Journal of Neurochemistry, 2006, **99**, 1494–1504 doi:10.1111/j.1471-4159.2006.04176.x

Mediation of adenylyl cyclase sensitization by PTX-insensitive $G\alpha_{oA}$, $G\alpha_{i1}$, $G\alpha_{i2}$ or $G\alpha_{i3}$

Mary J. Clark and John R. Traynor

Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan USA

Abstract

Chronic activation of mu-opioid receptors, which couple to pertussis toxin-sensitive $G\alpha_{i/o}$ proteins to inhibit adenylyl cyclase (AC), leads to a compensatory sensitization of AC. Pertussis toxin-insensitive mutations of $G\alpha_{i/o}$ subtypes, in which the pertussis toxin-sensitive cysteine is mutated to isoleucine ($G\alpha^{CI}_{i/o}$), were used to determine whether each of the $G\alpha_{i/o}$ subtypes is able to mediate sensitization of AC. $G\alpha^{CI}_{oA}$, $G\alpha^{CI}_{i1}$, $G\alpha^{CI}_{i2}$ or $G\alpha^{CI}_{i3}$ were individually transiently transfected into C6 glioma cells stably expressing the mu-opioid receptor, or transiently co-expressed with the mu-opioid receptor into human embryonic kidney (HEK)293T cells. Cells were treated with pertussis toxin to uncouple endogenous $G\alpha_{i/o}$ proteins, followed by acute or chronic treatment with the mu-opioid agonist, [D-Ala^2,N-Me-Phe^4, Gly^5-ol]enkephalin (DAMGO). Each $G\alpha_{i/o}$ subtype mediated acute DAMGO inhibition of AC and DAMGO-induced sensitization of AC. The potency for DAMGO to stimulate sensitization was independent of the $G\alpha_{i/o}$ subtype, but the level of sensitization was increased in clones expressing higher levels of $G\alpha_{i/o}$ subunits. Sensitization of AC mediated by a component of fetal bovine serum, which was also dependent on the level of functional $G\alpha_{i/o}$ subunits in the cell, was observed. This serum-mediated sensitization partially masked mu-opioid-mediated sensitization when expressed as percentage overshoot due to an apparent increase in AC activity.

Keywords: adenylyl cyclase, cAMP overshoot, $G\alpha_{i/o}$ subtypes, mu-opioid, pertussis toxin, sensitization.

J. Neurochem. (2006) **99**, 1494–1504.

The long-term use of mu-opioid agonists for the treatment of pain is complicated by the development of dependence, demonstrated by withdrawal upon removal of the agonist, and tolerance, a reduced response with the same amount of agonist. Although the development of dependence and tolerance usually coincide, they have separate mechanisms of development (Bohn *et al*. 2000; Yoshimura *et al*. 2000). At the cellular level, dependence is indicated by a sensitization of adenylyl cyclase (AC) (Sharma *et al*. 1975), also referred to as cAMP overshoot or heterologous sensitization of AC (Watts 2002). This follows chronic $G\alpha_{i/o}$ inhibition and is measured as an increase in $G\alpha_s$- or forskolin-stimulated cAMP accumulation upon removal of agonist. The mechanisms of the development of sensitization following chronic $G\alpha_{i/o}$-mediated inhibition are slowly being unraveled (Watts 2002).

Of the nine AC isoforms, only ACI, ACV, ACVI and ACVIII are strongly sensitized (Watts and Neve 1996; Avidor-Reiss *et al*. 1997; Nevo *et al*. 1998, 2000; Rhee *et al*. 2000; Cumbay and Watts 2001). Enhanced $G\alpha_s$-AC coupling has been proposed as a mechanism for sensitization (Watts and Neve 1996; Vortherms *et al*. 2004). Evidence for the intermediaries involved in the mediation of sensitization includes a requirement for $G\beta\gamma$ (Avidor-Reiss *et al*. 1996; Rhee *et al*. 2000; Rubenzik *et al*. 2001), a role for Raf-1 (Varga *et al*. 1999, 2002, 2003), and a selective role for protein kinase A (PKA) (Johnston *et al*. 2002) and protein kinase C (PKC) (Varga *et al*. 2003; Beazely and Watts 2005; Beazely *et al*. 2005).

Received February 4, 2006; revised manuscript received June 20, 2006; accepted July 27, 2006.

Address correspondence and reprint requests to John R. Traynor, Department of Pharmacology, 1301 MSRB III, 1150 W. Medical Center Drive, Ann Arbor, MI 48109–0632, USA. E-mail: jtraynor@umich.edu

Abbreviations used: AC, adenylyl cyclase; C6μ, C6 glioma cells stably expressing the mu-opioid receptor; DAMGO, [D-Ala^2,N-Me-Phe^4,Gly^5-ol]enkephalin; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FGF, fibroblast growth factor; HEK, human embryonic kidney; IBMX, 3-isobutyl-1-methylxanthine; IGF-1, insulin-like growth factor-1; PDGF, platelet-derived growth factor; PKA, protein kinase A; PKC, protein kinase C; PTX, pertussis toxin; RTK, receptor tyrosine kinase.

Although all of the Gα subtypes of the $G\alpha_{i/o}$ family ($G\alpha_{oA}$, $G\alpha_{oB}$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_z$) are able to mediate acute inhibition of AC (Gerhardt and Neubig 1991), the ability of a single $G\alpha_{i/o}$ subtype to mediate AC sensitization has only been demonstrated for $G\alpha_o$ (Watts *et al*. 1998; Clark *et al*. 2004), $G\alpha_{i2}$ (Nakagawa *et al*. 1999), and $G\alpha_z$ with ACV but not ACVI (Ammer and Christ 2002). In other studies, $G\alpha_{i1}$, $G\alpha_{i2}$ or $G\alpha_{i3}$ have not been able to mediate sensitization alone (Watts *et al*. 1998; Tso and Wong 2000; Tso and Wong 2001), leading to the suggestion that multiple Gα subtypes are required for opioid-induced sensitization (Tso and Wong 2000). This is supported by a decreased level of sensitization in cells expressing only one functional Gα subtype compared with wild-type cells expressing multiple Gα subtypes (Watts *et al*. 1998; Nakagawa *et al*. 1999; Clark *et al*. 2004). Therefore, it can be predicted that the different $G\alpha_{i/o}$ subtypes have different efficiencies in mediating sensitization, which may not correlate with efficiencies for acute inhibition of AC and could be AC isoform dependent. There is evidence for a subtype selective effect on acute inhibition of AC. For example, dopamine D_{2S} receptor-mediated inhibition of forskolin-stimulated AC is mediated by $G\alpha_{i2}$ and not $G\alpha_{i3}$, but prostaglaudin E_1-stimulated AC is mediated by $G\alpha_{i3}$ and not $G\alpha_{i2}$ in murine Ltk- cells that contain ACI, ACVI and other isoforms (Ghahremani *et al*. 1999); α_2-adrenergic inhibition of PKC-stimulated ACII in Sf9 cells can be mediated by $G\alpha_o$ but not $G\alpha_{i1}$ (Näsman *et al*. 2002).

The aim of this study was to determine whether $G\alpha_{i1}$ and $G\alpha_{i3}$, like $G\alpha_{i2}$ and $G\alpha_{oA}$, are able to mediate sensitization, and to investigate factors which may influence the degree of sensitization, including Gα expression levels and the degree of acute AC inhibition. For this purpose, Gα subunits with an Ile replacement of the pertussis toxin (PTX)-sensitive Cys close to the C-terminus ($G\alpha_{oA}^{CI}$, $G\alpha_{i1}^{CI}$, $G\alpha_{i2}^{CI}$, $G\alpha_{i3}^{CI}$) were expressed in C6 glioma cells stably expressing the mu-opioid receptor (C6μ), or transiently co-expressed in HEK293T cells with the mu-opioid receptor. Cells were treated with PTX to inactivate endogenous G proteins before acute or chronic treatment with mu-opioid agonist. Sensitization following agonist treatment was determined by measuring forskolin-stimulated cAMP accumulation in the presence of naloxone, an opioid antagonist. The results demonstrated that $G\alpha_{oA}$, $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$ are each able to mediate mu-opioid agonist-induced AC sensitization in C6μ and HEK293T cells.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, LipofectAMINE Plus Reagent, Geneticin and Zeocin were purchased from Invitrogen (Carlsbad, CA, USA). Pertussis toxin (PTX) was purchased from List Biological Laboratories Inc. (Campbell, CA, USA). [D-Ala^2, N-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO), guanosine 5′-diphosphate (GDP), 3-isobutyl-1-methylxanthine (IBMX), forskolin, Trizma base and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human PTX-insensitive $G\alpha_{o/i}^{CI}$ pDNA (with a cysteine to isoleucine mutation at the PTX-sensitive cysteine near the C-terminus) was purchased from UMA cDNA Resource Center (http://www.cDNA.org). Rat PTX-insensitive $G\alpha_{o/i}^{CG}$ and mouse PTX-insensitive $G\alpha_{oA}^{CG}$ pDNA were obtained from Steve Ikeda (Guthrie Research Institute, Sayre, PA, USA). QIAGEN Plasmid Maxi Kits were purchased from QIAGEN, Inc. (Valencia, CA, USA).

