Alterations in the expression of G-proteins and regulation of adenylate cyclase in human neuroblastoma SH-SY5Y cells chronically exposed to low-efficacy μ-opioids

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Western-blot analysis of human neuroblastoma SH-SY5Y cells (μ- and δ-receptors) revealed the presence of the following G-protein subunits: $G_i\alpha1$, $G_i\alpha2$, $G_s\alpha$, $G_o\alpha$, $G_z\alpha$, and $G\beta$, a pattern resembling that observed in central nervous tissue. Chronic treatment of differentiated [all-*trans*-retinoic acid (10 μM; 6 days)] SH-SY5Y cells with D(−)-morphine (10 μM; 3 days) significantly increased the abundance of all G-protein subunits identified. Co-incubation of morphine-exposed cells together with naloxone (10 μM; 3 days) or the μ-selective opioid antagonist CTOP (10 μM; 3 days), but not with the δ-selective antagonist ICI-174,864 (10 μM; 3 days), completely abolished this effect, suggesting that the increase in G-protein abundance is specifically mediated by μ-receptors. Moreover, the biologically inactive enantiomer L(+)-morphine (10 μM; 3 days) failed to produce a similar effect. G-protein up-regulation developed in a time- and dose-dependent manner and is most likely due to enhanced protein synthesis *de novo*, since concomitant treatment of the cells with cycloheximide (100 μg/ml; 3 days) prevented this effect. Chronic treatment with the low-efficacy μ-selective opioid peptide morphiceptin (10 μM; 3 days), but not with the highly potent μ-agonist DAGO (0.1 μM; 3 days) produced a comparable increase in G-protein abundance. Coincident with quantitative effects on G-protein levels in morphine-tolerant/dependent SH-SY5Y cells, we found elevated levels of basal, forskolin (1 μM)- and prostaglandin-E_1 (1 μM)-stimulated adenylate cyclase activities. Reconstitution experiments using S49 *cyc*$^-$ lymphoma-cell membranes suggest that this increase is most likely due to elevated levels of functionally intact G_s. Chronic treatment with both morphine and DAGO induces high degrees of tolerance in this cell line. However, the intrinsic activity of G_i was unchanged, as assessed in functional studies with low-nanomolar concentrations of guanosine 5′-[$\beta\gamma$-imido]triphosphate. Our data demonstrate that chronic treatment of SH-SY5Y cells with low-efficacy μ-opioids increases G-protein abundance, a phenomenon which might contribute to the biochemical mechanisms underlying opioid tolerance/dependence.

INTRODUCTION

The molecular mechanisms underlying opioid tolerance and dependence are still poorly understood, although several adaptations have been reported on receptor level, such as down-regulation, internalization and degradation of opioid-binding sites (Law et al., 1982, 1983; Vachon et al., 1987). However, these regulatory mechanisms can hardly explain the phenomenon of opioid dependence, which is believed to be linked to post-receptor events (Collier, 1984; Wüster et al., 1985; Johnson and Fleming, 1989).

Recent investigations implicate a critical role of pertussis-toxin-sensitive G-proteins in the development of chronic opioid actions (Kurose et al., 1983; Lux and Schulz, 1986), as prolonged activation of μ-receptors results in tissue-specific alteration of G-protein abundance. In particular, the opioid-tolerant/dependent guinea-pig myenteric plexus (Lang and Schulz, 1989; Ammer et al., 1991), the rat locus coeruleus (Nestler et al., 1989) and the rat amygdala (Terwilliger et al., 1991) display increased levels of certain G-protein subunits, but other nerve tissues remain unaffected or, like the rat nucleus accumbens, exhibit even decreased levels of G-proteins (Terwilliger et al., 1991). Furthermore, studies with the δ-opioid-receptor-bearing neuroblastoma × glioma NG108-15 hybrid cell line (Chang and Cuatrecasas, 1979) revealed no change in the abundance of pertussis-toxin-sensitive G-proteins during the state of opioid tolerance/dependence (Lang and Costa, 1989; Boyd et al., 1992).

In view of these diverse data, the present study was designed to examine adaptive processes on G-protein levels in human neuroblastoma SH-SY5Y cells (Biedler et al., 1973) chronically exposed to μ-opioids. This cell clone expresses predominantly μ- and, to a minor extent, δ-type opioid receptors (Kazmi and Mishra, 1986). The μ-receptors comprise a single population of binding sites (Toll, 1990), and are coupled to the inhibition of adenylate cyclase (Yu and Sadée, 1988). This mechanism is considerably potentiated after differentiation of the cells towards a neuronal-like phenotype by retinoic acid (Påhlman et al., 1984). Chronic exposure of differentiated SH-SY5Y cells to morphine results in the development of tolerance/dependence, which is indicated by desensitization of μ-receptors from adenylate cyclase inhibition (Yu and Sadée, 1988). However, this adaptation is not associated with changes in the capacity or affinity of μ-binding sites (Yu et al., 1990). To investigate chronic opioid effects on G-proteins, we first characterized G-protein distribution in this cell line by Western-blot analysis and compared the pattern obtained with that displayed by a typical preparation of central nervous tissue. Thereafter, we analysed acute and chronic actions of morphine as well as of the

Abbreviations used: CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (where Pen is penicillamine); DAGO, [D-Ala^2, MePhe^4,Gly-ol^5]-enkephalin; p[NH]ppG, guanosine 5′-[$\beta\gamma$-imido]triphosphate; GTP[S], guanosine 5′-[γ-thio]triphosphate; ICI-174,864, *NN*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (where Aib is α-aminoisobutyric acid); morphiceptin, H-Tyr-Pro-Phe-Pro-NH_2; PGE_1, prostaglandin E_1; RA, all-*trans*-retinoic acid; TBS, Tris-buffered saline.

