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Tolerance to μ-opioid agonists in human neuroblastoma SH-SY5Y cells as determined by changes in guanosine-5′-O-(3-[^{35}S]-thio)triphosphate binding

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1 The agonist action of morphine on membranes prepared from human neuroblastoma SH-SY5Y cells was measured by an increase in the binding of the GTP analogue [^{35}S]-GTPγS. Morphine increased the binding of [^{35}S]-GTPγS to SH-SY5Y cell membranes by 30 fmol mg^{-1} protein with an EC_{50} value of 76 ± 10 nM.

2 Incubation of SH-SY5Y cells with 10 μM morphine for 48 h caused a tolerance to morphine manifested by a 2.5 fold shift to the right in the EC_{50} value with a $31 \pm 6\%$ decrease in the maximum stimulation of [^{35} S]-GTPγS binding. The response caused by the partial agonist pentazocine was reduced to a greater extent.

3 Chronic treatment of the cells with the more efficacious μ-ligand [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (DAMGO, 10 μM) for 48 h afforded a greater effect than treatment with morphine. The maximal agonist effect of morphine was reduced to $58.9 \pm 6\%$ of that seen in control cells while the maximal effect of DAMGO was reduced to $62.8 \pm 4\%$. There was a complete loss of agonist activity for pentazocine.

4 The development of tolerance was complete within 24 h and was blocked by naloxone and by the nonselective protein kinase inhibitor H7, but not by the putative β-adrenoceptor kinase (β-ARK) inhibitor suramin.

5 The observed tolerance effect was accompanied by a down-regulation of μ-opioid receptors determined by a decrease in the maximal binding capacity for the opioid antagonist [^{3}H]-diprenorphine of $66 \pm 4\%$, but with no change in binding affinity. Binding of the agonist [^{3}H]-DAMGO was similarly reduced.

6 The modulation of [^{35}S]-GTPγS binding in SH-SY5Y cell membranes by opioids provides a simple method for the study of opioid tolerance at a site early in the signal transduction cascade.

Keywords: μ-Opioid receptor agonists; morphine; DAMGO; [^{35}S]-GTPγS binding; tolerance; SH-SY5Y cells

Introduction

μ-Opioid receptors belong to the family of 7-transmembrane domain receptors which couple to heteromeric G proteins (Uhl *et al*., 1993). Continual exposure of opioid receptors to agonists leads to a tolerance caused by an initial desensitization of the receptor due to uncoupling, followed by down-regulation as the number of cell surface receptors decreases. The actual molecular mechanisms governing these processes are unclear, though phosphorylation is believed to play an important role (Nestler, 1993).

μ-Opioid receptors are expressed, along with a lesser number of δ-opioid receptors, on SH-SY5Y human neuroblastoma cells (Kazmi & Mishra, 1987; Yu & Sadee, 1988) and are negatively coupled to adenylyl cyclase (Yu & Sadee 1988). Differentiation of SH-SY5Y cells with retinoic acid leads to an up-regulation of these receptors (Yu & Sadee, 1988). Chronic treatment of such differentiated cells with morphine leads to a decrease in the capacity of μ-opioid agonists to inhibit the accumulation of adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP) (Yu & Sadee, 1988; Carter & Medzihradsky 1993a) and to mediate inhibition of the N-type Ca^{2+} current (Kennedy & Henderson, 1991). These effects are accompanied by a decrease in the maximal binding capacity of μ-ligands in cell membranes (Zadina *et al*., 1994). Similar findings have been obtained in undifferentiated SH-SY5Y cells (Carter & Medzihradsky, 1992; Prather *et al*., 1994).

The agonist action of μ-opioids can be functionally demonstrated in membranes from undifferentiated SH-SY5Y cells by an increase in the level of binding of the guanosine 5′-triphosphate (GTP) analogue [^{35}S]-guanosine-5′-*O*-(γ-thiotriphosphate) ([^{35}S]-GTPγS) (Traynor & Nahorski, 1995). This follows coupling of agonist-occupied receptors to G_o and G_i proteins, as evidenced by the sensitivity of the system to pertussis toxin, and allows differentiation of opioids of varying intrinsic activity. In this study changes induced in this coupling mechanism following chronic morphine treatment were assessed as a reduction in the ability of opioid agonists to stimulate [^{35}S]-GTPγS binding. The changes observed are a small (2- to 3 fold) shift in potency accompanied by a reduction in the maximal agonist-stimulated binding of [^{35}S]-GTPγS, even with the highly efficacious agonist [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (DAMGO). The system provides a suitable method to investigate mechanisms of tolerance occuring at the level of receptor-G protein. A preliminary account of part of this work has been presented (Elliott & Traynor, 1994).