Cell culture and expression of PTX-insensitive Gα

C6μ and HEK293T cells were grown in DMEM with 10% FBS under 5% CO_2. Geneticin (0.25 mg/mL) was included with the C6μ cells to maintain expression of the mu-opioid receptor (Clark *et al*. 2003). Human PTX-insensitive $G\alpha_{o/i}^{CI}$, rat PTX-insensitive $G\alpha_i^{CG}$ or mouse PTX-insensitive $G\alpha_{oA}^{CG}$ DNA was inserted into pcDNA3.1zeo vector (Zeocin-resistant). Stable transfection of the PTX-insensitive $G\alpha_{o/i}$ constructs into C6μ cells was accomplished using LipofectAMINE Plus Reagent, and selected using 0.4 mg/mL Zeocin, as described previously (Clark *et al*. 2003). PTX-insensitive $G\alpha_{o/i}$ was transiently transfected (4 μg/10 mL plate) in C6μ cells using LipofectAMINE Plus Reagent. HEK293T cells were transiently transfected with mu-opioid receptor (Lee *et al*. 1999), with or without co-transfection with PTX-insensitive $G\alpha_{o/i}$ (3 μg plus 3 μg/10 mL plate).

Western blots

C6μ cell membrane homogenates were prepared as previously described (Clark *et al*. 2003) and separated (20 μg protein) by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 12% gel (Protogel, National Diagnostics, Inc., Atlanta, GA, USA). Proteins were transferred to a nitrocellulose membrane (45 μm; Osmonics, Inc., Minnetonka, MN, USA) and probed with 1 : 6000 anti-$G\alpha_{i2}$ (Calbiochem, San Diego, CA, USA) in 5% non-fat dry milk or 1 : 200 anti-$G\alpha_o$ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 5% non-fat dry milk. Bands were visualized using horseradish peroxidase-conjugated goat anti-rabbit IgG in 5% non-fat dry milk, followed by enhanced chemiluminescence (West Pico Super Signal, Pierce, Rockford, IL, USA) and quantification using an Eastman Kodak Image Station 440 (Eastman Kodak, Rochester, NY, USA).

cAMP accumulation assay

C6μ cells expressing endogenous Gα proteins, or stably expressing PTX-insensitive $G\alpha_{o/i}$ proteins, were plated to 80–90% confluency in 24-well plates with DMEM plus 10% FBS and immediately treated with 100 ng/mL PTX. Transiently transfected cells (HEK293T and C6μ) were plated into 24-well plates with DMEM and 10% FBS 24 h after transfection, and immediately treated with 100 ng/mL PTX. After 6 h, varying concentrations of DAMGO were added, without removing the PTX, and 18 h later cells were assayed for forskolin-stimulated cAMP accumulation. Cell media were replaced with serum-free DMEM containing 5 μM forskolin, 1 mM IBMX and 10 μM naloxone (for sensitization), or 0–10 μM DAMGO (for acute inhibition), and allowed to incubate for 10 min at 37°C. The reaction was stopped by replacing the media with ice-cold 3% perchloric acid. Accumulated cAMP was measured using a

[^{3}H]cAMP liquid phase assay kit (Diagnostic Products Corp., Los Angeles, CA, USA) as previously described (Clark *et al*. 2003). Sensitization (overshoot) was determined as the percentage increase or as the increase in picomoles of cAMP per microgram of protein in forskolin-stimulated cAMP accumulation, over that in the absence of an 18 h DAMGO pre-treatment. Acute inhibition was determined as the percentage decrease in forskolin-stimulated cAMP accumulation compared with that in the absence of opioid agonist.

Data analysis

Data from each experiment were fitted to sigmoidal concentration-response curves, using GraphPad prism (San Diego, CA, USA), for calculation of mean maxima and EC_{50} values ± SEM from at least three separate experiments. For studies on transiently transfected cells, results are means ± SEM from at least three separate transfections. Values were compared using one-way ANOVA with Tukey's post hoc test or, when appropriate, by two-way ANOVA. Figure representations of the data are presented as means ± SEM from at least three separate experiments.

Results

PTX-insensitive $G\alpha_{i/o}$ subunits rescue DAMGO-mediated sensitization of AC in PTX treated cells

Forskolin at a concentration of 5 μM strongly activates AC in C6μ cells and allows observation of a robust overshoot of cAMP accumulation following chronic mu-opioid treatment (Clark *et al*. 2004). DAMGO caused a strong inhibition (81 ± 6%) of 5 μM forskolin-stimulated AC, with an EC_{50} value of 17 ± 9 nM, in C6μ cells expressing endogenous Gα subtypes (Fig. 1a). Treatment of C6μ cells for 18 h with DAMGO, followed by removal of agonist and addition of the opioid antagonist, naloxone, resulted in an increase in forskolin-stimulated cAMP accumulation of 226%, equivalent to an increase of 5.2 ± 0.5 pmol cAMP/μg protein (Fig. 1b). A similar percentage increase (206%) was seen when overshoot was measured in the absence of forskolin, although this only represented 0.11 pmol cAMP/mg protein or 2.1% of the forskolin-stimulated increase (see legend to Fig. 1). The EC_{50} for DAMGO to induce this sensitization was 6.8 ± 1.2 nM (Fig. 1b). Treatment of cells with 100 ng/mL PTX for 24 h before DAMGO completely eliminated both DAMGO-mediated acute inhibition and sensitization (Fig. 1), in agreement with our findings that this level of PTX uncouples endogenous Gαi/o proteins in these cells (Clark *et al*. 2006). Similarly, in HEK293T cells transiently transfected with the mu-opioid receptor, acute inhibition of forskolin-stimulated AC by DAMGO (50 ± 11%) was completely eliminated by treatment with 100 ng/mL PTX for 24 h (1 ± 3%), and sensitization following overnight treatment with DAMGO (14 ± 1%), although small in those cells transiently transfected with mu-opioid receptor alone, was also completely eliminated by PTX treatment.

Transient transfection with individual PTX-insensitive Cys-Ile mutant Gα subtypes ($G\alpha^{CI}$) rescued both acute inhibition of forskolin-stimulated cAMP accumulation by DAMGO and DAMGO-mediated sensitization following PTX treatment in C6μ cells (Fig. 2a). In HEK293T cells transiently co-transfected with the mu-opioid receptor together with the PTX-insensitive Gαi/o subunit proteins, $G\alpha^{CI}$ subtypes rescued both acute inhibition of forskolin-stimulated cAMP accumulation by DAMGO and DAMGO-mediated sensitization following PTX treatment (Fig. 2b).