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Table 1 Characterization of antibodies against G-proteins

Antisera were characterized by Western-blot analysis employing recombinant (r) G-protein α-subunits expressed in *E. coli*. Anti-α_i1/2 antiserum strongly reacts with $rG_i\alpha1$ and $rG_i\alpha2$. In addition, a weak cross-reactivity to $rG_i\alpha3$ is observed when large amounts of the protein were loaded on the gel. Anti-α_s antiserum selectively binds to $rG_s\alpha$, whereas antiserum anti-α_o recognizes both $rG_o\alpha1$ and $rG_o\alpha2$ to the same extent. This pattern is expected, since both $G_o\alpha$ isoforms share an identical N-terminal amino acid sequence, to which the antibody binds. Antiserum anti-α_z does not react with any of the recombinant α-subunits. However, in bovine brain cortical membranes this antiserum strongly reacts with a 40 kDa protein. Moreover, antibody binding is completely blocked in the presence of a 100-fold molar excess of the corresponding peptide used for immunization. Anti-β antiserum was raised against an amino acid sequence deduced from β_1 cDNA. Since there is only a single amino acid substitution between β_1 and β_2 cDNA within the corresponding region, this antiserum recognizes both the 35 kDa as well as the 36 kDa form of Gβ.

Antiserum	Corresponding G-protein sequence* (amino acids)	Reactivity in Western blot†						
		$rG_i\alpha1$	$rG_i\alpha2$	$rG_i\alpha3$	$rG_o\alpha1$	$rG_z\alpha2$	$rG_s\alpha$	$\beta\gamma$
Anti-α_i1/2	349–354	+++	+++	+	−	−	−	−
Anti-α_s	385–394	−	−	−	−	−	+++	−
Anti-α_o	22–35	−	−	−	+++	+++	−	−
Anti-α_z	346–355	−	−	−	−	−	−	−
Anti-β	130–145	−	−	−	−	−	−	+++

* Amino acid sequence of corresponding G-protein cDNAs (Fong et al., 1987; Matsuoka et al., 1988).

† As determined against recombinant G-protein α-subunits or bovine brain $\beta\gamma$-preparations. Reactivity in Western blot: −, no reaction; +, weak reaction; +++, strong reaction.

μ-selective opioid peptides DAGO and morphiceptin on the regulation of G-protein abundance and function in SH-SY5Y cells.

We report here that SH-SY5Y cells contain several G-protein subunits ($G_o\alpha$, $G_i\alpha1$ and $G_z\alpha$) whose expression is characteristic of central nervous tissue (Sternweis and Robishaw, 1984; Mumby et al., 1988; Casey et al., 1990). Prolonged exposure of this cell clone to morphine as well as to the low-efficacy μ-opioid morphiceptin (Chang et al., 1983) results in elevated steady-state levels of G-proteins, leaving their intrinsic activities unchanged. In contrast, the high-affinity μ-agonist DAGO (Kosterlitz and Paterson, 1981), fails to similarly affect the abundance of G-proteins in tolerant SH-SY5Y cells.

MATERIALS AND METHODS

Materials

G-protein fragments were either purchased from American Peptide Co. (Santa Clara, CA, U.S.A.) or from Multiple Peptide Systems (San Diego, CA, U.S.A.). Cell culture reagents were from Gibco BRL (Karlsruhe, Germany) or from PAN Systems (Aidenbach, Germany). The following compounds were employed: DAGO and morphiceptin (Bachem Biochemica, Heidelberg, Germany), CTOP (Peninsula Laboratories, Heidelberg, Germany), ICI-174,864 (Research Biochemicals Inc., Köln, Germany), Naloxone hydrochloride and ^{125}I-labelled cyclic AMP tracer (DuPont–New England Nuclear, Dreieich, Germany). Poly(vinylidene difluoride) membranes were from Millipore (Eschborn, Germany), and all other electrophoresis reagents were from Bio-Rad Laboratories (Munich, Germany). Sheep anti-rabbit IgG (Fc) coupled to alkaline phosphatase (Promega Biotech, Heidelberg, Germany) and ^{125}I-labelled donkey $F(ab')_2$ anti-rabbit IgG (sp. radioactivity 5–20 μCi/μg) (Amersham Buchler, Braunschweig, Germany) were used as secondary antibodies. Anti-(cyclic AMP) antiserum was from Bio-Yeda (Rehovot, Israel). All other reagents were from Sigma (Munich, Germany).

Cell culture and membrane preparation

Human neuroblastoma SH-SY5Y cells were grown as described by Yu and Sadée (1988). Murine S49 *cyc*$^-$ lymphoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated horse serum. SH-SY5Y cells were harvested in PBS (pH 7.4) [Ca^{2+}/Mg^{2+}-free, containing 0.04% (w/v) EDTA], and membranes were prepared as described by Vachon et al. (1987). Membranes were resuspended in 20 mM Tris/HCl (pH 7.4) containing 1 mM EDTA and 1 mM dithiothreitol, and stored at a concentration of 2 mg of protein/ml at −70 °C until use.

Cell differentiation and chronic drug treatment

SH-SY5Y cells were differentiated in the presence of all-*trans*-retinoic acid (RA) (10 μM; 6 days) towards a neuronal-like phenotype as described by Påhlman et al. (1984). Chronic treatment of the cells with either morphine or the μ-selective opioid peptides DAGO and morphiceptin was performed as described by Yu and Sadée (1988). Incubation periods and drug concentrations are indicated in the text. Each experiment was performed with subconfluent monolayers of an individual cell passage. Untreated RA-differentiated SH-SY5Y cells from identical cell passages served as controls.

Production of antisera and immunoblotting

A panel of G-protein-specific antisera was generated in rabbits each immunized with a conjugate of a synthetic peptide coupled to keyhole-limpet haemocyanin. Anti-α_s antiserum was raised against a C-terminal decapeptide (RMHLRQYELL) of $G_s\alpha$ as described by Milligan and Unson (1989). Anti-α_z antiserum was raised against a decapeptide resembling the extreme C-terminus of $G_z\alpha$ (QNNLKYIGLC) (Simonds et al., 1989). For production of anti-α_i1/2 antiserum, a heptapeptide (LKDCGLF) common to the C-termini of $G_i\alpha1$ and $G_i\alpha2$ served as hapten. Coupling of the peptide to the carrier and development of antibodies followed essentially the protocol of Goldsmith et al. (1987). Antisera against $G_o\alpha$ (anti-α_o; CNLKEDGISAAKDVK) and Gβ (anti-β; CEGNVRVSRELAGHTGY) were produced as originally described by Mumby et al. (1986). All antisera were characterized by Western-blot analysis using recombinant G-proteins expressed in *Escherichia coli* as well as of G-proteins from bovine brain cortical membranes. A summary of the properties of the antisera used in this study is given in Table 1.