Methods

Cell culture and membrane preparation

Undifferentiated human neuroblastoma SH-SY5Y cells (passage number 78–100) were cultured in Minimum Essential Medium supplemented with 10% foetal calf serum and antibiotics, as described previously (Traynor & Nahorski, 1995). Briefly, cells were grown in monolayers to confluency at 37°C

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in a humidified 5% CO_2 atmosphere. The cells were harvested in HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid], 20 mM, pH 7.4)-buffered saline containing 1 mM EDTA, dispersed by agitation and collected by centrifugation at 500 × *g*. For [^{35}S]-GTPγS assays the cell pellet was suspended in a buffer of 20 mM HEPES, pH 7.4, 100 mM NaCl and 10 mM $MgCl_2$; $6H_2O$ (buffer A) and homogenized with a tissue tearor (Biospec Products). The resultant homogenate was centrifuged at 50,000 × *g* and the pellet collected, washed in buffer A and recentrifuged. The pellet was finally resuspended in buffer A to give a protein concentration of between 100–200 μg ml^{-1} (Lowry *et al*., 1951). All procedures were performed at 4°C and membranes freshly prepared. In experiments to determine opioid binding in membranes, buffer A was replaced with 50 mM Tris-HCl buffer, pH 7.4, in all procedures and the final pellet was resuspended to a protein concentration of approximately 100 μg ml^{-1}. To examine the effect of chronic opioid exposure confluent cells were fed five days after passaging, and morphine (10 μM), DAMGO (10 μM) or sterile vehicle (water), was added to the medium. Two days later, unless otherwise stated, the cells were harvested and membranes prepared as above. Morphine (10 μM) or DAMGO (10 μM) was added to control cells 5 min before harvesting to control for any effects due to residual opioid (Yu & Sadee, 1988; Zadina *et al*., 1993).

[^{35}S]-GTPγS binding assays

SH-SY5Y cell membranes (100–200 μg protein), prepared as described above, were incubated in buffer A containing [^{35}S]-GTPγS (80 pM), GDP (3 μM) and varying concentrations of opioid (1–10,000 nM) in a total volume of 1 ml, for 60 min at 30°C as described previously (Traynor & Nahorski, 1995). Nonspecific binding was defined with unlabelled [^{35}S]-GTPγS (10 μM). Bound and free [^{35}S]-GTPγS were separated by vacuum filtration through GF/B filters and quantified by liquid scintillation counting. EC_{50} values were determined by use of Graphpad Prism, version 1.02 (GraphPad, San Diego, CA, U.S.A.) and compared as log EC_{50} values by Student's paired *t* test.

Opioid binding assays

Cell membranes (100 μg of protein) prepared as described above were incubated in 50 mM Tris-HCl buffer, pH 7.4, with varying concentrations (0.04–10 nM) of the μ-opioid agonist [^{3}H]-DAMGO, or the nonselective antagonist [^{3}H]-diprenorphine, in a total volume of 1 ml, for 40 min at 25°C, as described previously (Traynor & Wood, 1989). Competition assays with [^{3}H]-diprenorphine (0.48 nM) were performed at 37°C for 60 min in buffer A containing GDP (3 μM), or, for whole cell assays, in Krebs/HEPES buffer composition in mM: $NaHCO_3$ 25, NaCl 118, KCl 4.7, $CaCl_2$. $2H_2O$ 2.6, KH_2PO_4 1.17, $MgSO_4.7H_2O$ 1.18, glucose 11.7 and HEPES 10, pH 7.4 for 60 min at 37°C. In all opioid binding experiments nonspecific binding was defined with naloxone (10 μM), and bound and free ligand were separated by vacuum filtration and quantified by liquid scintillation counting. Total numbers of binding sites (B_{max}) and affinities (K_d) were determined by the program LIGAND (Munsen & Rodbard, 1980) and affinities from competition assays (K_i) calculated with the Cheng and Prussoff equation (Cheng & Prussoff, 1973).

Chemicals and drugs

[^{35}S]GTPγS (guanosine-5′-O-(3-[^{35}S]-thiotriphosphate 46.1-51.5 TBq mmol^{-1}) was from New England Nuclear (Stevenage, U.K.) and [^{3}H]-DAMGO ([^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin 1.49 TBq mmol^{-1}) and [^{3}H]-diprenorphine (1.78 TBq mmol^{-1}) from Amersham International (Aylesbury, U.K.). Unlabelled DAMGO, naloxone and pentazocine were purchased from Sigma (Poole, U.K.) and H7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine) from Calbiochem-Novabiochem (Nottingham, U.K.). Morphine sulphate (MacFarlan Smith, Edinburgh, U.K.), suramin (Bayer plc, Newbury, U.K.) and TIPP (TyrTicPhePhe where Tic = tetrahydroisoquinoline-3-carboxylic acid; Dr. P. Schiller, Clinical Research Institute, Montreal, Canada) were gifts. Minimal essential medium, foetal calf serum, fungizone, penicillin/streptomycin and L-glutamine were from GIBCO (Paisley, Scotland). Pertussis toxin and all other chemicals were from Sigma and were of analytical grade.