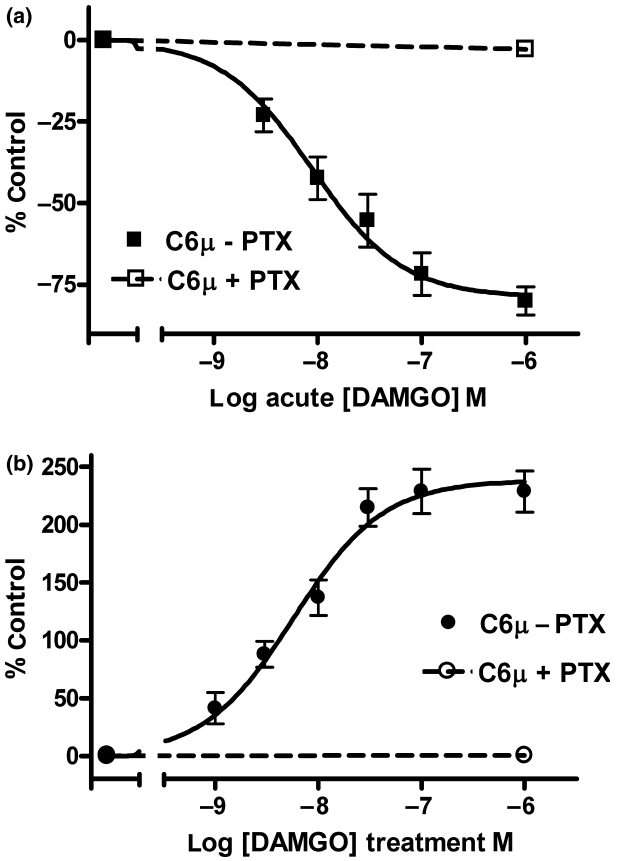

Fig. 1 Effects of acute and chronic DAMGO on forskolin-stimulated cAMP accumulation in C6μ cells. C6μ cells expressing endogenous Gα proteins were plated into 24-well plates and treated with or without PTX 24 h before the assay. (a) Acute inhibition by DAMGO. (b) Overshoot following an 18 h treatment with 0–10 μM DAMGO. To start the cAMP assay, medium was replaced with DMEM without FBS and containing 5 μM forskolin, 1 mM IBMX and 0–10 μM DAMGO (a), or 10 μM naloxone (b). After 10 min at 37°C, the assay was stopped and cAMP levels were measured as described in Materials and methods. Data are presented as percentage of control (without DAMGO) values. Shown are the combined data ± SEM from three experiments, each performed in duplicate. Forskolin-stimulated cAMP levels in DAMGO-naïve cells are given in Table 3. Maximum cAMP overshoot was 226 ± 21% and equivalent to an increase of 5.2 ± 0.5 pmol cAMP/μg protein (Table 2). Basal (in absence of forskolin stimulation) levels of cAMP accumulation in control and chronic DAMGO-treated C6μ cells were 0.054 ± 0.027 and 0.165 ± 0.031 pmol cAMP/μg protein, respectively ($n = 3$), representing an overshoot of 206%.

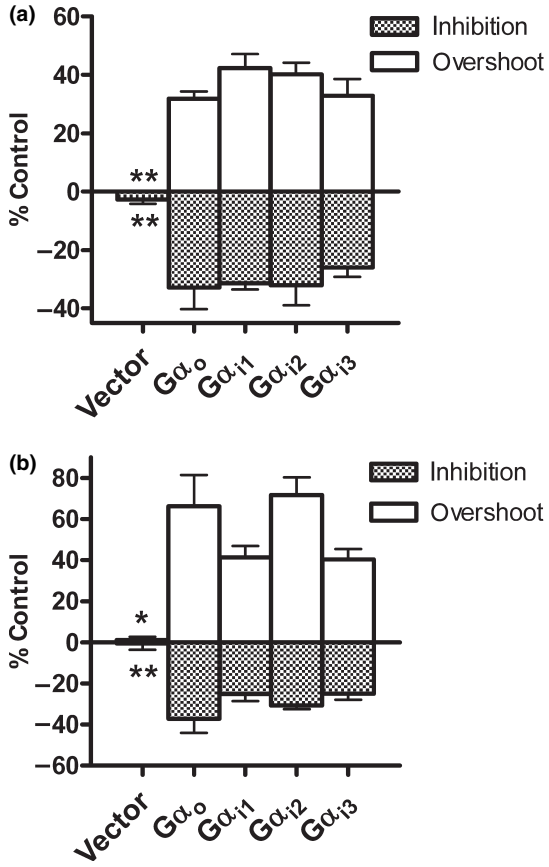

Fig. 2 Rescue of DAMGO-mediated inhibition and sensitization of adenylyl cyclase by PTX-insensitive $G\alpha^{CI}_{i/o}$ in PTX-treated cells. (a) C6μ cells transiently transfected with PTX-insensitive $G\alpha^{CI}_{i/o}$ or (b) HEK293T cells co-transfected with mu-opioid receptor and PTX-insensitive $G\alpha^{CI}_{i/o}$ were treated with 100 ng/mL PTX for 24 h. The mu-opioid inhibition (filled bars) of forskolin (5 μM)-stimulated cAMP accumulation was measured with 10 μM DAMGO. Overshoot (open bars) of forskolin-stimulated cAMP accumulation was measured with 10 μM naloxone following an 18 h treatment with 1 μM DAMGO. Data are presented as percentage of control (without DAMGO). Details are given in Materials and methods. Shown are the combined data from three separate experiments, each performed in duplicate; $^*p < 0.05$ or $^{**}p < 0.01$ compared with cells transfected with any $G\alpha_{i/o}$ subunit protein.

The ratio of the degree to which sensitization was rescued compared with the degree to which inhibition was rescued was similar for each $G\alpha^{CI}$ subtype (for HEK293T cells, this ranged from 1.6 ± 0.2 for $G\alpha_{i3}$ to 2.3 ± 0.4 for $G\alpha_{i2}$; for C6μ cells, the range was 1.1 ± 0.3 for $G\alpha_{oA}$ to 1.8 ± 0.5 for $G\alpha_{i1}$).

The PTX-insensitive mutant of $G\alpha_{i1}$ in which the Cys at residue 351 is replaced by Ile is very efficient at coupling to receptor compared with Cys itself, or compared with replacement of this Cys with Ser or Gly, when activated by α_{2A}-adrenergic agonists (Bahia *et al*. 1998). Similarly, the Cys-Ile mutant is activated by mu-opioid agonists at least six times more efficiently than the Cys-Gly mutant, as measured by stimulation of [^{35}S]GTPγS binding, for each of the Gα subtypes used in this study (data not shown). With the very efficiently coupled $G\alpha^{CI.}$ Subtypes, there is a concern that selectivity of coupling could be lost. To address this, stable clones of C6μ cells expressing the less efficiently coupled Cys-Gly mutant Gα subtypes ($G\alpha^{CG}$) were tested. DAMGO-mediated overshoot was rescued in PTX-treated cells, with $G\alpha^{CG}_{i1}$ (18 ± 11%, EC_{50} = 180 ± 60 nM), $G\alpha^{CG}_{i2}$ (26 ± 10%, EC_{50} = 22 ± 18 nM) and $G\alpha^{CG}_{oA}$ (31 ± 8%, EC_{50} = 55 ± 33 nM; Clark *et al*. 2004). Maximal acute inhibition by DAMGO was 44 ± 5% in PTX-treated $G\alpha^{CG}_{i2}$ cells and 29 ± 3% in PTX-treated $G\alpha^{CG}_{i1}$ cells.

DAMGO-mediated sensitization of AC is dependent on expression level of PTX-insensitive $G\alpha^{CI}_{oA}$ or $G\alpha^{CI}_{i2}$ in PTX-treated C6μ cells

The degree of DAMGO-mediated inhibition or sensitization of AC was similar with transient transfection of each of the PTX-insensitive $G\alpha^{CI}$ subtypes, but less than that seen in cells expressing only endogenous $G\alpha_{o/i}$ proteins. Consequently, it would appear that Gα expression level may be more important than Gα subtype in determining the degree of inhibition or sensitization of AC. If so, a single Gα subtype should be able to restore inhibition and sensitization levels to those seen in non-PTX-treated C6μ cells expressing only endogenous Gα proteins, without a need for the involvement of multiple Gα subtypes.

To test this, clones of C6μ cells stably expressing high or low levels of PTX-insensitive $G\alpha_{oA}$ or $G\alpha_{i2}$, as determined by western blot (Fig. 3), were prepared. As with their transiently transfected counterparts, there was rescue of PTX-sensitive AC inhibition and sensitization. Following PTX treatment, clones expressing the higher levels of PTX-insensitive $G\alpha_{o/i}$ had increased percentage inhibition of forskolin-stimulated cAMP accumulation by DAMGO, comparable with the levels seen in untreated C6μ cells expressing only endogenous Gα proteins, with no significant change in DAMGO potency (Table 1). In contrast, there was

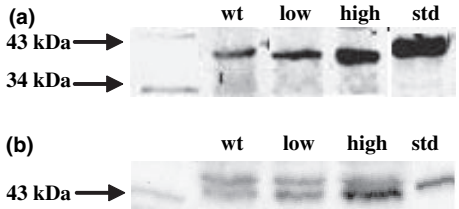

Fig. 3 Western blots of $G\alpha^{CI}_{oA}$ or $G\alpha^{CI}_{i2}$ stably expressed in C6μ cells. Membrane proteins (20 μg) from C6μ cells stably expressing (a) $G\alpha^{CI}_{oA}$ or (b) $G\alpha^{CI}_{i2}$ were separated by SDS–PAGE, transferred to a nitrocellulose membrane and probed with anti-$G\alpha_o$ or anti-$G\alpha_{i2}$, as described in Materials and methods. His^6-$G\alpha_o$ (Dr R. Neubig) and $G\alpha_{i2}$ (Santa Cruz Biotechnology) were used as standards. Shown is a representative blot from three experiments.