Identification of G-protein subunits in SH-SY5Y cells was performed in Western-blot experiments using whole cell prepar-

ations. Cells (approx. 10^7 cells/500 μl) were sonicated in 20 mM Tris/HCl buffer (pH 7.4) containing 2% (w/v) SDS, and subsequently centrifuged for 15 min at 10000 *g*. Solubilized proteins (supernatant) were collected and measured for protein content by the method of Peterson (1983) with BSA as standard. Proteins were precipitated with ice-cold acetone, dried, and resuspended in electrophoresis sample buffer (Laemmli, 1970). Samples were subjected to SDS/PAGE [10% (w/v) polyacrylamide/0.25% (w/v) bisacrylamide] as described by Lang and Schulz (1989). Separation of $G_i\alpha1$ from $G_i\alpha2$ was performed by SDS/PAGE with 12.5% polyacrylamide and 0.065% bisacrylamide in resolving gels (McKenzie and Milligan, 1990). Proteins were transferred to poly(vinylidene difluoride) membranes, by using a semi-dry-blotting apparatus (Pharmacia–LKB, Freiburg, Germany; 0.8 mA/cm² for 2 h). The blots were blocked for 1 h with 4% (w/v) BSA and 0.1% (v/v) Tween 20 in TBS (pH 8.0), consisting of 20 mM Tris/HCl and 190 mM NaCl. The filters were then incubated overnight with primary antibodies diluted in TBS supplemented with 0.1% BSA and 0.05% Tween 20. After three washes with TBS/T [TBS containing 0.05% Tween 20], the blots were incubated with anti-rabbit IgG coupled to alkaline phosphatase (25 ng/ml in TBS/T) for 2 h. Finally, the blots were washed as mentioned above, dried, and the colour reaction was developed with 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium as substrate.

Quantification of immunoblots

For quantification of G-proteins, blots were incubated in a mixture of alkaline-phosphatase-conjugated anti-rabbit IgG (33 ng/ml) and ^{125}I-labelled IgG (300 ng/ml) as secondary-antibody solution for 2 h (Lang and Schulz, 1989). After they had been made visible, the bands were cut according to the staining, and incorporated radioactivity was measured in a γ-counter (Clinigamma; LKB, Freiburg, Germany). In preliminary experiments, standard curves for various amounts of cell protein were obtained for each G-protein subunit tested. Within the range of linearity, the amount of incorporated radioactivity was directly proportional to the amount of cell protein used in the assay (results not shown).

S49 *cyc*⁻ reconstitution assay

SH-SY5Y membranes were extracted with sodium cholate (1%, w/v) for 1 h at 4 °C as described by Milligan and Klee (1985). Solubilized proteins were adjusted to a protein concentration of 1 mg/ml in 50 mM Tris/HCl (pH 8.0) containing 1 mM dithiothreitol, 1 mM EDTA and 1% sodium cholate. Portions (10 μg) of G_s-deficient S49 *cyc*⁻ membranes were reconstituted with 5 μl portions of sodium cholate extracts (final sodium cholate concn. 0.05%), and guanosine 5′-[γ-thio]triphosphate (GTP[S]; 100 μM)-stimulated adenylate cyclase activity was determined as described below. Under these experimental conditions, a linear increase in GTP[S]-stimulated enzyme activity has been demonstrated when 0.5–10 μg of extracted membrane protein was used for reconstitution (results not shown).

Adenylate cyclase assay

Adenylate cyclase activity in particulate membrane preparations was measured as described by Vachon et al. (1987). The amount of cyclic AMP generated was determined by radioimmunoassay after acetylation of the samples as described by Frandsen and Krishna (1977). The ability of low-nanomolar concentrations of guanosine 5′-[βγ-imido]triphosphate (p[NH]ppG) to attenuate forskolin (1 μM)-amplified adenylate cyclase activity in the absence of inhibitory receptor agonists (Strassheim et al., 1990) was used to determine intrinsic activity of G_i in SH-SY5Y membranes.

RESULTS

A panel of G-protein-specific polyclonal antibodies was used to analyse G-protein expression in RA-differentiated SH-SY5Y cells. The pattern obtained was compared with that displayed for bovine brain cortical membranes (Figure 1). In both samples, antiserum anti-$\alpha_i1/2$ reacts with two proteins of 41 ($G_i\alpha1$) and 40 ($G_i\alpha2$) kDa respectively. Antiserum anti-α_s identifies two forms of $G_s\alpha$ (45 and 52 kDa) in bovine cortex, with the smaller form being more prevalent. In contrast, the larger form of $G_s\alpha$ migrates slightly faster (48 kDa) in SH-SY5Y cells, whereas the 45 kDa

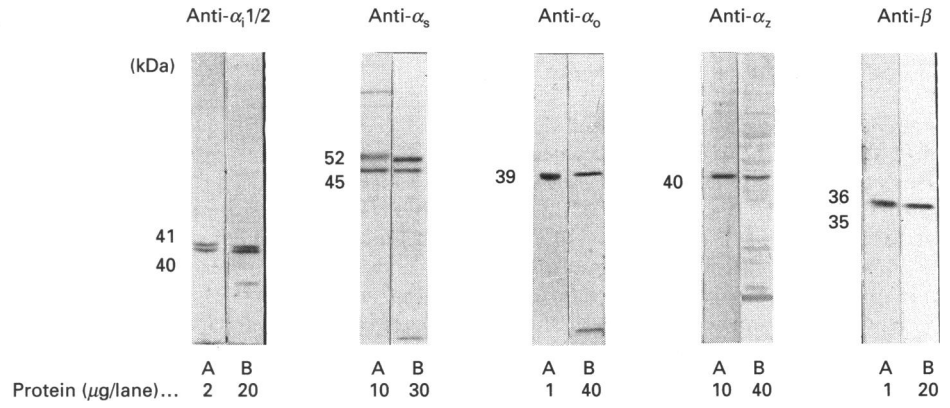

Figure 1 Expression of G-protein subunits in human SH-SY5Y cells and bovine brain cortex

Bovine cortical brain extracts (A) as well as SDS lysates of SH-SY5Y cells (B) were electrophoretically resolved by SDS/PAGE (10% gels). For separation of $G_i\alpha1$ from $G_i\alpha2$, 12.5% (w/v) polyacrylamide gels, containing low concentrations of bisacrylamide, were used. Due to the different gel composition, R_F values of $G_i\alpha1$ and $G_i\alpha2$ differ from those of other G-protein subunits. The molecular sizes (kDa) of the labelled bands are indicated for each G-protein subunit. The amount of protein applied to the gel is given below each lane. Proteins were transferred to poly(vinylidene difluoride) membranes and made to react overnight with the following subtype-specific anti-G-protein antisera: anti-$\alpha_i1/2$ (used at a dilution of 1:5000); anti-α_s (1:4000); anti-α_o (1:2000); anti-α_z (1:2000); and anti-β (1:1000). The blots were further processed as described in the Materials and methods section.

