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The effects of recombinant rat μ-opioid receptor activation in CHO cells on phospholipase C, $[Ca^{2+}]_i$ and adenylyl cyclase

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1 The rat μ-opioid receptor has recently been cloned, yet its second messenger coupling remains unclear. The endogenous μ-opioid receptor in SH-SY5Y cells couples to phospholipase C (PLC), increases $[Ca^{2+}]_i$ and inhibits adenylyl cyclase (AC). We have examined the effects of μ-opioid agonists on inositol(1,4,5)trisphosphate ($Ins(1,4,5)P_3$), $[Ca^{2+}]_i$ and adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation in Chinese hamster ovarian (CHO) cells transfected with the cloned μ-opioid receptor.

2 Opioid receptor binding was assessed with [^{3}H]-diprenorphine ([^{3}H]-DPN) as a radiolabel. $Ins(1,4,5)P_3$ and cyclic AMP were measured by specific radioreceptor assays. $[Ca^{2+}]_i$ was measured fluorimetrically with Fura-2.

3 Scatchard analysis of [^{3}H]-DPN binding revealed that the B_{max} varied between passages. Fentanyl (10 pM – 1 μM) dose-dependently displaced [^{3}H]-DPN, yielding a curve which had a Hill slope of less than unity (0.6 ± 0.1), and was best fit to a two site model, with pK_i values (% of sites) of 9.97 ± 0.4 ($27 \pm 4.8\%$) and 7.68 ± 0.07 ($73 \pm 4.8\%$). In the presence of GppNHp (100 μM) and Na^+ (100 mM), the curve was shifted to the right and became steeper (Hill slope $= 0.9 \pm 0.1$) with a pK_i value of 6.76 ± 0.04.

4 Fentanyl (0.1 nM – 1 μM) had no effect on basal, but dose-dependently inhibited forskolin (1 μM)-stimulated, cyclic AMP formation ($pIC_{50} = 7.42 \pm 0.23$), in a pertussis toxin (PTX; 100 ng ml^{-1} for 24 h)-sensitive and naloxone-reversible manner ($K_i = 1.7$ nM). Morphine (1 μM) and [D-Ala^2, MePhe^4, gly(ol)^5]-enkephalin (DAMGO, 1 μM) also inhibited forskolin (1 μM)-stimulated cyclic AMP formation, whilst [D-Pen^2, D-Pen^5], enkephalin (DPDPE, 1 μM) did not.

5 Fentanyl (0.1 nM – 10 μM) caused a naloxone (1 μM)-reversible, dose-dependent stimulation of $Ins(1,4,5)P_3$ formation, with a pEC_{50} of 7.95 ± 0.15 ($n = 5$). PTX (100 ng ml^{-1} for 24 h) abolished, whilst Ni^{2+} (2.5 mM) inhibited (by 52%), the fentanyl-induced $Ins(1,4,5)P_3$ response. Morphine (1 μM) and DAMGO (1 μM), but not DPDPE (1 μM), also stimulated $Ins(1,4,5)P_3$ formation. Fentanyl (1 μM) also caused an increase in $[Ca^{2+}]_i$ (80 ± 16.4 nM, $n = 6$), reaching a maximum at 26.8 ± 2.5 s. The increase in $[Ca^{2+}]_i$ remained elevated until sampling ended (200 s) and was essentially abolished by the addition of naloxone (1 μM). Pre-incubation with naloxone (1 μM, 3 min) completely abolished fentanyl-induced increases in $[Ca^{2+}]_i$.

6 In conclusion, the cloned μ-opioid receptor when expressed in CHO cells stimulates PLC and inhibits AC, both effects being mediated by a PTX-sensitive G-protein. In addition, the receptor couples to an increase in $[Ca^{2+}]_i$. These findings are consistent with the previously described effector-second messenger coupling of the endogenous μ-opioid receptor.

