexamine, β -CNA} revealed β -CNA as

a full inverse agonist. Relative efficacies of these compounds will be reported separately. Dependent upon the incubation conditions, naloxone either had little or no effect or displayed low partial-agonist activity. Since the addition of GDP largely suppressed naloxone-stimulated GTP[³⁵S] binding, it appears that naloxone has at best very low efficacy as an agonist [35]. Yet, a partial agonist effect of naloxone is consistent with our previous observations that naloxone decreases cAMP levels in untreated SH-SY5Y cells [26] and HEK- μ cells [36]. Since SH-SY5Y cells express relatively low levels of OP₃ [37], it appears that the partial agonist properties of naloxone may not necessarily require high levels of OP₃ expression. Thus the partial agonist activity of naloxone may account for the 'morphine-like' effects of naloxone seen in early studies [38]. We are currently studying the behaviour of naloxone after morphine pretreatment – resulting in an apparent shift toward inverse agonism in morphine-dependent tissues [26].

Dose-response curves at optimal time points for morphine (after 1 min) and naloxone (after 30 min) produced similar EC₅₀ values for activation of G₁₁ α /G₁₂ α , G₀ α and G₁₃ α , suggesting that both morphine and naloxone activated each of these G-proteins with equal potency. Previous studies have suggested that different G-proteins can be activated with different potencies by OP₃ agonists in Chinese-hamster ovary cell membranes [39]. This was not found in the present study in HEK cell membranes, which agrees with studies on δ - and κ -opioid receptors, suggesting that commonly used agonists of these receptors can activate multiple G-protein subtypes with similar potency [10,11,40]. These discrepant results might be resolved if one considers the different experimental conditions.

In conclusion, this study supports the hypothesis that OP₃ receptors are constitutively active. It remains to be seen how such basal activity affects signalling pathways in intact cells. We have previously proposed that constitutive activation of OP₃ following chronic agonist treatment played a role in narcotic tolerance and dependence [26]. We are currently investigating the effect of chronic opiate treatment on OP₃ coupling to G-proteins, including possible changes in basal activation of these G-proteins.

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