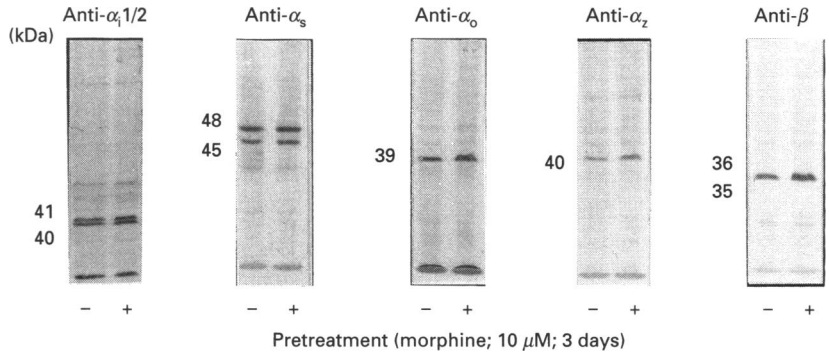

Figure 2 Western-blot analysis of G-protein subunits in SH-SY5Y cells chronically exposed to morphine

RA-differentiated SH-SY5Y cells were cultured for 3 days in either the absence (−) or presence (+) of morphine (10 μM). Whole cell lysates were separated by SDS/PAGE and immunoblotted for $G_i\alpha1/2$ (10 μg of cell protein/lane), $G_s\alpha$ (20 μg/lane), $G_o\alpha$ (50 μg/lane), $G_z\alpha$ (50 μg/lane), and $G\beta$ (20 μg/lane) as indicated in Figure 1. A more intense staining throughout is observed in SH-SY5Y cells chronically pretreated with morphine.

form is only expressed to a minor extent. Anti-α_o antiserum was raised against a sequence common to both $G_o\alpha$ cDNAs (Hsu et al., 1990). This antiserum recognizes a protein of 39 kDa present in both SH-SY5Y cells and bovine brain cortex. Anti-α_z antiserum labels a single polypeptide of 40 kDa ($G_z\alpha$) which is also present in bovine cortex as well as in SH-SY5Y cells. Finally, $G\beta$ subunits (35 and 36 kDa forms) are also expressed in both tissues, as identified by anti-β antiserum.

Prolonged exposure of RA-differentiated SH-SY5Y cells to morphine (10 μM; 3 days) has been reported to result in tolerance/dependence, as indicated by the loss of maximal inhibition of adenylate cyclase activity (Yu and Sadée, 1988; Yu et al., 1990). Immunoblotting of total cell lysates derived from chronically D(−)-morphine-treated cells revealed an overall more intense staining of all G-protein subunits tested (Figure 2). Relative quantification of G-protein immunoreactivity by using a ^{125}I-labelled anti-rabbit IgG as secondary antibody discloses a significant increase in cellular levels of $G_s\alpha$/48 kDa (39.3 ± 13.8 %; mean ± S.E.M., $n = 4$), $G_s\alpha$/45 kDa (25.1 ± 9.1 %; $n = 4$), $G_i\alpha1/2$ (26.2 ± 10.3 %; $n = 4$), $G_o\alpha$ (20.5 ± 0.7 %; $n = 3$), $G_z\alpha$ (26.7 %; mean value, $n = 2$), and $G\beta$ (33.9 ± 3.2 %; $n = 4$). In control experiments, a linear relationship between the binding of ^{125}I-labelled secondary antibody and the amount of cell lysate loaded on the gel has been established separately for each G-protein subunit analysed (results not shown).

The finding that chronic morphine treatment increases all G-protein subunits identified here raises the question as to whether this effect reflects a specific opioid-receptor action. This issue was investigated (*a*) by chronic treatment of the cells with the pharmacologically inactive enantiomer L(+)-morphine (10 μM; 3 days) and (*b*) by the combined exposure to D(−)-morphine (10 μM; 3 days) and the non-selective opioid antagonist naloxone (10 μM; 3 days). Both treatments completely prevented D(−)-morphine-induced G-protein up-regulation. Since SH-SY5Y cells express both μ- and δ-opioid receptors in the ratio approx. 4.5:1 (Kazmi and Mishra, 1986), more detailed studies were performed to define the type of opioid receptor involved in the generation of morphine-induced G-protein alteration. Because our special interest focuses on the signal-transduction mechanisms involved in regulation of intracellular cyclic AMP levels, these and all further results relate to the G-proteins mediating either stimulation ($G_s\alpha$) or inhibition ($G_i\alpha1/2$) of adenylate cyclase activity. In a representative experiment, the μ-selective opioid antagonist

Table 2 Pharmacology of morphine-induced G-protein elevation in SH-SY5Y cells

For time-course experiments, RA-differentiated SH-SY5Y cells were cultured either in the absence (none) or in the presence of morphine (10 μM) for 12, 24, 36 or 72 h. Dose–response experiments were conducted with cells which had been exposed for 3 days to various concentrations of morphine (0.1, 1 and 10 μM). Whole cell lysates were prepared and quantitative immunoblotting was carried out with anti-α_s and anti-$\alpha_i1/2$ antiserum. Specific antibody binding is expressed as the amount of ^{125}I-IgG bound in c.p.m. Values in parentheses indicate percentage change as compared with controls. The data shown are taken from a representative experiment. Two further experiments produced qualitatively similar data.