Keywords: μ-Opioid receptor; phospholipase C; adenylyl cyclase; transfected CHO cells; $[Ca^{2+}]_i$; inositol(1,4,5)trisphosphate; cyclic AMP

Introduction

The main therapeutic use of opioid drugs, for example morphine and fentanyl, is for the management of pain. However, these compounds can also produce side-effects, particularly respiratory depression, and are highly addictive. Both these beneficial and detrimental effects are predominately mediated by the μ-opioid receptor (Reisine, 1995), and therefore a better understanding of the structure and functional coupling of this receptor would aid the development of improved opioid analgesics.

A major advance to this end was the cloning of the μ-opioid receptor by several independent groups (Chen *et al*., 1993; Thompson *et al*., 1993; Bunzow *et al*., 1995), with probes based on the sequence of the δ-opioid receptor cloned by Evans *et al*. (1992) and Kieffer *et al*. (1992). All of the μ-opioid receptor clones found consist of 398 amino acids and have identical sequences, except that in some clones amino acid 245 is valine (Thompson *et al*., 1993), whilst in others it is isoleucine (Chen *et al*., 1993; Bunzow *et al*., 1995). However, as the conversion of valine-245 to isoleucine-245 is conservative, this is unlikely to be functionally relevant (Bunzow *et al*., 1995).

Use of these μ-, as well as the δ- and κ-, clones to study opioid receptor structure-function relationships has been extensive (for reviews see Uhl *et al*., 1994; Reisine, 1995; Knapp *et al*., 1995). However, their use to study opioid-induced signal transduction has been more limited, even though the benefits of using an homogeneous population of receptors in a well defined system are obvious, especially as expression levels can be varied (Chakrabarti *et al*., 1995a). Most studies have examined the opioid-induced inhibition of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation in cells expressing the cloned receptors, including the cloned μ-opioid receptor (Bunzow *et al*., 1995; Chakrabarti *et al*., 1995a). Activation of the cloned μ-opioid receptor has also been shown to inhibit voltage-sensitive Ca^{2+} channels (VSCCs) (Piros *et al*., 1995; Morikawa *et al*., 1995) and activate K^+ channels (Chen & Yu, 1994; Kovoor *et al*., 1995; Mestek *et al*., 1995). However, the coupling of the cloned μ-opioid receptor to phospholipase C (PLC) remains controversial with one group finding both an inhibition (Johnson *et al*., 1994) and a stimulation (Johnson *et*

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al., 1995) of inositol(1,4,5)triphosphate (Ins(1,4,5)P_3) in cells expressing the μ-opioid receptor. Another group has also shown a cloned μ-opioid receptor-mediated increase in Ins(1,4,5)P_3 formation, but only at high doses of D-Ala^2,Me-Phe^4,gly(ol)^5]-enkephalin (DAMGO) (Zimprich *et al*., 1995). It is worth noting that the cloned δ-opioid receptor also couples to phospholipase C (PLC) (Tsu *et al*., 1995; Ueda *et al*., 1995a), as does the endogenous δ-opioid receptor in NG108-15 cells (Smart & Lambert, 1996a).

Endogenous μ-opioid receptors in SH-SY5Y human neuroblastoma cells also inhibit N-type VSCCs (Seward *et al*., 1991), as well as basal and forskolin-stimulated cyclicAMP formation (Lambert *et al*., 1993; Smart *et al*., 1995a). Furthermore, the μ-opioid receptor also stimulates Ins(1,4,5)P_3 formation in these cells (Smart *et al*., 1994; 1995a), by opening L-type VSCCs (Smart & Lambert, 1995; Smart *et al*., 1995a) via a pertussis toxin (PTX)-sensitive G-protein (Smart *et al*., 1994), and so allowing Ca^{2+} influx (Wandless *et al*., 1996) which activates PLC.

The present study was undertaken to examine the second messenger coupling of the cloned μ-opioid receptor (Bunzow *et al*., 1995) expressed in Chinese hamster ovarian (CHO) (CHOμ) cells, in order to clarify whether these cloned receptors couple to PLC, as seen with the endogenous receptors.

A preliminary account of part of these data was presented to the British Pharmacological Society (Lambert *et al*., 1996).