Table 1 Maximal inhibition of forskolin-stimulated cAMP accumulation by DAMGO is dependent on the expression levels of PTX-insensitive $G\alpha_{i/o}^{CI}$ in PTX-treated C6 μ cells

Expressed Gα protein	Maximum inhibition %	EC_{50} nM
Low $G\alpha_{oA}^{CI}$	45 ± 3%***	27 ± 14 nM
High $G\alpha_{oA}^{CI}$	92 ± 4%	14 ± 4 nM
Low $G\alpha_{i2}^{CI}$	43 ± 3%***	57 ± 18 nM
High $G\alpha_{i2}^{CI}$	83 ± 2%	8 ± 2 nM
Endogenous Gα – PTX	81 ± 6%	17 ± 9 nM
Endogenous Gα + PTX	3 ± 2%***	–

cAMP accumulation in the presence of 5 μM forskolin and 0–10 μM DAMGO was measured in C6μ cells expressing endogenous Gα proteins or in PTX-treated C6μ cells stably transfected with PTX-insensitive $G\alpha^{CI}$ as described in Materials and methods. Maximal inhibition and EC_{50} values were derived from concentration–response curves. Shown are the means ± SEM from three separate experiments, each performed in duplicate. ***$p < 0.001$, compared with cells expressing only endogenous Gαproteins not treated with PTX. Forskolin-stimulated cAMP levels in the absence of DAMGO treatment are given in Table 3.

no difference between the high and low expressing $G\alpha o^{CI}$ cells, and only a twofold difference between the $G\alpha_{i2}^{CI}$ in the percentage overshoot of cAMP induced by chronic DAMGO treatment (Figs 4a and c). Surprisingly, if overshoot was expressed as the difference in picomoles of cAMP per microgram of protein between DAMGO-treated and -untreated cells, there was a 4-fold or 11-fold increase in cells expressing the higher levels of PTX-insensitive $G\alpha_{oA}^{CI}$ or $G\alpha_{i2}^{CI}$, respectively, with no change in the potency of DAMGO (Figs 4b and d). This discrepancy would appear to be due to different levels of forskolin-stimulated cAMP accumulation in the DAMGO-naïve cells (Figs 4b and d) and is addressed below. The levels of sensitization in the PTX-treated $G\alpha_{oA}^{CI}$- or $G\alpha_{i2}^{CI}$-expressing cells were lower than those seen in C6μ cells expressing endogenous Gα proteins, measured as percentage increase or picomoles per microgram of protein (Fig. 4).

Combined effects of exogenous PTX-insensitive Gα and endogenous Gα for mu-opioid-mediated sensitization or inhibition of forskolin-stimulated cAMP accumulation

One reason for incomplete recovery of sensitization by expression of $G\alpha_{oA}^{CI}$ or $G\alpha_{i2}^{CI}$ to levels seen in C6μ cells expressing only endogenous Gα proteins could be competition between the exogenous mutated $G\alpha_{i/o}$ proteins and endogenous $G\alpha_{i/o}$ proteins. To determine whether the effects of the exogenously-expressed PTX-insensitive Gα subunits were additive or competitive with the endogenously-expressed Gα, inhibition and sensitization of cAMP accumulation were examined without PTX treatment. DAMGO inhibition of forskolin-stimulated cAMP accumulation in $G\alpha_{oA}^{CI}$- or $G\alpha_{i2}^{CI}$-expressing C6μ cells was not significantly different from that in the wild-type cells ($p = 0.13$), although the higher expressing clones tended to have greater inhibition (Table 2). There was no difference between any of the clones in the potency of DAMGO to inhibit forskolin-stimulated AC. DAMGO-mediated sensitization in the $G\alpha_{oA}^{CI}$- or $G\alpha_{i2}^{CI}$-expressing cells was also similar to that of cells expressing endogenous Gα proteins (Table 2), with no change in potency. Sensitization in the absence of PTX treatment was not significantly different than that in C6μ cells expressing endogenous Gα proteins in any of the clones, except low $G\alpha_{oA}^{CI}$, but only if expressed as percentage overshoot rather

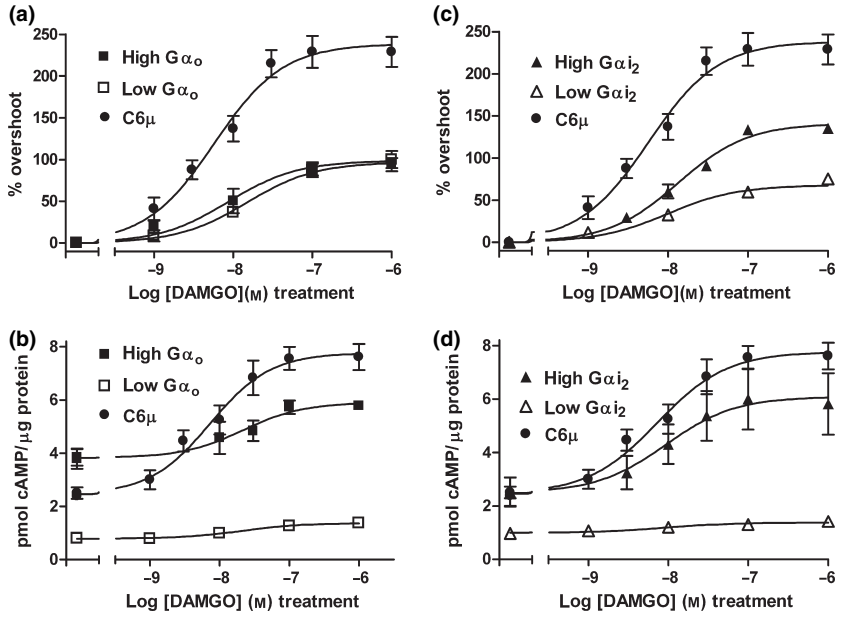

Fig. 4 DAMGO-induced cAMP overshoot in C6μ cells expressing different amounts of PTX-insensitive $G\alpha_o$ or $G\alpha_{i2}$. C6μ cells expressing low or high levels of $G\alpha_{oA}^{CI}$ or $G\alpha_{i2}^{CI}$ (Fig. 3), or only endogenous Gα proteins (C6μ), were plated into 24-well plates then treated with PTX for 24 h followed by 0–10 μM DAMGO for 18 h. To start the assay, media were replaced with DMEM without FBS and containing 5 μM forskolin, 1 mM IBMX and 10 μM naloxone. After 10 min at 37°C, the assay was stopped and cAMP levels were measured as described in Materials and methods. Data are presented as percentage of control (non-DAMGO-treated cells) (a and c) or as pmol cAMP/μg protein (b and d). Shown are the combined data ± SEM from three experiments, each performed in duplicate.

Table 2 DAMGO-mediated inhibition and overshoot of forskolin-stimulated cAMP accumulation without PTX treatment in C6μ cells expressing PTX-insensitive $G\alpha_{i/o}^{CI}$

	Acute inhibition		Overshoot		
Expressed Gα protein	Maximum %	EC_{50} nM	Δ pmol cAMP/ μg protein	%	EC_{50} nM
Low $G\alpha_{oA}^{CI}$ – PTX	84 ± 2	9 ± 1	6.5 ± 0.9	365 ± 46*	12 ± 3
High $G\alpha_{oA}^{CI}$ – PTX	99 ± 1	15 ± 3	4.7 ± 1.0	160 ± 14	5 ± 1
Low $G\alpha_{i2}^{CI}$ – PTX	89 ± 2	18 ± 6	5.3 ± 1.0	169 ± 11	7 ± 1
High $G\alpha_{i2}^{CI}$ – PTX	97 ± 1	20 ± 1	5.6 ± 0.5	173 ± 35	8 ± 2
Endogenous Gα – PTX	81 ± 6	17 ± 9	5.2 ± 0.5	226 ± 21	7 ± 1

In the absence of PTX treatment, inhibition and overshoot of 5 μM forskolin-stimulated cAMP accumulation by DAMGO (0–10 μM) was measured in C6μ cells stably expressing PTX-insensitive $G\alpha^{CI}$ or in C6μ cells only expressing endogenous Gα proteins as described in Materials and methods. Maximal responses and EC_{50} values were derived from dose–response curves. Shown are the means ± SEM from three separate experiments, each performed in duplicate; $^*p < 0.05$ compared with C6μ cells expressing only endogenous Gα proteins in the absence of PTX treatment. Forskolin-stimulated cAMP levels in non-DAMGO treated cells are given in Table 3.

than the increase in picomoles of cAMP per microgram of protein, again due to differences in forskolin-stimulated cAMP accumulation in untreated cells (Table 3). These results demonstrate that the exogenously-expressed, PTX-insensitive Gα subunits and the endogenous Gα do not interfere with each other functionally.