		G-protein immunoreactivity: ^{125}I-IgG bound (c.p.m.)	
Experiment	Pretreatment	$G_i\alpha1/2$	$G_s\alpha$/48
Time course	None	1751	4642
	D(−)-Morphine (10 μM)		
	12 h	1821 (+4%)	4456 (−6%)
	24 h	1942 (+11%)	5012 (+8%)
	48 h	2066 (+18%)	6452 (+39%)
	72 h	2136 (+22%)	6638 (+43%)
Dose–response	None	1205	3702
	D(−)-Morphine (3 days)		
	0.1 nM	1183 (−2%)	3924 (+6%)
	1 μM	1374 (+14%)	4776 (+29%)
	10 μM	1494 (+24%)	5193 (+40%)

CTOP (10 μM) (Pelton et al., 1986) completely abolished the D(−)-morphine-mediated increase in the levels of $G_s\alpha$/48 kDa [4 %, versus 39 % increase in D(−)-morphine-treated cells] and $G_i\alpha1/2$ (5 % versus 27 %). Exposure of the cells to CTOP (10 μM) alone had no effect. In contrast, selective blockade of δ-receptors by ICI-174,864 (10 μM) during the course of chronic morphine exposure failed to prevent G-protein up-regulation (43 % versus 39 % for $G_s\alpha$/45 kDa and 22 % versus 27 % for $G_i\alpha1/2$).

Subsequent experiments demonstrated that this chronic morphine effect is dependent on the time and D(−)-morphine concentration used for pretreatment (Table 2). Exposure of SH-SY5Y cells to D(−)-morphine (10 μM) for 12 h was not associated with detectable changes in the abundance of $G_s\alpha$ (48 kDa form) and $G_i\alpha1/2$, whereas exposure for 24 h slightly increased

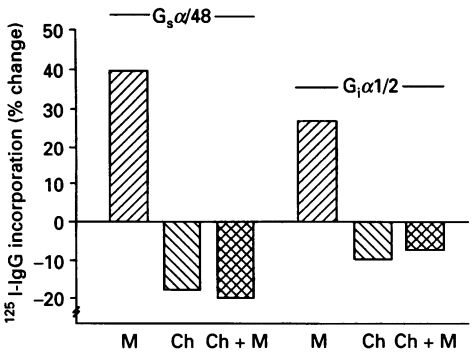

Figure 3 Co-incubation with cycloheximide prevents G-protein up-regulation in SH-SY5Y cells

RA-differentiated SH-SY5Y cells were kept in either the absence or presence of 10 μM morphine (M) for 3 days. Cells of an identical passage received the same treatment, but were additionally exposed to cycloheximide (Ch; 100 μg/ml). Changes in G-protein immunoreactivity are expressed as percentage change from control levels. The data shown are means of two independent experiments.

the amounts of both subunits. After 48 h G-protein quantities were almost maximally increased. The morphine concentration required to induce G-protein adaptation was also examined in dose–response experiments. Treatment of the cells for 3 days with morphine concentrations above 0.1 μM elevates $G_s\alpha$ (48 kDa form) and $G_i\alpha1/2$ levels, reaching maximal values at a concentration of 10 μM of the opioid alkaloid.

The question as to whether the increase in G-protein levels is based on enhanced synthesis of the proteins *de novo* was investigated in experiments by which total protein synthesis was blocked by growing the cells in the presence of cycloheximide (100 μg/ml; 3 days). This treatment results in a moderate decrease in the steady-state levels of $G_s\alpha$/48 kDa (17%) and $G_i\alpha1/2$ (10%) (mean values of two separate experiments). However, in the same set of experiments, concomitant exposure of the cells to D(−)-morphine (10 μM; 3 days) failed to produce G-protein up-regulation (Figure 3).

We also examined quantitative G-protein regulation in SH-SY5Y cells chronically exposed to highly μ-selective opioid peptide agonists. These studies included the extremely potent enkephalin analogue DAGO (0.1 μM; 3 days) as well as the low-efficacy μ-agonist morphiceptin (10 μM; 3 days). In drug-naive cells, these peptide concentrations have been demonstrated to result in almost maximal inhibition of adenylate cyclase (results not shown). Western-blot experiments revealed that, unlike chronic morphine exposure, prolonged DAGO treatment fails to affect the level of any G-protein subunit analysed (Figure 4). In contrast, sustained exposure of the cells to morphiceptin causes a comparable increase in $G_s\alpha$/48 kDa and $G_i\alpha1/2$ immunoreactivity of 35% and 30% (mean values of two separate experiments) respectively, which resembles the data obtained for the chronic D(−)-morphine treatment. Again, this effect was completely blocked by naloxone (10 μM) (Figure 5).

Possible functional consequences of morphine-modulated G-protein levels on signal transduction in SH-SY5Y cells were investigated by using hormone-regulated adenylate cyclase activity as indicator. In membranes from untreated RA-differentiated control cells, both morphine and DAGO decrease prostaglandin E_1 (PGE_1; 1 μM)-stimulated adenylate cyclase activity by approx. 35%. The maximal effective concentrations of morphine and DAGO to inhibit enzyme activity were 100 and 1 μM respectively (Table 3). Coincident with elevated $G_s\alpha$ levels, as displayed in SH-SY5Y cells chronically exposed to morphine (10 μM; 3 days), we observed an increase in both basal and PGE_1-stimulated enzyme activity in morphine-tolerant/dependent cells. However, the ability of morphine (100 μM) to attenuate cyclic AMP generation in these cells is largely decreased (14% of maximal inhibition). In contrast, basal as well as PGE_1-stimulated adenylate cyclase activities remain unaffected in DAGO (0.1 μM; 3 days)-pretreated cells, although a high degree of tolerance developed (10% of maximal adenylate cyclase inhibition at 1 μM DAGO) (Table 3).