Methods

Cell culture and harvesting

Chinese hamster ovary cells transfected with the cloned rat μ-opioid receptor, TS11, (CHOμ, passage 11–22; Bunzow *et al*., 1995) were cultured in Hams F12 medium supplemented with 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2.5 μg ml^{-1} fungizone, and 10% foetal calf serum. Stock (but not experimental) cultures also contained 100 μg ml^{-1} G418. Some cells were cultured with pertussis toxin (PTX, 100 ng ml^{-1}) for 24 h before being harvested (Smart *et al*., 1994).

Cells were harvested with 10 mM HEPES-buffered saline/0.02% EDTA, pH 7.4, washed twice with, and resuspended in Krebs/HEPES buffer, pH 7.4, of the following composition (in mM): Na^+ 143.3, K^+ 4.7, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 125.6, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2, glucose 11.7 and HEPES 10.

Measurement of opioid binding

This was carried out as described previously (Lambert *et al*., 1993). Briefly, the saturation and displacement studies were performed at 20°C for 60 min in a 1 ml assay volume, with [^{3}H]-diprenorphine ([^{3}H]-DPN) and CHOμ membranes in 50 mM Tris-HCl, pH 7.4. Non-specific binding was defined in the presence of 10 μM naloxone. Displacement studies used a fixed concentration of [^{3}H]-DPN (~0.2 nM) and various concentrations of fentanyl (10 pM–1 μM) in the presence and absence of the non-hydrolysable guanosine 5′-triphosphate (GTP) analogue guanylylimidodiphosphate (GppNHp, 100 μM) and Na^+ (100 mM). Bound and free radioactivity were separated by rapid vacuum filtration.

Measurement of cyclicAMP

Whole cell suspensions (0.3 ml) were incubated in the presence of isobutylmethylxanthine (IBMX, 1 mM), with forskolin (1 μM), fentanyl (0.1 nM–1 μM), DAMGO (1 μM), morphine (1 μM), [D-Pen^2, D-Pen^5]-enkephalin (DPDPE, 1 μM) or naloxone (1 nM–10 μM) in various combinations at 37°C for 15 min. Reactions were terminated as described previously (Hirst & Lambert, 1995). The concentration of cyclic AMP was measured in the supernatants by use of a specific radioreceptor assay, as described previously (Brown *et al*., 1971).

Measurement of Ins(1,4,5)P_3

Whole cell suspensions (0.3 ml) were preincubated at 37°C, with or without naloxone (1 μM) or Ni^{2+} (2.5 mM), for 15 min. The cells were then incubated with fentanyl (0.1 nM–10 μM), morphine (1 μM), DAMGO (1 μM) or DPDPE (1 μM) for 0–300 s. Reactions were terminated and Ins(1,4,5)P_3 extracted as described previously (Smart & Lambert 1995). Ins(1,4,5)P_3 was assayed by use of a specific radioreceptor mass assay, as described by Challiss *et al*. (1988).

Measurement of $[Ca^{2+}]_i$

This was performed fluorimetrically in cells loaded with the Ca^{2+} indicator dye Fura-2, as described previously (Smart *et al*., 1995b; Hirst & Lambert, 1995). Briefly, cells were incubated at 37°C with 3 μM Fura-2/AM for 30 min, washed (15 ml buffer) and then incubated at 20°C for a further 20 min. $[Ca^{2+}]_i$ was measured in 2 ml suspensions at 37°C in a Perkin-Elmer LS50B spectrofluorimeter set at 340/380 nm excitation with emission at 510 nm. $[Ca^{2+}]_i$ was then calculated from the 340/380 ratio according to Grynkiewicz *et al*. (1985), with R_{max} and R_{min} determined by use of Triton-X (0.1%) and EGTA (4.5 mM, pH > 8.0), respectively.

Data analysis

Unless otherwise stated, all data are given as mean ± s.e.mean of 3–10 individual experiments done once or in duplicate. pEC_{50} (log half maximal stimulation) and pIC_{50} (log half maximal inhibition) values were obtained by computer-assisted curve fitting of the individual curves with GRAPHPAD-PRISM. In displacement studies IC_{50} values are corrected for the competing mass of [^{3}H]-DPN. Statistical comparisons were made where appropriate by ANOVA and/or Student's *t* test, and were considered significant when $P < 0.05$.