Results

Morphine stimulated the binding of [^{35}S]-GTPγS (80 pM) to membranes from undifferentiated SH-SY5Y cells in a concentration-dependent manner, as shown previously (Traynor & Nahorski, 1995), affording an EC_{50} of 76.1 ± 9.7 nM (n = 9) and a maximal stimulation of 30 fmol of [^{35}S]-GTPγS bound mg^{-1} protein (Figure 1). The opioid partial agonist pentazocine afforded a maximum level of stimulation of only 30% of that seen with morphine, with an EC_{50} of 129 ± 51 nM (Figure 1).

Pretreatment of undifferentiated SH-SY5Y cells with 10 μM morphine for 48 h, conditions which have been shown to induce down-regulation of μ-opioid receptors in differentiated SH-SY5Y cells (Zadina *et al*., 1994), caused a significant (P < 0.01) change in the morphine EC_{50} value to 190.7 ± 36.8 nM (n = 9) and significantly (P < 0.01) reduced the maximal stimulation of [^{35}S]-GTPγS binding by morphine to 69.3 ± 6.3% (n = 9) of control values (Figure 1). A similar reduction (51.0 ± 7.9% n = 3) in the stimulation afforded by the partial agonist pentazocine was seen at the highest concentration tested (Figure 1) and in the stimulation of [^{35}S]-GTPγS binding caused by a maximal concentration of the more efficacious fentanyl, which was reduced to 69.7 ± 4.9% (n = 14) of control values. Following pretreatment of SH-SY5Y cells for various times with morphine (10 μM) the loss of maximum stimulant effect of subsequently added morphine occurred over 24 h, with no further loss of effect when cells were exposed to morphine for up to 72 h (Table 1).

When the cells were incubated with morphine (10 μM) for 48 h in the presence of naloxone (10 μM) there was no change

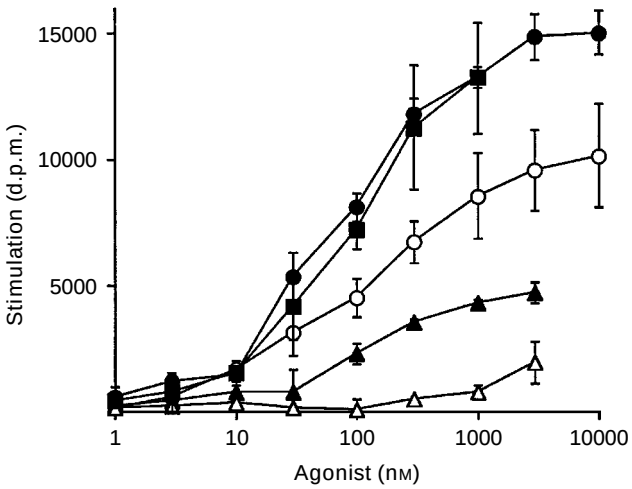

Figure 1 Stimulation of [^{35}S]-GTPγS binding by morphine (circles) and pentazocine (triangles) in membranes from control (solid symbols) or morphine (10 μM, 48 h)-pretreated (open symbols) SH-SY5Y cells. The response to morphine in cells pretreated with morphine (10 μM, 48 h) in the presence of naloxone (10 μM) is represented by the squares. Incubations were performed at 30°C for 1 h as described in Methods. Values represent means and vertical lines show s.e. from at least three separate experiments (nine separate experiments for morphine) performed in duplicate. Maximal stimulation by morphine was equivalent to 30.0 ± 1.7 fmol [^{35}S]-GTPγS bound mg^{-1} protein.

Table 1 Stimulating effects of morphine on the binding of [^{35}S]-GTPγS to membranes prepared from SH-SY5Y cells exposed to morphine (10μM) for varying times

Time of exposure (h)	*Maximal effect* (%)	*EC_{50}* (nM)
0*	100	76.1±9.7
1.5	96.3±2.4	134±42
3	87.6±4.9	131±30
6	77.8±6.2	140±35
12	83.7±3.7	101±26
24	72.8±6.8	129±19
48*	69.3±6.3**	191±37**
72	70.4±1.2	157±14

SH-SY5Y cells were incubated with morphine (10 μM) for the stated times. Membranes from these cells were prepared as in the Methods section and incubated with [^{35}S]-GTPγS (100 pM) and GDP (3 μM) for 60 min at 30°C, with increasing concentrations of morphine. Maximal effect and EC_{50} values were determined from concentration-effect curves of the data. Values are means±s.e. from three (or *nine) experiments performed in triplicate. (**$P<0.01$, compared to control cells).

in the morphine-induced stimulation of [^{35}S]-GTPγS binding compared to control, naive, cells (Figure 1). However, naloxone itself had a small effect on the basal level of [^{35}S]-GTPγS binding in both control and morphine-treated cells. In control cells naloxone (10 μM) stimulated binding of [^{35}S]-GTPγS by 3.0±0.4 fmol [^{35}S]-GTPγS bound mg^{-1} protein but in morphine pretreated cells inhibited binding by 2.4±0.1 fmol [^{35}S]-GTPγS bound mg^{-1} protein ($n=3$). This compares with the increase in binding induced by morphine (10 μM) in control cells of 30.0±1.7 fmol [^{35}S]-GTPγS bound mg^{-1} protein and in pretreated cells of 20.2±3.9 fmol [^{35}S]-GTPγS bound mg^{-1} protein. The inhibitory effect of naloxone was not due to residual morphine since this was added to control cells immediately before the assay.