To assess whether an altered intrinsic activity of G_i accounts for the observed desensitization from adenylate cyclase after chronic morphine and DAGO treatment, we measured receptor-independent inhibition of forskolin (1 μM)-amplified enzyme activity in the presence of 10 nM p[NH]ppG, a concentration which brings about maximal inhibition of adenylate cyclase in this cell system. Apparently, chronic morphine, but not DAGO, pretreatment largely enhances forskolin-stimulated adenylate cyclase activity. Regardless of the opioid given chronically, p[NH]ppG produced a similar degree of enzyme inhibition as

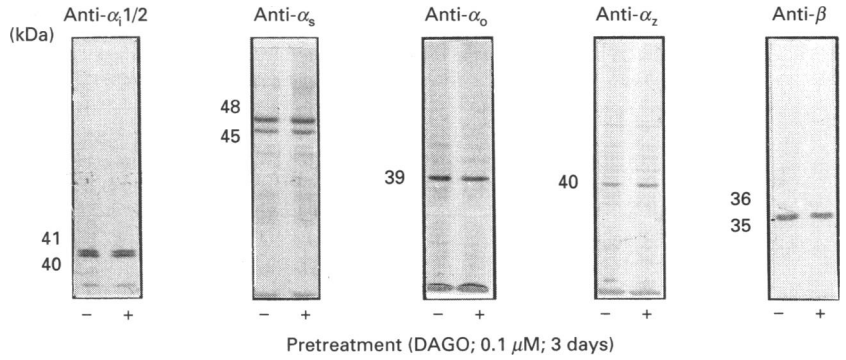

Figure 4 Effect of chronic DAGO treatment on G-protein abundance in SH-SY5Y cells

Cells were grown for 3 days either in the absence (−) or in the presence (+) of the highly potent μ-opioid DAGO (0.1 μM). Whole cell lysates were prepared and further processed as in Figure 2. No change in intensity of the colour reaction is observed.

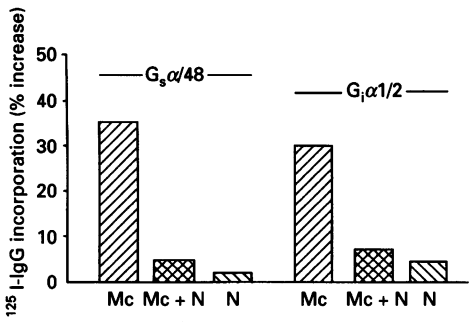

Figure 5 Chronic exposure of SH-SY5Y cells to morphiceptin increases the abundance of G-proteins

RA-differentiated cells were exposed for 3 days to morphiceptin (Mc), morphiceptin together with naloxone (Mc + N), or naloxone alone (N) at a concentration of 10 μM each. Cell lysates were prepared and quantitative immunoblotting was carried out as described in the Materials and methods section. Increase in G-protein immunoreactivity is expressed as percentage change from control levels. The data shown are means of two independent experiments.

compared with untreated control cells (Table 4). These findings suggest that the functional activity of inhibitory G-proteins does not change during the state of opioid tolerance/dependence.

We also examined whether chronic μ-opioid treatment alters intrinsic G_s activity. This was investigated by reconstitution of S49 *cyc*$^-$ membranes with sodium cholate extracts derived from particulate membrane preparations of control or chronically pretreated SH-SY5Y cells. In the presence of GTP[S] (100 μM), extracts from control cells produced cyclic AMP levels of 131.1 ± 1.2 pmol/min per mg (mean ± S.E.M., $n = 3$) (Table 5). Extracts derived from morphine-tolerant/dependent cells cause an increase in cyclic AMP production of 42% (186.8 ± 10.2; $n = 3$) over control levels. This effect was abolished upon concomitant exposure of the cells to naloxone (10 μM) (132.9 ± 7.4; $n = 3$). In addition, extracts derived from cells chronically pretreated with the pharmacologically inactive enantiomer L(+)-morphine (10 μM; 3 days) or DAGO (0.1 μM; 3 days) produced similar levels of reconstituted S49 *cyc*$^-$ adenylate cyclase activity to those observed for untreated cells. These data suggest that the increase in steady-state levels of $G_s\alpha$ in chronically morphine-

Table 3 Effect of chronic opioid exposure on adenylate cyclase activity in SH-SY5Y cells

RA-differentiated SH-SY5Y cells were kept either in he absence (no pretreatment) or in the presence of morphine (10 μM) or DAGO (0.1 μM) for 3 days before cells were harvested and membranes were prepared. Basal and PGE_1 (1 μM)-stimulated adenylate cyclase activities, as well as the ability of maximal effective concentrations of morphine (100 μM) and DAGO (1 μM) to inhibit PGE_1-stimulated enzyme activity, were determined as described in the text. Adenylate cyclase activity is expressed in pmol of cyclic AMP formed/min per mg of membrane protein. The data shown are means ± S.E.M. ($n = 3$). Values in parentheses indicate percentage of μ-receptor-mediated inhibition of PGE_1-stimulated adenylate cyclase activity.

Pretreatment	Assay conditions	Adenylate cyclase activity (pmol of cyclic AMP/min per mg of membrane protein)
None	Basal	28.6 ± 1.2
	PGE_1 (1 μM)	119.6 ± 2.1
	PGE_1 (1 μM) + morphine (100 μM)	77.2 ± 2.6 (−35.5%)
	PGE_1 (1 μM) + DAGO (1 μM)	78.6 ± 3.1 (−34.3%)
Morphine (10 μM; 3 days)	Basal	39.8 ± 0.9
	PGE_1 (1 μM)	235.9 ± 8.8
	PGE_1 (1 μM) + morphine (100 μM)	202.3 ± 7.9 (−14.2%)
DAGO (0.1 μM; 3 days)	Basal	30.5 ± 2.3
	PGE_1 (1 μM)	108.7 ± 4.1
	PGE_1 (1 μM) + DAGO (1 μM)	97.1 ± 9.2 (−10.7%)

Table 4 Receptor-independent inhibition of adenylate cyclase activity in SH-SY5Y cells

RA-differentiated SH-SY5Y cells were chronically treated with morphine (10 μM) or DAGO (0.1 μM) for 3 days. Untreated cells of an identical passage served as controls (no pretreatment). Basal and forskolin (1 μM)-amplified activity of membrane adenylate cyclase was determined as described in the text. Receptor-independent attenuation of forskolin-amplified adenylate cyclase was assessed in the presence of 10 nM p[NH]ppG, a concentration which brings about maximal adenylate cyclase inhibition in this cell system. Enzyme activity is expressed in pmol of cyclic AMP formed/min per mg of membrane protein. The data shown are means ± S.E.M. ($n = 3$). Values in parentheses indicate percentage inhibition of forskolin-amplified adenylate cyclase activity.