Forskolin-stimulated cAMP accumulation is dependent on the level of functional Gα

The discrepancy in changes in sensitization with Gα expression between percentage over untreated values and values expressed as an increase in picomoles of cAMP per microgram of protein (Fig. 4) can be explained by a difference in the degree of forskolin-stimulated cAMP accumulation in the absence of DAMGO treatment. In PTX-treated cells expressing higher levels of $G\alpha_{oA}^{CI}$ or $G\alpha_{i2}^{CI}$, forskolin-stimulated cAMP accumulation was significantly greater than in cells expressing lower levels of the Gα proteins, or in PTX-treated wild-type cells. In the absence of PTX treatment, forskolin-stimulated cAMP accumulation was higher than in the PTX-treated cells in each of the clones tested (Table 3). This was significant for each of the clones, except high $G\alpha_{oA}^{CI}$. These findings indicate that increasing levels of functional $G\alpha_{i/o}$ increased forskolin-stimulated cAMP accumulation. This was unexpected because intuitively, increased expression of inhibitory Gα subunits should decrease forskolin-stimulated cAMP accumulation. A possible explanation for these results is that there is a tonic level of sensitization, independent of the DAMGO-mediated sensitization, which is exposed during the assay. This most likely occurs with the replacement of the maintenance medium, containing FBS, with medium without FBS to start the cAMP accumulation assay. Therefore, the sensitizing agonist would be a component of FBS or a compound that is released by the cells.

Table 3 Forskolin-stimulated cAMP accumulation is dependent on the level of functional $G\alpha_{i/o}$

	Forskolin-stimulated cAMP (pmol/μg protein)	
Expressed Gα protein	+ PTX	– PTX
Low $G\alpha_{oA}^{CI}$	0.8 ± 0.1	1.8 ± 0.1#
High $G\alpha_{oA}^{CI}$	3.7 ± 0.3*	4.2 ± 0.5
Low $G\alpha_{i2}^{CI}$	0.9 ± 0.1	3.0 ± 0.9#
High $G\alpha_{i2}^{CI}$	2.2 ± 0.3*	4.2 ± 0.7#
Endogenous Gα[a]	0.8 ± 0.2	2.4 ± 0.2#

Forskolin (5 μM)-stimulated cAMP accumulation in C6μ cells stably expressing PTX-insensitive $G\alpha^{CI}$ or C6μ cells expressing only endogenous Gα proteins with or without PTX treatment as described in Materials and methods. Shown are the means ± SEM from at least three separate experiments, each performed in duplicate; $^*p < 0.05$ compared with C6μ cells expressing only endogenous Gα proteins with PTX treatment; $^\#p < 0.05$ compared with same clone with PTX treatment. [a]Basal values in the absence of forskolin were 0.022 ± 0.004 pmol/μg protein in the presence of PTX treatment and 0.027 ± 0.01 pmol/μg protein in the absence of PTX treatment.

To determine whether a component of FBS does indeed cause sensitization, forskolin-stimulated cAMP accumulation was measured in C6μ cells, expressing only endogenous Gα proteins, immediately after removal of FBS. In these cells, there was a large increase (0.95 pmol/μg protein equivalent to an overshoot of 100%) in the level of forskolin-stimulated cAMP accumulation compared with cells maintained in the presence of FBS, indicating a sensitization of AC by the FBS (Fig. 5a). However, when forskolin-stimulated cAMP accumulation was measured after the cells had been treated with serum-free FBS for 30 min, the increase in forskolin-stimulated cAMP accumulation was no longer evident. This increased level of forskolin-stimulated cAMP accumulation immediately following removal of FBS was also seen in cells

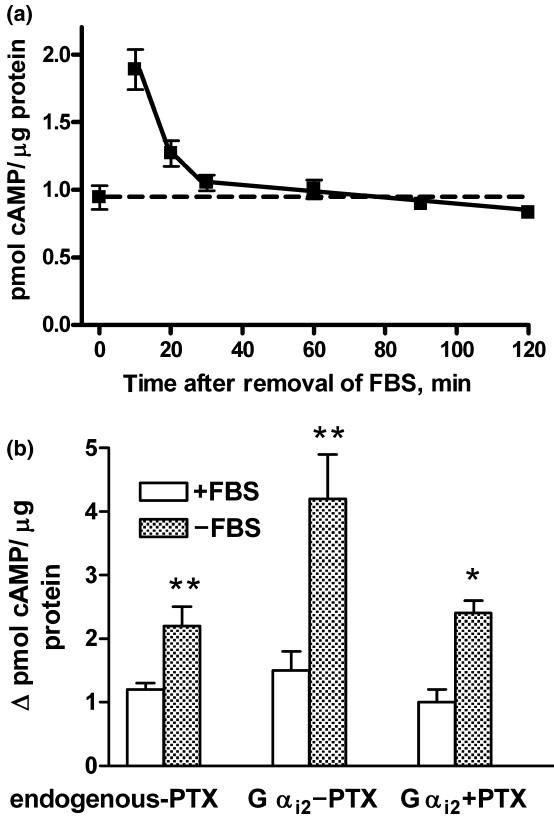

Fig. 5 Effect of removal of FBS on forskolin-stimulated cAMP in naïve C6μ cells. Cell media (DMEM with 10% FBS) were replaced with serum-free DMEM for varying times, before (a) or simultaneously with (b) stimulation of cAMP with forskolin, in C6μ cells expressing endogenous Gα proteins (a and b) and stably expressing PTX-insensitive $G\alpha_{i2}$, with or without PTX-treatment (b). Accumulated cAMP was determined 10 min after addition of 5 μM forskolin and 1 mM IBMX, as described in Materials and methods. Shown are the combined data from three separate experiments, each performed in duplicate. In (a) the value at time zero and dashed line represent the level of forskolin-stimulated cAMP production when serum was not removed from the media; $^{**}p < 0.005$, $^{*}p < 0.05$ vs. with FBS.

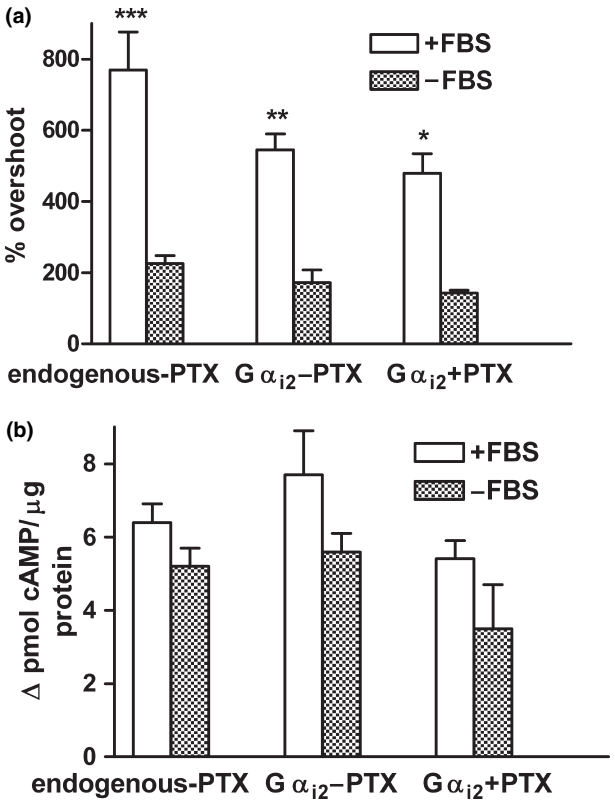

Fig. 6 Effect of FBS on cAMP overshoot in chronic DAMGO-treated C6μ cells. Cells expressing only endogenous Gα proteins or PTX-insensitive $G\alpha_{i2}$ proteins (higher expressing cells shown in Fig. 3) were grown in DMEM with 10% FBS, then treated for 24 h with or without PTX followed by 18 h in the presence of 1 μM DAMGO. Media were then replaced with DMEM in the presence or absence of 10% FBS and 10 μM naloxone to induce overshoot. cAMP levels were assayed after addition of 5 μM forskolin and 1 mM IBMX as described in Materials and methods. Data are expressed as percentage increase in cAMP in DAMGO-treated cells over untreated cells (a), or as the increase in pmol cAMP/μg protein over untreated cells (b). Shown are the mean ± SEM from three separate experiments, each performed in duplicate. The levels of forskolin-stimulated cAMP in naïve cells (pmol/μg protein) are shown in Fig. 5(b); $^{***}p < 0.001$, $^{**}p < 0.005$, $^{*}p < 0.05$ compared with absence of FBS. There are no significant differences between the presence and absence of FBS in Δ pmol cAMP/μg protein (b).

expressing PTX-insensitive $G\alpha_{i2}$, with or without PTX treatment (Fig. 5b). There was also an increase in endogenous cAMP levels in C6μ cells (in the absence of forskolin) on removal of FBS, although in absolute terms this was very small compared with the overshoot in the presence of forskolin. Thus, C6μ cells in the presence of FBS contained a basal level of 0.012 ± 0.003 pmol/cAMP/μg protein, which was increased to 0.027 ± 0.011 pmol cAMP/μg protein when assayed immediately after removal of the FBS, representing an overshoot of 125%. This effect was lost on PTX treatment of the cells (with FBS = 0.022 ± 0.005 pmol cAMP/μg protein; without FBS = 0.022 ± 0.004 pmol cAMP/μg protein). Addition of naloxone in the presence of FBS had no effect on forskolin-stimulated cAMP accumulation levels in any of the clones tested (data not shown), indicating that if tonic sensitization was caused by a component of FBS this did not involve the mu-opioid receptor.