Exposure of SH-SY5Y cells to the highly efficacious μ-agonist, DAMGO (10 μM), for 48 h caused a greater loss of responsiveness of [^{35}S]-GTPγS binding compared to morphine (10 μM)-pretreated cells. Thus the maximal morphine-mediated stimulation of [^{35}S]-GTPγS binding was reduced to 58.6±6.6% ($P<0.01$) of the level seen in naive cells (Figure 2a), with a shift in the EC_{50} to 341±68.5 nM ($P<0.05$). Chronic DAMGO treatment of the cells also reduced the effectiveness of DAMGO itself to stimulate [^{35}S]-GTPγS binding, reducing the maximum effect to 62.8±3.95% ($P<0.01$) of the response in control cells and affording EC_{50} values of 46.2±8.1 nM and 240±50.4 nM ($P<0.01$) in membranes from naive and tolerant cells, respectively (Figure 2b). The maximal response to fentanyl was reduced to 42.5±5.9% ($n=12$) of the control level. The increased effectiveness of chronic DAMGO over chronic morphine pretreatment was particularly marked for the partial agonist pentazocine, where a complete loss of the stimulant effect was observed (Figure 2c). Chronic incubation with morphine or DAMGO had no effect on the basal level of [^{35}S]-GTPγS binding.

Incubation of SH-SY5Y cells with the non-specific protein kinase inhibitor H7 (50 μM) (Hidaka *et al*., 1984), during chronic exposure to morphine (10 μM, 48 h), largely prevented the reduction in agonist modulation of [^{35}S]-GTPγS binding (Figure 3). H7 treatment alone did not alter the maximum level of stimulation of [^{35}S]-GTPγS binding over basal binding afforded by DAMGO, morphine or pentazocine, nor did acute H7 have any effect on the response of the [^{35}S]-GTPγS binding to morphine. However, H7 has been shown to compete directly with opioid receptor binding (Aloyo, 1995). To determine if H7 was acting by interfering with the binding of morphine to μ-opioid receptors in whole SH-SY5Y cells competition experiments against [^{3}H]-diprenorphine in Krebs-HEPES buffer were performed. However, the affinity (K_i) of H7