Results

The specific binding of [^{3}H]-DPN to CHOμ membranes was dose-related and saturable (Figure 1). Scatchard analysis (Figure 1) revealed B_{max} and K_d values of 266 fmol mg^{-1} protein and 0.19 nM, respectively, at passage 12.

The B_{max} varied between passages (Figure 2), but this did not affect the second messenger coupling. For example, comparing passages 14 and 17 there was a 3.7 fold difference in receptor expression (326 vs 88 fmol mg^{-1} protein), yet the pIC_{50} values for the fentanyl-induced inhibition of cyclic AMP formation were essentially the same (7.57 ± 0.10 and 7.50 ± 0.13, respectively, $n = 3$ each), as was the peak fentanyl (100 nM)-induced Ins(1,4,5)P_3 response (80.6 ± 6.9 and 78.2 ± 11.1 pmol mg^{-1} protein, respectively, $n = 3$ each). [^{3}H]-DPN binding was dose-dependently displaced by fentanyl (Figure 3), and analysis showed that the curves were shallow (Hill slope 0.6 ± 0.1) and best fit to two sites yielding a pK_i of 9.97 ± 0.4 (0.1 nM) at the high affinity site (27.0 ± 4.8%) and a pK_i of 7.68 ± 0.07 (20.7 nM) at the low affinity site (73 ± 4.8%). In the presence of GppNHp (100 μM) and Na^+ (100 mM), fentanyl also displaced [^{3}H]-DPN in a dose-dependent manner. However, the curves were steeper (Hill slope = 0.9 ± 0.1) and best fit to a one site model with a pK_i value of 6.76 ± 0.04 (184 nM), (Figure 3).

Fentanyl (1 μM) had no effect on basal cyclic AMP formation (data not shown), but significantly ($P < 0.05$) inhibited (I_{max} = 36.4 ± 6.1%, $n = 4$) forskolin (1 μM)-stimulated cyclic-AMP formation. The inhibition of forskolin (1 μM)-induced adenylyl cyclase activity by fentanyl was dose-dependent (Figure 4a), with pIC_{50} of 7.42 ± 0.23 (38 nM). Naloxone reversed fentanyl (1 μM) inhibition of forskolin (1 μM) stimulated cyclic AMP formation in a dose-dependent manner with a pIC_{50} of 6.67 ± 0.1 (210 nM; $n = 4$), which when corrected for

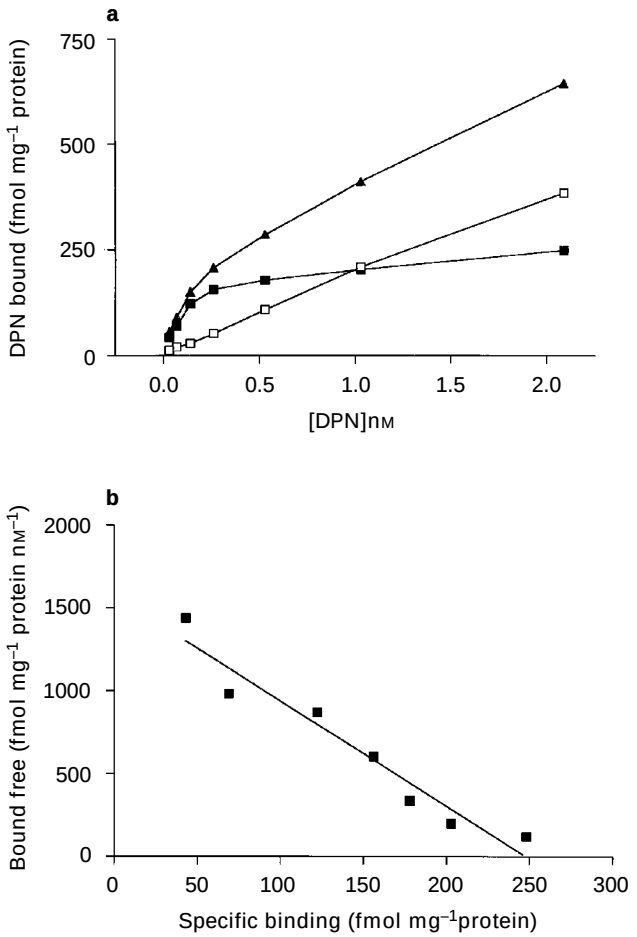

Figure 1 (a) ([^{3}H]-DPN) binding in CHOμ membranes was dose-dependent and saturable. (▲) Total binding, (□) non-specific and (■) specific binding are shown. (b) The associated Scatchard plot. Studies were performed at 20°C in 1 ml volumes for 60 min, with [^{3}H]-DPN (0.03–2.0 nM). Non-specific binding was defined in the presence of 10 μM naloxone. Data are from a single experiment, typical of $n=3$ at passage 12.