Forskolin-stimulated cAMP accumulation following DAMGO treatment was measured in the continued presence of FBS to examine whether a larger DAMGO-mediated overshoot could be revealed by eliminating the FBS-mediated overshoot. The level of DAMGO-mediated overshoot was increased significantly in the presence of FBS in cells expressing endogenous Gα proteins, or heterologously expressing $G\alpha_{i2}$ proteins, if measured as percentage overshoot (Fig. 6a). In contrast, cAMP overshoot was increased

Table 4 Abilities of PTX-insensitive Gα subunits to rescue adenylyl cyclase sensitization following PTX treatment

PTX-insensitive Gα protein and mutation	Receptor	Cell type	Rescue	Reference
$G\alpha_o$				
CI	mu	C6	Yes	This paper
CG	mu	C6	Yes	Clark *et al.* (2004)
CG	D_{2L}	NS20Y	Yes	Watts *et al.* (1998)
$G\alpha_{i1}$				
CI	mu	C6	Yes	This paper
CI	mu	HEK293	Yes	This paper
CG	D_{2L}	NS20Y	No	Watts *et al.* (1998)
	kappa	HEK293	No	Tso and Wong (2001)
	mu	HEK293	No	Tso and Wong (2001)
	delta	HEK293	Yes	Tso and Wong (2001)
$G\alpha_{i2}$				
CI	mu	C6	Yes	This paper
CI	mu	HEK293	Yes	This paper
CG	D_{2L}	NS20Y	No	Watts *et al.* (1998)
$G\alpha_{i2}/G\alpha_z$ chimera	mu	HEK293	No	Tso and Wong (2000)
	delta	HEK293	No	Tso and Wong (2000)
	kappa	HEK293	No	Tso and Wong (2000)
$G\alpha_{i2}/G_q$ chimera	kappa	CHO	Yes	Nakagawa *et al.* (1999)
$G\alpha_{i3}$				
CI	mu	C6	Yes	This paper
CI	mu	HEK293	Yes	This paper
CG	mu	HEK293	No	Tso and Wong (2001)
	kappa	HEK293	No	Tso and Wong (2001)
	D_{2L}	NS20Y	No	Watts *et al.* (1998)
$G\alpha_{i2}$ + $G\alpha_{i3}$				
Wild-type	mu with ACVI	COS-7	Yes	Ammer and Christ (2002)
	mu with ACV	COS-7	Yes	Ammer and Christ (2002)
$G\alpha_z$				
Wild-type	mu with ACV	COS-7 HEK293	Yes	Ammer and Christ (2002)
Wild-type	mu with ACVI	COS-7 HEK293	No	Ammer and Christ (2002)

only slightly (and not significantly) in the presence of FBS when measured as change in picomoles of cAMP per microgram of protein, and did not reach significance (Fig. 6b), showing that the AC sensitization by DAMGO is not inherently altered by FBS, or that there is a ceiling to the degree of sensitization that can be achieved. Maximal acute inhibition of AC by 1 μM DAMGO was not significantly affected by the presence of FBS. In C6μ cells, this was 89 ± 2% and in high expressing $G\alpha_{i2}^{CI}$ cells, it was 94 ± 1% or, after PTX-treatment, 78 ± 2%.

Discussion

PTX-insensitive mutations of the inhibitory Gα subtypes were expressed in C6 glioma cells stably expressing the mu-opioid receptor to determine whether $G\alpha_{oA}$, $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$ are all able to rescue the chronic mu-opioid-mediated sensitization of adenylyl cyclase (AC) lost on treatment with PTX. Although we cannot directly compare expression levels of the different $G\alpha^{CI}$ subtypes based on the ratio between the level of rescue of sensitization and inhibition of AC, there would appear to be no significant differences between the $G\alpha^{CI}$ subtypes in mediating inhibition or sensitization of AC in either C6μ or HEK293 cells.

Results from previous studies that examined the abilities of PTX-insensitive Gα proteins to rescue sensitization of AC have been conflicting (Table 4). One explanation for some of these divergent findings could be that different AC isoforms are expressed in the various cell lines, and that there is Gα selectivity for mediation of sensitization. Indeed, $G\alpha_z$ is able to mediate sensitization with overexpression of ACV, but not ACVI, in COS-7 or HEK293 cells expressing the mu-opioid receptor (Ammer and Christ 2002). C6 cells predominately express ACVI (Debernardi *et al.* 1993). However, differential AC isoform requirements do not explain the discrepancy between our findings with $G\alpha^{CI}$ mutants in HEK293 cells and those of Tso and Wong (2000) with the $G\alpha^{CG}$ mutants in the same cell type. These conflicting results may be explained by the use of different PTX-insensitive mutations that are quantitatively differently activated by agonist-

occupied receptor. The Cys-Ile PTX-insensitive mutant is at least six times more efficiently activated than the Cys-Gly mutant in DAMGO-mediated stimulation of [^{35}S]GTPγS binding for each of the subtypes, and in α_{2A}-adrenoceptor-mediated stimulation of [^{35}S]GTPγS binding with $G\alpha_{i1}$ (Bahia *et al*. 1998). Although it is possible that the high efficiency of the Cys-Ile mutation has masked any selectivity there may be in the ability of the $G\alpha_{i/o}$ subtypes to mediate sensitization of AC in both the stable and transiently transfected cells, we have been able to measure low levels of DAMGO-mediated sensitization with stably expressed $G\alpha_{i1}^{CG}$ or $G\alpha_{i2}^{CG}$ (this study), or $G\alpha_{oA}^{CG}$ (Clark *et al*. 2004), to confirm that this is not the case.

The mu-opioid sensitization of AC was routinely measured as the forskolin-stimulated activity due to the low basal levels of cAMP. Sensitization was seen in the absence of forskolin, but this represented no more then 2% of the level seen in the presence of forskolin. Sensitization expressed as the difference in picomoles of cAMP per microgram of protein between control and DAMGO-treated cells was greater with increased expression levels of $G\alpha_{oA}^{CI}$ or $G\alpha_{i2}^{CI}$. Higher levels of mu-opioid receptor also increase sensitization levels (Moulédous *et al*. 2005). Maximal levels of sensitization were not significantly increased with the combination of endogenous and exogenous Gα as measured in the absence of PTX treatment compared with sensitization with endogenous Gα alone. This suggests that sensitization in the C6μ cells expressing only endogenous Gα proteins was near the maximum possible effect under those particular assay conditions. Similarly, maximal acute DAMGO-mediated inhibition of forskolin-stimulated cAMP accumulation with the combination of endogenous and exogenous Gα as measured in the absence of PTX treatment tended to be higher than with endogenous Gα alone, but because this was close to the maximum possible effect, the increase was not statistically significant. Together, these data demonstrate that there was no competition between the exogenous and endogenous Gα. In further support of this lack of competition, the expression level of $G\alpha_{oA}^{CI}$ or $G\alpha_{i2}^{CI}$ had no significant effect on the potency of DAMGO to inhibit AC or to mediate sensitization in non-PTX-treated cells.