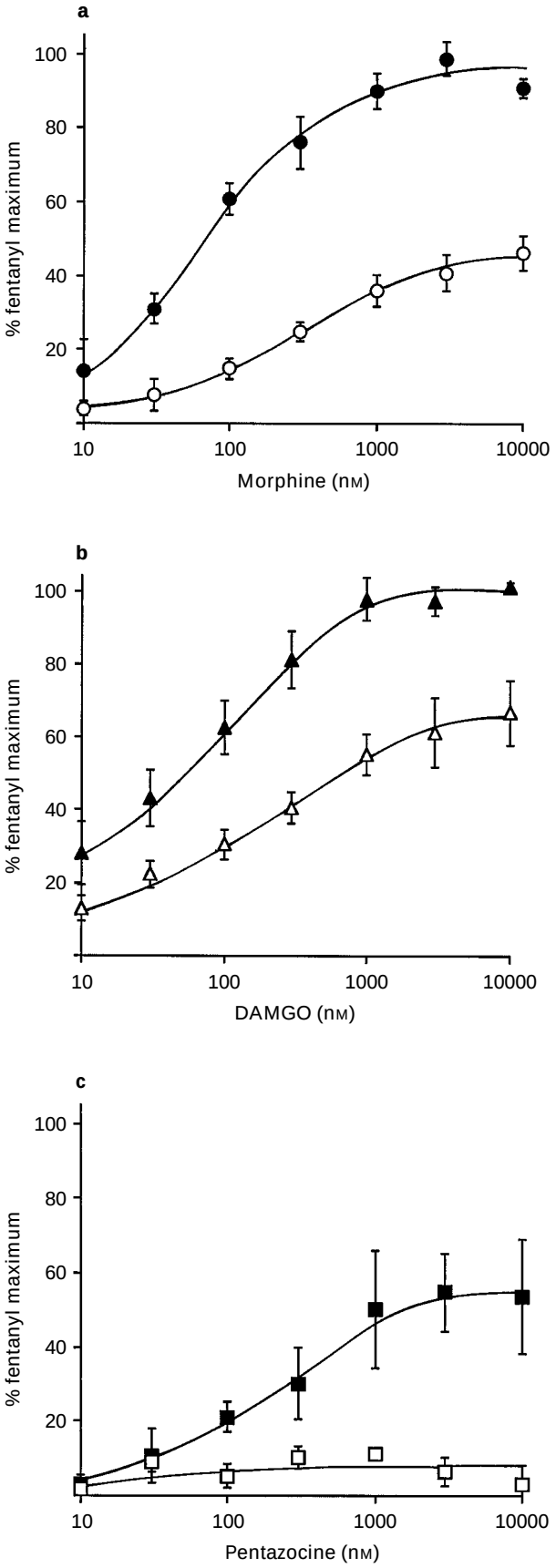

Figure 2 Concentration-effect curves for the stimulation of [^{35}S]-GTPγS binding by (a) morphine, (b) DAMGO and (c) pentazocine to membranes from naive SH-SY5Y cells (solid symbols) and cells pretreated for 48 h with DAMGO (10 μM) (open symbols). Values are given as a percentage of the maximal response to fentanyl in naive cells. Assays were conducted at 30°C for 1 h and values represent means with vertical lines showing s.e. from at least three separate experiments performed in duplicate.

for the μ-opioid receptor was >500 μM compared to the K_i for morphine, determined under the same conditions, of 566 ± 38 nM (Hill coefficient of 0.89 ± 0.10). In contrast to H7, incubation of SH-SY5Y cells with the putative β-adrenoceptor kinase (β-ARK) inhibitor suramin (Miller *et al.*, 1993) (10 μM) during exposure of the cells to morphine (10 μM for 48 h), did not prevent the loss in μ-agonist-modulation of [^{35}S]-GTPγS binding by either morphine, DAMGO or pentazocine (Figure 3).

The undifferentiated SH-SY5Y cells used in this study expressed μ-opioid receptors at a level (B_{max}) of 140 ± 11 fmol mg^{-1} protein, as measured with [^{3}H]-DAMGO, with an affinity (K_d) for [^{3}H]-DAMGO of 1.45 ± 0.31 nM. Chronic DAMGO treatment (10 μM, 48 h) of the cells caused a reduction in the B_{max} for [^{3}H]-DAMGO to 71 ± 7 fmol mg^{-1} protein, but no significant change in the measured affinity (K_d, 0.88 ± 0.18 nM). By use of the antagonist [^{3}H]-diprenorphine to label the opioid sites the number of binding sites in naive cells was determined as 206 ± 36 fmol mg^{-1} protein, and dropped to 65 ± 14 fmol mg^{-1} protein in DAMGO-pretreated cells (Figure 4). The ability of the agonist DAMGO to compete for [^{3}H]-diprenorphine (0.5 nM) binding sites in SH-SY5Y cell membranes in buffer A, as used for [^{35}S]-GTPγS assays, was similar in membranes from both control and morphine-treated (10 μM, 48 h) cells affording K_i values of 79 ± 12 and 60.3 ± 27 nM, respectively (Figure 5). These K_i values are high when compared to expected values obtained in low ionic strength buffers (e.g. Tris) since the assays were performed under the conditions of the [^{35}S]-GTPγS binding assay, which promotes the formation of lower affinity states of the μ-receptor (Carroll *et al.*, 1988; Traynor & Nahorski, 1995). The selective δ-ligand TIPP at a concentration of 10 nM, that is 30 times its affinity for δ-receptors (Schiller *et al.*, 1992), displaced only $19.8 \pm 1.5\%$ ($n=3$) of specifically bound [^{3}H]-diprenorphine (0.5 nM).

Discussion

Binding the μ-opioid agonists morphine and DAMGO to membranes from undifferentiated SH-SY5Y cells caused an increase in the binding of the GTP analogue [^{35}S]-GTPγS. The maximal stimulation induced by pentazocine was less, confirming the partial agonist nature of this opioid. These observations are similar to those previously obtained (Traynor & Nahorski, 1995) but the compounds were less potent, for example the EC_{50} value for morphine was 2.7 fold lower and for pentazocine was 1.4 fold lower. However, binding assays with the selective μ-opioid agonist [^{3}H]-DAMGO revealed a lower number of opioid μ-receptors in the SH-SY5Y cells used in these studies (140 fmol mg^{-1} protein) compared to previous studies (254 fmol mg^{-1} protein). The shift to lower potency with a reduction in receptor number agrees with accepted theories of receptor occupancy (Kenakin, 1993) and confirms the usefulness of the system for investigating pharmacological concepts. The tritiated antagonist [^{3}H]-diprenorphine did afford a maximal number of binding sites (206 fmol mg^{-1} protein) higher than the number of sites labelled by the μ-agonist [^{3}H]-DAMGO. This suggests that the antagonist [^{3}H]-dipre-