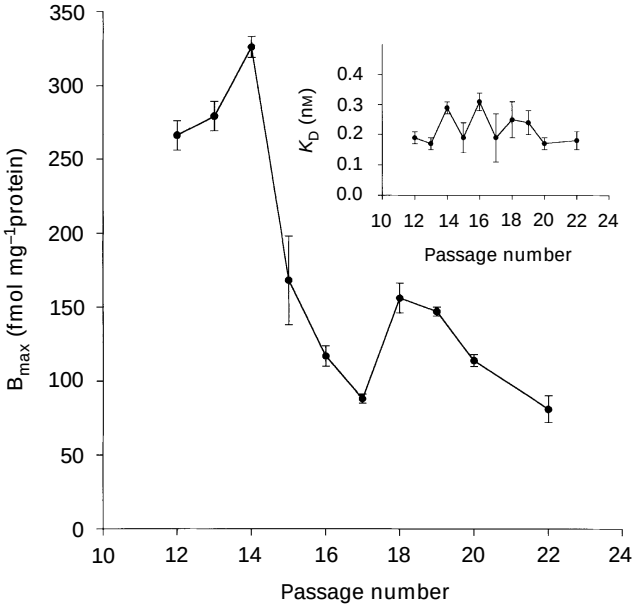

Figure 2 The B_{max} for [^{3}H]-diprenorphine ([^{3}H]-DPN) binding varied between passages. Studies were performed as in Figure 1, with cells from various passages. Data are mean ± s.e.mean (vertical lines), where $n=3-6$, except for passages 17 and 22 where the mean and range of $n=2$ are given.

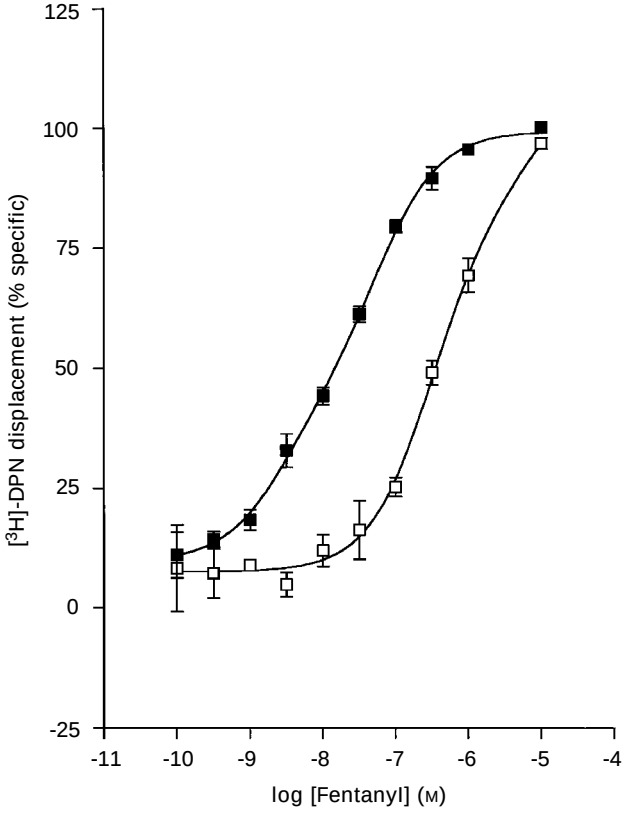

Figure 3 Fentanyl dose-dependently displaced [^{3}H]-diprenorphine ([^{3}H]-DPN) in CHOμ membranes in the absence and presence of GppNHp (100 μM) and Na^+ (100 mM). Studies were performed at 20°C in 1 ml volumes for 60 min, in the absence (■) or presence (□) of GppNHp (100 μM) and Na^+ (100 mM) with a fixed (~0.2 nM) concentration of [^{3}H]-DPN, and fentanyl (10 pM–1 μM) as the displacer. Non-specific binding was defined in the presence of 10 μM naloxone. Data are mean ± s.e.mean (vertical lines), where $n=3$.