Surprisingly, maximal DAMGO-mediated sensitization expressed as percentage cAMP overshoot did not reflect maximal sensitization expressed as the increase in picomoles of cAMP per microgram of protein. The cause of this discrepancy proved to be a difference in the level of forskolin-stimulated cAMP accumulation in the naïve cells, which therefore altered the derived percentage values but not the absolute picomoles of cAMP per microgram of protein values. The forskolin-stimulated cAMP accumulation levels in naïve cells were consistently higher in the high-expressing clones and also higher in the absence of PTX treatment, indicating a positive correlation with the level of functional $G\alpha_{i/o}$. It might have been predicted that an increased level of inhibitory $G\alpha_{i/o}$ proteins would reduce forskolin-stimulated cAMP accumulation, similar to the decrease in isoprenaline- or GTP-stimulated adenylyl cyclase activity in membranes from cardiac myocytes overexpressing $G\alpha_{i2}$ (Rau *et al*. 2003). Our results can be explained by an increase in AC sensitization with increased levels of functional $G\alpha_{i/o}$, caused by chronic activation of the inhibitory Gα. An overshoot was revealed, even in the absence of DAMGO, by removal of FBS, suggesting activation of an endogenous $G\alpha_{i/o}$-coupled receptor by a component of FBS. Preventing this serum-mediated overshoot by retaining FBS in the medium during the assay decreased forskolin-stimulated cAMP accumulation and revealed a large increase in the percentage DAMGO-mediated overshoot. However, sensitization expressed as picomoles of cAMP per microgram of protein was not significantly higher with FBS in the assay than without, indicating that the intrinsic sensitization due to DAMGO was not altered. Alternatively there could be a ceiling effect in the level of cAMP overshoot in these cells.

FBS contains several components that could potentially activate $G\alpha_{i/o}$-coupled cell-surface receptors to induce sensitization of AC. $G\alpha_{i/o}$-coupled receptors expressed in C6 cells are known to include $P2Y_{12}$ purinergic receptors (Valeins *et al*. 1992), metabotropic glutamate receptors (Albasanz *et al*. 1997), cannabinoid receptors (Sánchez *et al*. 2001), 5-hydroxytryptamine 2A receptors (Elliott *et al*. 1995) and lysophosphatidic acid A1 receptors (Cechin *et al*. 2005). In addition, FBS contains growth factors, and C6 cells express receptors for fibroblast growth factor (FGF), insulin-like growth factor (IGF-1) and platelet-derived growth factor (PDGF), in addition to vascular endothelial growth factor receptors; they also secrete these growth factors (Okumura *et al*. 1989; Chernausek 1993; Hamel and Westphal 2000). Growth factors could activate receptor tyrosine kinases (RTKs) to affect signaling and therefore, sensitization, via mu-opioid receptor activation. Mu-opioid receptor-mediated, extracellular signal-regulated kinase (ERK) phosphorylation was seen to be partially dependent on epidermal growth factor (EGF) receptor transactivation in HEK293 cells (Della Rocca *et al*. 1997; Belcheva *et al*. 2001), and in C6 cells, mu-opioid agonist-stimulated ERK phosphorylation was partially reversed by inhibition of FGF receptor tyrosine phosphorylation (Belcheva *et al*. 2002). The mu-opioid receptor–RTK interactions, and their convergence in the ERK pathway, may be particularly important for sensitization of AC. Thus, mu- and delta-opioid-mediated sensitization of AC in Chinese hamster ovary (CHO) cells is reduced by inhibition of Raf-1, an intermediate in the ERK signaling pathway (Varga *et al*. 2002; Yue *et al*. 2006). The delta-opioid-mediated sensitization has been shown to be sensitive to inhibition of both RTKs and PKC (Varga *et al*. 2003), and both PKC and EGF enhance ACVI activity in a Raf-1-dependent manner (Beazely *et al*. 2005). In support of this, RTK activation by IGF-1 stimulated phosphorylation of

ACVI, and this was reduced by an inhibitor of Raf-1 kinase (Tan *et al*. 2001).

In summary, the effect of FBS on mu-opioid-mediated sensitization demonstrates that the overall level of sensitization of AC can be determined by the combined effects of multiple receptors, possibly by a convergence of separate pathways. FBS- and mu-opioid-mediated sensitization of AC were both affected by the expression level of functional $G\alpha_{i/o}$, and each of the inhibitory Gα subtypes $G\alpha_{oA}^{CI}$, $G\alpha_{i1}^{CI}$, $G\alpha_{i2}^{CI}$ or $G\alpha_{i3}^{CI}$ was able to mediate mu-agonist-mediated sensitization. Together, these results suggest that receptor and G protein stoichiometry may be more important than Gα subtype in determining the parameters of acute and chronic effects of mu-opioids on adenylyl cyclase.

Acknowledgements

This work was supported by National Institutes of Health grant DA04087. We thank Dr Huda Akil for the mu-opioid receptor DNA.

References

Albasanz J. L., Ros M. and Martın M. (1997) Characterization of metabotropic glutamate receptors in rat C6 glioma cells. *Eur. J. Pharmacol.* **326**, 85–91.

Ammer H. and Christ T. E. (2002) Identity of adenylyl cyclase isoform determines the G protein mediating chronic opioid-induced adenylyl cyclase supersensitivity. *J. Neurochem.* **83**, 818–827.

Avidor-Reiss T., Nevo I., Levy R., Pfeuffer T. and Vogel Z. (1996) Chronic opioid treatment induces adenylyl cyclase V superactivation. *J. Biol. Chem.* **271**, 21 309–21 315.

Avidor-Reiss T., Nevo I., Saya D., Bayewitch M. and Vogel Z. (1997) Opiate-induced adenylyl cyclase superactivation is isozyme-specific. *J. Biol. Chem.* **272**, 5040–5047.

Bahia D. S., Wise A., Faelli F., Lee M., Rees S. and Milligan G. (1998) Hydrophobicity of residue^{351} of the G protein $G_{i1}\alpha$ determines the extent of activation by the α_{2A}-adrenoceptor. *Biochemistry* **37**, 11 555–11 562.

Beazely M. A. and Watts V. J. (2005) Activation of a novel PKC isoform synergistically enhances D_{2L} dopamine receptor-mediated sensitization of adenylate cyclase type 6. *Cell. Signalling* **17**, 647–653.

Beazely M. A., Alan J. K. and Watts V. J. (2005) Protein kinase C and epidermal growth factor stimulation of raf-1 potentiates adenylyl cyclase type 6 activation in intact cells. *Mol. Pharmacol.* **67**, 250–259.

Belcheva M. M., Szùcs M., Wang D., Sadee W. and Coscia C. J. (2001) μ-Opioid receptor-mediated ERK activation involves calmodulin-dependent epidermal growth factor receptor transactivation. *J. Biol. Chem.* **276**, 33 847–33 853.

Belcheva M. M., Haas P. D., Tan Y., Heaton V. M. and Coscia C. J. (2002) The fibroblast growth factor receptor is at the site of convergence between μ-opioid receptor and growth factor signaling pathways in rat C6 glioma cells. *J. Pharmacol. Exp. Ther.* **303**, 909–918.

Bohn L. M., Galnetdinov R. R., Lin F.-T., Lefkowitz R. J. and Caron M. G. (2000) μ-Opioid receptor desensitization by β-arrestin-2 determines morphine tolerance but not dependence. *Nature* **408**, 720–723.

Cechin S. R., Dunkley P. R. and Rodnight R. (2005) Signal transduction mechanism involved in the proliferation of C6 glioma cells induced by lysophosphatic acid. *Neurochem. Res.* **30**, 603–611.

Chernausek S. D. (1993) Insulin-like growth factor-1 (IGF-1) production by astroglial cells: regulation and importance for epidermal growth factor-induced cell replication. *J. Neurosci. Res.* **34**, 189–197.

Clark M. J., Harrison C., Zhong H., Neubig R. R. and Traynor J. R. (2003) Endogenous RGS protein action modulates μ-opioid signaling through $G\alpha_o$. Effects of adenylyl cyclase, extracellular signal-regulated kinases, and intracellular calcium pathways. *J. Biol. Chem.* **278**, 9418–9425.

Clark M. J., Neubig R. R. and Traynor J. R. (2004) Endogenous regulator of G protein signaling proteins suppress $G\alpha_o$-dependent, μ-opioid agonist-mediated adenylyl cyclase supersensitization. *J. Pharmacol. Exp. Ther.* **310**, 215–222.

Clark M. J., Furman C. A., Gilson T. D. and Traynor J. R. (2006) Comparison of the relative efficacy and potency of μ-opioid agonists to activate Gαi/o proteins containing a pertussis toxin-insensitive mutation. *J. Pharmacol. Exp. Ther.* **317**, 858–864.

Cumbay M. G. and Watts V. J. (2001) Heterologous sensitization of recombinant adenylate cyclases by activation of D_2 dopamine receptors. *J. Pharmacol. Exp. Ther.* **297**, 1201–1209.