Pretreatment	Assay conditions	Adenylate cyclase activity (pmol of cyclic AMP/min per mg of membrane protein)
None	Basal	24.9 ± 2.5
	Forskolin (1 μM)	125.1 ± 6.1
	Forskolin (1 μM) + p[NH]ppG (10 nM)	95.7 ± 3.1 (−23.4%)
Morphine (10 μM; 3 days)	Basal	45.4 ± 6.2
	Forskolin (1 μM)	250.4 ± 5.4
	Forskolin (1 μM) + p[NH]ppG (10 nM)	196.1 ± 2.5 (−21.7%)
DAGO (0.1 μM; 3 days)	Basal	21.2 ± 4.7
	Forskolin (1 μM)	119.6 ± 9.5
	Forskolin (1 μM) + p[NH]ppG (10 nM)	92.4 ± 4.3 (−22.7%)

Table 5 Reconstitution of S49 *cyc*⁻ membrane adenylate cyclase by SH-SY5Y cell extracts

RA-differentiated SH-SY5Y cells were kept either in the absence (no pretreatment) or in the presence of various drugs or substances for 3 days as indicated. RA-differentiated cells of an identical passage served as controls. Membranes were prepared and extracted with sodium cholate (1%, w/v) for 1 h at 4 °C. Solubilized proteins (5 μg/assay) were reconstituted with G_s-deficient S49 *cyc*⁻ lymphoma-cell membranes (10 μg/assay) and GTP[S] (100 μM)-stimulated adenylate cyclase was determined for 10 min at 37 °C. In the absence of GTP[S], no detectable enzyme activity was observed. Adenylate cyclase activity is expressed in pmol of cyclic AMP formed/min per mg of membrane protein. The data shown are means ± S.E.M. ($n = 3$). Values in parentheses indicate percentage change in GTP[S]-stimulated adenylate cyclase activity.

Pretreatment	GTP[S] (100 μM)-stimulated adenylate cyclase (pmol of cyclic AMP/min per mg of membrane protein)	
None	131.1 ± 1.2	
D(−)-Morphine (10 μM; 3 days)	186.8 ± 10.2	(+42.4%)
D(−)-Morphine + naloxone (10 μM; 3 days)	132.9 ± 7.4	
L(+)-Morphine (10 μM; 3 days)	127.3 ± 0.4	
DAGO (0.1 μM; 3 days)	134.5 ± 9.5	
DAGO + naloxone (10 μM; 3 days)	122.4 ± 15.1	
Cycloheximide (100 μg/ml; 3 days)	51.9 ± 2.2	(−60.4%)
Cycloheximide + D(−)-morphine (10 μM; 3 days)	50.6 ± 4.1	(−61.4%)

exposed SH-SY5Y cells is due to an increase in the quantity of the G-protein rather than of altered intrinsic activity.

In extension to these experiments, we investigated whether inhibition of protein synthesis *de novo* would abolish the effect of morphine-reconstituted S49 *cyc*⁻ adenylate cyclase activity. Membrane extracts derived from SH-SY5Y cells pretreated with cycloheximide (100 μg/ml; 3 days) decreased enzyme activity by 60% (to 51.9 ± 2.2 pmol of cyclic AMP/min per mg; mean ± S.E.M., $n = 3$). Under these experimental conditions, extracts from untreated as well as chronically morphine (10 μM)-exposed cells revealed similar amounts of reconstituted S49 *cyc*⁻ adenylate cyclase activity (50.6 ± 4.1; $n = 3$) (Table 5). It is therefore suggested that the increase in adenylate cyclase activity of reconstituted S49 *cyc*⁻ membranes, as observed for extracts derived from morphine-tolerant/dependent SH-SY5Y cells, is indeed due to an increase in the abundance of functional active $G_s\alpha$.

DISCUSSION

Chronic exposure of human neuroblastoma SH-SY5Y cells (Biedler et al., 1973) to low-efficacy μ-opioids elevates the abundance of a variety of G-protein subunits. Coincident with this increase is the development of opioid tolerance/dependence as assessed by receptor-regulated adenylate cyclase activity. The data reported here demonstrate that quantitative adaptations in G-protein content develop in a defined neuronal cell line as consequence of long-term activation of μ-opioid receptors. This finding, together with data from opioid-tolerant/dependent brain areas, suggests that adaptational processes at the level of G-proteins may play an important role in the regulatory mechanisms underlying chronic opioid action.

The use of human neuroblastoma SH-SY5Y cells to study chronic opioid effects is favoured by the fact that they express μ-opioid receptors (Kazmi and Mishra, 1986) which are negatively coupled to adenylate cyclase via pertussis-toxin-sensitive G-proteins (Yu and Sadée, 1988). Moreover, chronic exposure of RA-differentiated SH-SY5Y cells to morphine results in tolerance with respect to μ-receptor-mediated adenylate cyclase inhibition (Yu and Sadée, 1988), and withdrawal of the drug results in adenylate cyclase supersensitivity (Yu et al., 1990). Because there is little information on G-protein distribution in this cell line (Klinz et al., 1987), we first characterized G-protein content by polyclonal antibodies and found a pattern of G-protein subunits typically displayed by the brain [$G_o\alpha$ (Sternweis and Robishaw, 1984); $G_i\alpha 1$ (Mumby et al., 1988); $G_z\alpha$ (Casey et al., 1990)]. Therefore, the μ-receptor-expressing SH-SY5Y cell clone may represent a suitable model *in vitro* to investigate cellular aspects of chronic opioid effects.

Prolonged activation of μ-receptors has been described to affect G-protein concentrations in a number of opioid-responsive tissues (Lang and Schulz, 1989; Nestler et al., 1989; Ammer et al., 1991; Terwilliger et al., 1991). However, the results obtained were rather heterogeneous, depending on the tissue examined. Here we report that chronic exposure of RA-differentiated SH-SY5Y cells to the prototype low-efficacy μ-opioids D(−)-morphine and morphiceptin results in a clear-cut increase in the abundance of all G-protein subunits investigated. This up-regulation is dose- and time-dependent and is prevented by both naloxone and the selective μ-receptor antagonist CTOP, but not by the δ-selective antagonist ICI-174,864. Moreover, the biologically inactive enantiomer L(+)-morphine failed to produce a similar effect.