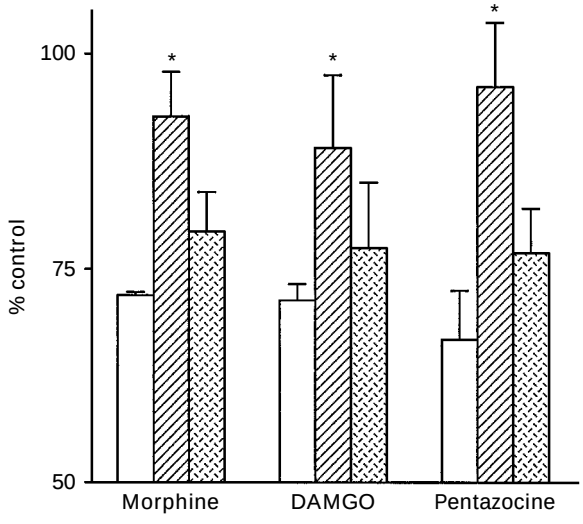

Figure 3 Effect of a single concentration (3 μM) for each of the agonists morphine, DAMGO and pentazocine on the stimulation of [^{35}S]-GTPγS binding to membranes of SH-SY5Y cells pretreated for 48 h with morphine (10 μM) alone (open columns) or in the presence of H7 (50 μM; hatched columns) or suramin (10 μM; stippled columns). Values are given as a percentage of the maximal response to each agonist in naive cells. Assays were conducted at 30°C for 1 h and values represent means ± s.e. from at least three separate experiments performed in duplicate. *Not significantly different from control values ($P<0.05$, Student's t test).

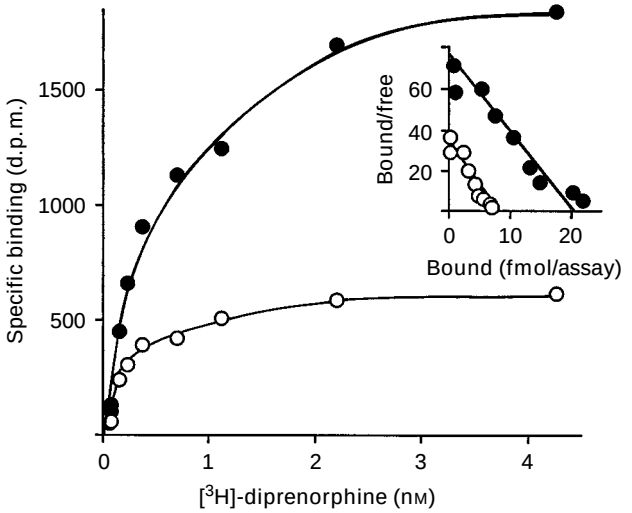

Figure 4 Saturation binding of [^{3}H]-diprenorphine to control (●) and DAMGO-treated (10 μM, 48 h) (O) SH-SY5Y cells. Binding assays were performed in SH-SY5Y cell membranes in Tris buffer (pH 7.4, 50 mM) for 40 min at 25°C as described in Methods. This is a representative experiment which was replicated three times. Inset shows the Scatchard transformation of the data.

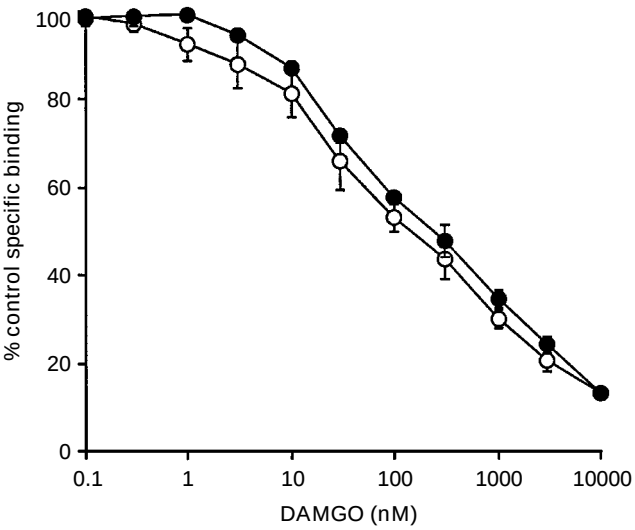

Figure 5 Displacement of the specific binding of [^{3}H]-diprenorphine (0.49 nM) by DAMGO in control (●) and morphine-treated (10 μM, 24 h) (O) SH-SY5Y membranes, conducted in buffer A (20 mM HEPES, 100 mM NaCl and 10 mM $MgCl_2.6H_2O$, pH 7.4) in the presence of 3 μM GDP at 30°C for 1 h as described in Methods. Values represent means with vertical lines showing s.e. from at least three separate experiments performed in duplicate.

norphine labels both high and low affinity states of the opioid μ-receptor, whereas [^{3}H]-DAMGO will only bind to μ-opioid receptors in a high affinity state (Carroll *et al*., 1988; Traynor & Wood, 1989). However, there is a contribution to the observed [^{3}H]-diprenorphine binding from a δ-opioid receptor population since the selective δ-antagonist TIPP (Schiller *et al*., 1992) blocked 20% of the specific binding of [^{3}H]-diprenorphine, and others have suggested the presence of κ_3 binding sites on these cells (Cheng *et al*., 1995).