the concentration of fentanyl used, yielded a K_i of 1.7 nM for naloxone (Figure 4b). DAMGO (1 μM) and morphine (1 μM) also inhibited forskolin (1 μM)-stimulated cyclic AMP formation, whilst DPDPE (1 μM) did not (Table 1). PTX (100 ng ml^{-1} for 24 h) abolished the fentanyl-induced inhibition of forskolin-stimulated cyclicAMP formation (Table 1).

Fentanyl (1 μM) stimulated Ins(1,4,5)P_3 formation, which rose 2.4 fold from basal levels (35.8 ± 4.0 pmol mg^{-1} protein, $n=6$) over 30 s, and was then sustained until sampling ended at 300 s (Figure 5a). This stimulation of Ins(1,4,5)P_3 formation by fentanyl was dose-dependent (Figure 5b), with a pEC_{50} of 7.95 ± 0.15 (11.2 nM). The fentanyl-induced Ins(1,4,5)P_3 response was naloxone (1 μM)-reversible (Table 2), and morphine (1 μM) and DAMGO (1 μM) also stimulated Ins(1,4,5)P_3 formation, whilst DPDPE (1 μM) did not (Table 1). Furthermore Ni^{2+} (2.5 mM) inhibited (52%) the fentanyl (1 μM)-induced Ins(1,4,5)P_3 response (Table 2), suggesting that the μ-opioid-induced activation of PLC involves a Ca^{2+}-entry driven component. Pretreatment with PTX (100 ng ml^{-1} for 24 h) abolished the fentanyl (1 μM)-induced Ins(1,4,5)P_3 response (Table 1), indicating the response was mediated by G_i/G_o.

Fentanyl (1 μM) also increased $[Ca^{2+}]_i$, which rose from basal levels (106 ± 20 nM, $n=6$) to a maximum (186 ± 16.4 nM, $n=6$) at 26.8 ± 2.5 s, and then remained elevated until sampling ended (200 s). Pre-incubation with naloxone (1 μM, 3 min) completely blocked fentanyl-induced increases in $[Ca^{2+}]_i$ (Figure 6, $n=6$). The increase in $[Ca^{2+}]_i$ remained elevated until sampling ended (200 s) and was essentially abolished by the addition of naloxone (1 μM, 200 s, Figure 6 inset).