Quantitative regulation of G-protein levels upon long-term activation of inhibitory receptors is a well-documented phenomenon. For instance, chronic exposure of isolated adipocytes to an A_1-adenosine-receptor agonist leads to a decrease in inhibitory G-proteins (Green et al., 1990) with a time course similar to that observed in our cell system. Our finding that chronic morphine treatment augments the levels of all G-protein subunits, regardless of their proposed functional activity, implicates that not only G-proteins directly coupled to μ-receptors (G_o/G_i) (Ueda et al., 1988) appear to be affected. In addition, the uniform increases in both $G_s\alpha$ and $G_i\alpha 1/2$ is in contrast with data obtained from primary cultures of rat striatal neurons, which respond to chronic morphine treatment with an elevation of $G_s\alpha$ and a simultaneous decrease of $G_i\alpha$ levels (Van Vliet et al., 1991). This discrepancy may be explained by cross-regulation between adenylate cyclase-controlling receptor systems, as originally described for inhibitory A_1-adenosine receptors in DDT_1 MF-12 cells (Hadcock et al., 1991; Port et al., 1992), which in turn increases the expression of excitatory β_2-adrenergic receptors, and thus modifies stimulatory control of adenylate cyclase. Such a regulatory mechanism is likely to also occur in SH-SY5Y cells, since long-term treatment with morphine has been found to increase greatly the maximal extent of PGE_1-receptor-stimulated adenylate cyclase (Table 3). Moreover, activation of stimulatory PGE_1 receptors during the course of morphine withdrawal results in a considerable adenylate cyclase supersensitivity (Yu et al., 1990). In this context, our finding of elevated $G_s\alpha$ levels further supports the idea that chronic activation of inhibitory μ-opioid receptors leads to adaptational processes on the excitatory axis of the adenylate cyclase system. A similar regulation has been recently demonstrated for neuroblastoma × glioma NG108-15 hybrid cells following chronic activation of δ-opioid receptors (Ammer and Schulz, 1993).

The nature of quantitative G-protein regulation may originate either from alterations in the rate of protein synthesis or from changes in the turnover of the protein. Since a recent report emphasizes an increase in mRNA levels for $G_s\alpha$, $G_i\alpha 1$ and $G_i\alpha 2$ subunits in primary cultures of rat cerebral cortex upon chronic

μ-receptor activation (Eriksson et al., 1992), we addressed the question whether the increase in G-protein levels reported here is due to enhanced protein synthesis *de novo*. This was investigated by co-incubation of the cells with cycloheximide, which is known to abolish protein synthesis at concentrations used here (Mossman and Williamson, 1980). In these experiments, elevation of G-protein concentrations upon chronic morphine treatment was prevented, suggesting that the increase in G-protein abundance is due to enhanced synthesis. However, the possibility of further mechanisms regulating steady-state levels, such as an altered half-life of the proteins (Brabet et al., 1991; Hadcock et al., 1991), cannot be ruled out.

Functional consequences of altered G-protein levels in morphine-tolerant/dependent SH-SY5Y cells were investigated by using adenylate cyclase as a μ-receptor-controlled effector system. Besides the development of tolerance to adenylate cyclase inhibition, we also found markedly increased basal and PGE_1-amplified adenylate cyclase activities. The most simple explanation for this effect may be the presence of increased levels of functionally intact stimulatory G-proteins rather than alterations in the level of excitatory-acting receptors. In fact, receptor-independent activation of adenylate cyclase by forskolin produces a similar increase in cyclic AMP levels. Such a mechanism is strengthened by the notion that the functional activity of $G_s\alpha$ is unchanged in opioid-tolerant/dependent cells as assessed in S49 *cyc*$^-$ reconstitution experiments. Here, sodium cholate extracts derived from chronically morphine-pretreated cells, containing increased amounts of $G_s\alpha$, result in elevated enzyme activities as compared with extracts from control cells. Moreover, morphine tolerance is also unlikely to depend on impaired functional activities of inhibitory G-proteins, since intrinsic activity of G_i, as assessed by the ability of low-nanomolar concentrations of p[NH]ppG to inhibit adenylate cyclase (Strassheim et al., 1990), remains unchanged. This observation is in line with the notion that chronic morphine treatment induces homologous tolerance in SH-SY5Y cells, lacking cross-tolerance to α_2-adrenergic receptors (Lameh et al., 1992).

Our consistent finding that DAGO, a highly potent and selective μ-agonist, fails to bring about a similar adaptation in G-protein levels, as compared with the low-efficacy μ-ligands morphine and morphiceptin, may be explained by its ability to uncouple μ-receptors from their subsequent signal-transduction pathways. A comparable mechanism has been demonstrated for δ-opioid receptors in neuroblastoma × glioma NG108-15 hybrid cells exposed to high-efficacy δ-opioids, such as [D-Ala^2,D-Leu^5]enkephalin. In this cell line, receptor occupation results in rapid uncoupling of the receptors from low-K_m GTPase, desensitization from adenylate cyclase and down-regulation of δ-binding sites (Law et al., 1982, 1983; Vachon et al., 1987). Therefore, it could be speculated that the highly potent μ-ligand DAGO may also elicit similar mechanisms in SH-SY5Y cells, resulting in interruption of signal transduction, and thus preventing regulatory mechanisms on the post-receptor level. Direct demonstration of this possible adaptation by binding assays and low-K_m GTPase studies is currently in progress. In contrast with DAGO, the low-efficacy alkaloid morphine is less likely to produce alterations on the receptor level in SH-SY5Y cells, since no significant changes have been described in the density or affinity of μ-binding sites after chronic treatment of the cells (Yu et al., 1990). Moreover, morphine also fails to down-regulate μ-receptors in central nervous tissue (Nishino et al., 1990), suggesting a continuing ability of μ-receptors to activate their associated G-proteins during the state of tolerance/dependence. In general, most studies conducted to explain tolerance and dependence after chronic morphine treatment failed to correlate these phenomena with alterations on the level of μ-binding sites (for review, see Johnson and Fleming, 1989).

In conclusion, our results demonstrate that chronic activation of μ-receptors by means of low-efficacy opioids such as morphine and morphiceptin is associated with an increase in steady-state levels of G-proteins in human neuroblastoma SH-SY5Y cells. This adaptational process could represent at least one possible mechanism responsible for the development of opioid dependence. Moreover, the finding that DAGO, a highly potent μ-ligand, does not produce a similar effect implies that ligands with different intrinsic activities are able to induce distinct types of biochemical adaptations in a single cell, although acting at the same receptor population.

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