Incubation of the SH-SY5Y cells with morphine (10 μM) or DAMGO (10 μM) for 48 h led to a decrease in the ability of subsequently added μ-opioid agonists to stimulate the binding of [^{35}S]-GTPγS to membranes of SH-SY5Y cells. The effect of opioid pretreatment was manifest by a rightward shift in the EC_{50} value and a reduction in the maximal [^{35}S]-GTPγS binding, even for the efficacious μ-opioid DAMGO. This was most marked for the partial agonist pentazocine which completely lost the ability to stimulate [^{35}S]-GTPγS binding in membranes from cells which had been treated chronically with DAMGO. Both the loss of maximal effect and the alteration in EC_{50} were specific opioid-receptor mediated events, as indicated by the sensitivity of the processes to naloxone. Chronic treatment with the more efficacious DAMGO afforded a greater effect than that induced by chronic morphine treatment.

The loss of agonist activity following chronic exposure of the cells to morphine or DAMGO was accompanied by a reduction in the number of μ-opioid binding sites measured with either [^{3}H]-DAMGO or [^{3}H]-diprenorphine. A μ-opioid receptor antagonist such as [^{3}H]-diprenorphine which labels both coupled and uncoupled states of the μ-receptor measures total receptor numbers, and confirms an actual loss in membrane-bound receptor number, as shown in both undifferentiated (Carter & Medzihradsky, 1992; Prather *et al*., 1994) and retinoic acid-differentiated SH-SY5Y cells (Zadina *et al*., 1994), rather than just a desensitization and uncoupling from G proteins. The loss of receptor number decreased to a plateau level at approximately 70 fmol mg^{-1} protein, measured with either [^{3}H]-DAMGO or [^{3}H]-diprenorphine, suggesting that approximately 40% of the μ-opioid receptors are refractory to down-regulation. These 'refractory' receptors are confirmed to be of the μ-type, as indicated by the affinity of DAMGO (K_d 0.9 nM) determined from the saturation binding curve. This may indicate the presence of two pools of μ-opioid receptors, only one of which is sensitive to chronic opioid treatment. However, both pools appear to have very similar properties since μ-opioid receptors in naive and morphine pretreated cells were not distinguishable in saturation binding assays or in the displacement of [^{3}H]-diprenorphine binding by DAMGO and the EC_{50} values for stimulation of [^{35}S]-GTPγS binding differed only by 2–3 fold. It will be intriguing to discover if the μ-opioid binding site population resistant to down-regulation relates to the finding that the effects of opioids on pupil size and the constipating effects of opioids are generally resistant to tolerance (Adams & Holtzman, 1990).

A reduction in receptor number, as observed following morphine or DAMGO pretreatment of the cells, would be expected to result in a shift to the right in the concentration-effect curve for a full agonist, accompanied by a reduction in the maximal effect for a less efficacious compound (Kenakin, 1993). In the locus coeruleus μ-opioid receptors couple to an inwardly rectifying K^+ channel. Chronic treatment of rats with morphine results in a shift in the concentration curve for DAMGO acting on this channel, while for the less efficacious normorphine a flattening of the dose-effect curve occurs (Christie *et al*., 1987). Similarly, chronic morphine treatment of retinoic acid differentiated SH-SY5Y cells leads to a 7 fold shift in the ability of DAMGO to inhibit an N-type Ca^{2+} current in these cells, whereas morphine shows a reduction in the maximal effect (Kennedy & Henderson, 1991), and there is a reduction in the maximal inhibition of adenylyl cyclase by morphine (Yu & Sadee, 1988). In contrast, the present findings in undifferentiated SH-SY5Y cells show both a shift in the EC_{50} value and a decrease in the maximal response, not only for morphine but also for the highly efficacious agonists DAMGO and fentanyl. Although unexpected, similar findings have been obtained. Thus, in retinoic acid-differentiated SH-SY5Y cells chronically treated with DAMGO, a decreased maximal effect of DAMGO inhibition of adenylyl cyclase and stimulation of GTPase is seen (Carter & Medzihradsky, 1993a). However, this effect is not seen with chronic morphine pretreatment. On the other hand, chronic exposure of 7315c pituitary cells to morphine for 3 h results in a reduction in the maximal ability of the full-agonist [D-Ala^2, D-Leu^5]enkephalin to reduce adenylyl cyclase activity and, with longer exposure to morphine, a complete loss of the activity (Puttfarcken *et al*., 1988).