Debernardi M. A., Munshi R., Yoshimura M., Cooper D. M. F. and Brooker G. (1993) Predominant expression of type-VI adenylate cyclase in C6–2B rat glioma cells may account for inhibition of cyclic AMP accumulation by calcium. *Biochem. J.* **293**, 325–328.

Della Rocca G. J., van Biesen T., Daaka Y., Luttrell D. K., Luttrell L. M. and Lefkowitz R. J. (1997) Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors. *J. Biol. Chem.* **272**, 19 125–19 132.

Elliott J. M., Newberry N. R., Cholewinski A. J. *et al.* (1995) Characterization of the 5-hydroxytryptamine$_{2A}$ receptor-activated cascade in rat C6 glioma cells. *Neuroscience* **69**, 1119–1131.

Gerhardt M. A. and Neubig R. R. (1991) Multiple Gi protein subtypes regulate a single effector mechanism. *Mol. Pharmacol.* **40**, 707–711.

Ghahremani M. H., Cheng P., Lembo P. M. and Albert P. R. (1999) Distinct roles for Gαi2, Gαi3, and Gβγ in modulation of forskolin- or Gs-mediated cAMP accumulation and calcium mobilization by dopamine D2S receptors. *J. Biol. Chem.* **274**, 9238–9245.

Hamel W. and Westphal M. (2000) Growth factors in gliomas revisited. *Acta Neurochir.* **142**, 113–137.

Johnston C. A., Beazely M. A., Vancura A. F., Wang J. K. T. and Watts V. J. (2002) Heterologous sensitization of adenylate cyclase is protein kinase A-dependent in Cath.a differentiated (CAD)-D_{2L} cells. *J. Neurochem.* **82**, 1087–1096.

Lee K. O., Akil H., Woods J. H. and Traynor J. R. (1999) Differential binding properties of oripavines at cloned mu- and delta-opioid receptors. *Eur. J. Pharmacol.* **378**, 323–330.

Moulédous L., Neasta J., Uttenweiler-Joseph S., Stells A., Matondo M., Corbani M., Monsarrat B. and Meunier J.-C. (2005) Long-term morphine treatment enhances proteasome-dependent degradation of Gβ in human neuroblastoma SH-SY5Y cells: correlation with onset of adenylyl cyclase sensitization. *Mol. Pharmacol.* **68**, 467–476.

Nakagawa T., Ozawa T., Watanabe T., Minami M. and Satoh M. (1999) Sensitization of the adenylyl cyclase system in cloned κ-opioid receptor-transfected cells following sustained agonist treatment: a chimeric study using G protein α_{i2}/α_q subinits. *Jpn J. Pharmacol.* **81**, 353–361.

Näsman J., Kukkonen J. P., Holmqvist T. and Åkerman K. E. O. (2002) Different roles for G_i and G_o proteins in modulation of adenylyl cyclase type-2 activity. *J. Neurochem.* **83**, 1252–1261.

Nevo I., Avidor-Reiss T., Levy R., Bayewitch M., Heldman E. and Vogel Z. (1998) Regulation of adenylyl cyclases isozymes on acute and chronic activation of inhibitory receptors. *Mol. Pharmacol.* **54**, 419–426.

Nevo I., Avidor-Reiss T., Levy R. I., Bayewitch M. and Vogel Z. (2000) Acute and chronic activation of the μ-opioid receptor with the endogenous ligand endomorphin differentially regulates adenylyl cylcase isozymes. *Neuropharmacology* **39**, 364–371.

Okumura N., Takimoto K., Okada M. and Nakagawa H. (1989) C6 glioma cells produce basic fibroblast growth factor that can stimulate their own proliferation. *J. Biochem.* **106**, 904–909.

Rau T., Nose M., Remmers U., Weil J., Weissmüller A., Davia K., Harding S., Peppel K., Koch W. J. and Eschenhagen T. (2003) Overexpression of wild-type Gαi-2 suppresses β-adrenergic signaling in cardiac myocytes. *FASEB J.* **17**, 523–525.

Rhee M.-H., Nevo I., Avidor-Reiss T., Levy R. and Vogel Z. (2000) Differential superactivation of adenylyl cyclase isozymes after chronic activation of the CB1 cannabinoid receptor. *Mol. Pharmacol.* **57**, 746–752.

Rubenzik M., Varga E., Stropova D., Roeske W. R. and Yamamura H. I. (2001) Expression of α-transducin in Chinese Hamster Ovary cells stably transfected with the human δ-opioid receptor attenuates chronic opioid agonist-induced adenylyl cyclase superactivation. *Mol. Pharmacol.* **60**, 1076–1082.

Sánchez C., de Ceballos M. L., del Pulgar T. G., Rueda D., Corbacho C., Velasco G., Galve-Roperh I., Huffman J. W., Cajal S. R. and Guzmán M. (2001) Inhibition of glioma growth *in vivo* by selective activation of the CB_2 cannabinoid receptor. *Cancer Res.* **61**, 5784–5789.

Sharma S. K., Klee W. A. and Nirenberg M. (1975) Dual regulation of adenylyl cyclase accounts for narcotic dependence and tolerance. *Proc. Natl Acad. Sci. USA* **72**, 3092–3096.

Tan C. M., Kelvin D. J., Litchfield D. W., Ferguson S. S. G. and Feldman R. D. (2001) Tyrosine kinase-mediated serine phosphorylation of adenylyl cyclase. *Biochemistry* **40**, 1702–1709.

Tso P. H. and Wong Y. H. (2000) Deciphering the role of G_{i2} in opioid-induced adenylyl cyclase supersensitization. *Mol. Neurosci.* **11**, 3213–3217.

Tso P. H. and Wong Y. H. (2001) Opioid-induced adenylyl cyclase supersensitization in human embryonic kidney 293 cells requires pertussis toxin-sensitive G proteins other than G_{i1} and G_{i3}. *Neurosci. Lett.* **299**, 25–28.

Valeins H., Merle M. and Labouesse J. (1992) Pre-steady state study of β-adrenergic and purinergic receptor interaction in C6 cell membranes: undelayed balance between positive and negative coupling to adenylyl cyclase. *Mol. Pharmacol.* **42**, 1033–1041.

Varga E. V., Stropova D., Rubenzik M., Waite S., Roeske W. R. and Yamamura H. I. (1999) Phosphorylation of adenylyl cyclase VI upon chronic δ-opioid receptor stimulation. *Eur. J. Pharmacol.* **364**, R1–R2.

Varga E. V., Rubenzik M. K., Grife V., Sugiyama M., Stropova D., Roeske W. R. and Yamamura H. I. (2002) Involvement of Raf-1 in chronic delta-opioid receptor agonist-mediated adneylyl cyclase superactivation. *Eur. J. Pharmacol.* **451**, 101–103.

Varga E. V., Rubenzik M. K., Stropova D., Sugiyama M., Grife V., Hruby V. J., Rice K. C., Roeske W. R. and Yamamura H. I. (2003) Converging protein kinase pathways mediate adenylyl cyclase superactivation upon chronic δ-opioid agonist treatment. *J. Pharmacol. Exp. Ther.* **306**, 109–115.

Vortherms T. A., Nguyen C. H., Berlot C. H. and Watts V. J. (2004) Using molecular tools to dissect the role of $G\alpha_s$ in sensitization of AC1. *Mol. Pharmacol.* **66**, 1617–1624.

Watts V. J. (2002) Molecular mechanisms for heterologous sensitization of adenylate cyclase. *J. Pharmacol. Exp. Ther.* **302**, 1–7.

Watts V. J. and Neve K. A. (1996) Sensitization of endogenous and recombinant adenylate cyclase by activation of D_2 dopamine receptors. *Mol. Pharmacol.* **50**, 966–976.

Watts V. J., Wiens B. L., Cumbay M. G., Vu M. N., Neve R. L. and Neve K. A. (1998) Selective activation of $G\alpha_o$ by D_{2L} dopamine receptors in NS20Y neuroblastoma cells. *J. Neurosci.* **18**, 8692–8699.

Yoshimura M., Wu P. H., Hoffman P. L. and Tabakoff B. (2000) Overexpression of type 7 adenylyl cyclase in the mouse brain enhances acute and chronic actions of morphine. *Mol. Pharmacol.* **58**, 1011–1016.

Yue X., Varga E. V., Stropova D., Vanderah T. W., Yamamura H. I. and Roeske W. R. (2006) Chronic morphine-mediated adenylyl cyclase superactivation is attenuated by the raf-1 inhibitor, GW5074. *Eur. J. Pharmacol.* **540**, 57–59.