The efficacy of drugs is not only related to the drug but to tissue factors. For example, morphine is a full agonist in the guinea-pig ileum, a partial agonist in the mouse vas deferens, but an antagonist in the rat vas deferens (Traynor, 1994). These effects can be explained by differences in receptor reserve. A low μ-opioid receptor reserve in SH-SY5Y cells, as observed for the cloned μ-receptor expressed in CHO or BHK cells (Pak *et al*., 1996), could explain the present findings. However, there is evidence for a μ-receptor reserve in SH-SY5Y cells since the ratio of K_i to EC_{50} values for stimulation of [^{35}S]-GTPγS binding is >1 (Traynor & Nahorski, 1995). In addition, at least in retinoic acid treated cells, reduction in receptor number by 50% with the alkylating agent β-chlornaltexamine has no effect on the maximal inhibition of adenylyl cyclase and a 90% reduction in receptor number following chronic treatment with levorphanol only reduces the maximal response by a small amount (Carter & Medzihradsky, 1993a). Other tissue factors altered by the tolerance process may therefore be responsible for the reduction in maximal [^{35}S]-GTPγS binding. It is known that the μ-opioid receptor in undifferentiated SH-SY5Y cells activates several different, pertussis-toxin sensitive, heterotrimeric G proteins (Laugwitz *et al*., 1993). The [^{35}S]-GTPγS binding assay does not distinguish these different G proteins but rather represents a measure of all G proteins activated following agonist occupation of a particular receptor. The reduced maximal response to the full agonist DAMGO may indicate that the loss of receptors is accompanied by a reduction in associated G proteins or altered function of these G proteins, such that there is a reduced capacity for [^{35}S]-GTPγS binding. Indeed, chronic treatment of rat striatal neurones with morphine causes a reduction in the number of inhibitory G-proteins (Van Vliet *et al*., 1993) and chronic DAMGO treatment of retinoic acid differentiated SH-SY5Y cells decreases the content of $G_{o\alpha}$, the G protein responsible for coupling opioid μ-receptors to adenylyl cyclase in these cells (Carter & Medzihradsky, 1993b). However, any such G protein changes would have to be specific for the μ-opioid receptor since in SH-SY5Y cells tolerance to μ-opioid agonists appears to be homologous (Kennedy & Henderson, 1991; Lameh *et al*., 1992; Prather *et al*., 1994).

In naive SH-SY5Y cells naloxone showed a small degree of activity resulting in stimulation of [^{35}S]-GTPγS of 10% of that seen with morphine. In contrast, in morphine pre-treated cells naloxone caused a small inhibition of basal [^{35}S]-GTPγS binding, indicating a possible inverse agonist action. These results agree with findings at the β_2 receptor where, following desensitization, very weak agonists may show inverse agonist activity (Chidiac *et al*., 1996). Such an inverse agonist action of naloxone would lead to a reduction in the level of activated G protein and could be responsible for the overshoot in cyclic AMP production seen with naloxone following chronic treatment of SH-SY5Y cells with morphine (Wang *et al*., 1994).

Opioid receptors may be substrates for β-adrenoceptor kinase (β-ARK). Indeed, κ-receptors in cells with dominant negative mutations of β-ARK do not down-regulate or desensitize (Raynor *et al*., 1994). The present experiments have shown that the putative β-ARK inhibitor suramin at 10 μM, that is 100 times higher than its reported activity in A_{431} cells (Miller *et al*., 1993), did not block the development of tolerance as determined by changes in [^{35}S]-GTPγS binding. In contrast,

the non-specific protein kinase inhibitor H7 (Hidaka *et al*., 1984) was able to prevent the effects of chronic morphine pretreatment, supporting evidence that phosphorylation is a key step in receptor desensitization and the development of tolerance to opioids (Childers, 1991; Nestler, 1993; Raynor *et al*., 1994; Wang *et al*., 1994; Pei *et al*., 1995; Ueda *et al*., 1995). It is possible that the observed effects of H7 are unrelated to an inhibition of kinase enzymes, but are due to a direct action of the compound at opioid receptors (Aloyo, 1995). However, H7 displaced [^{3}H]-diprenorphine specifically bound to whole cells under physiological conditions (Krebs buffer) with a K_i of >500 μM, whilst the K_i for morphine was 566 nM, similar to values previously obtained in SH-SY5Y cells (Elliott *et al*., 1994) and guinea-pig brain membranes (Carroll *et al*., 1988). At the level of morphine (10 μM) used for the chronic studies it was determined, by use of the Cheng and Prussoff equation (Cheng & Prussoff, 1973), that approximately 5 mM H7 would be required to displace 50% of the morphine from opioid receptors. It is unlikely, therefore, that H7, at the concentration used, prevented the effects of chronic morphine treatment by direct competition at μ-opioid binding sites. Thus, unlike previous findings in non-physiological buffers (Aloyo, 1995), the results do indicate that H7 can be used to study phosphorylation processes associated with opioid receptor systems.

In conclusion, the results show that the stimulation of [^{35}S]-GTPγS binding mediated by μ-opioid receptors in SH-SY5Y cells is sensitive to long-term exposure to μ-agonists. A tolerance develops that is manifested by a small reduction in agonist potency together with a reduced maximal effect, even for a highly efficacious agonist such as DAMGO. The mechanism of this tolerance involves a reduction in receptor number and may require altered function of inhibitory G protein. This appears to involve phosphorylation of components within the receptor and/or G protein systems. Since guanine nucleotide exchange is the first measurable biochemical event following agonist occupation of seven-transmembrane domain G protein-linked receptors, the binding of [^{35}S]-GTPγS can be used as a functional measure to probe readily mechanisms behind the development of tolerance very early in the signal-transduction cascade.

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