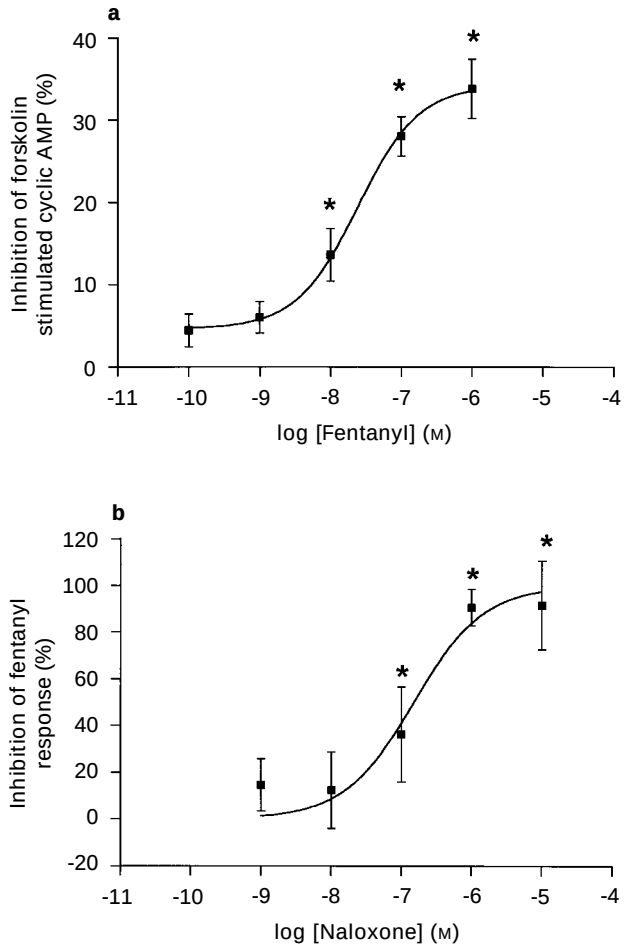

Figure 4 (a) Fentanyl dose-dependently inhibited forskolin-stimulated cyclic AMP formation in CHOμ cells, and this effect was reversed by naloxone. (b) Whole cell suspensions (0.3 ml) were incubated at 37°C in the presence of IBMX (1 mM) with forskolin (1 μM), naloxone (1 nM – 10 μM) and fentanyl (0.1 nM – 1 μM) for 60 s. Cyclic AMP was measured with a radioreceptor assay. Data are mean ± s.e.mean, where $n = 4-6$. Dose-response curves are $P < 0.05$ by ANOVA. *Denotes $P < 0.05$ (t test) different when compared to forskolin alone (a) or fentanyl + forskolin (b).

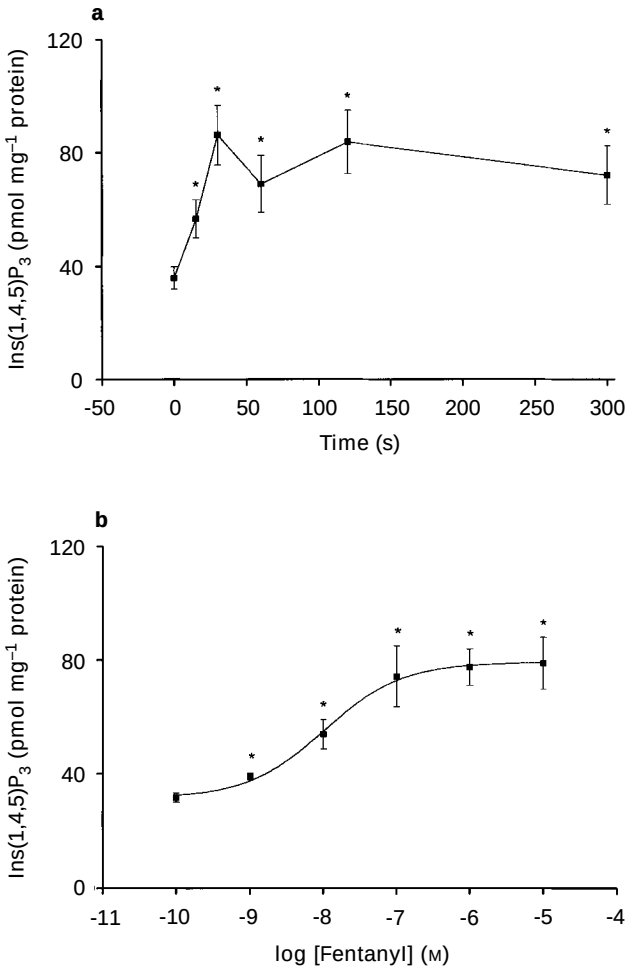

Figure 5 Fentanyl stimulated Ins(1,4,5)P_3 formation in a time-(a) and dose-(b) dependent manner in CHOμ cells. Whole cell suspensions (0.3 ml) were preincubated at 37°C for 15 min and then incubated with fentanyl (1 μM) for 0 – 300 s (a) or fentanyl (0.1 nM – 10 μM) for 60 s (b). Ins(1,4,5)P_3 was measured by a stereospecific radioreceptor mass assay. Data are mean ± s.e.mean (vertical lines), where $n = 5-6$. Whole time course and dose-response curve are $P < 0.05$ by ANOVA. *Denotes $P < 0.05$ (t test) increased compared to basal.

Discussion

We show here that the TS11 rat μ-opioid receptor clone when expressed in CHO cells couples to PLC, as well as coupling to AC (Bunzow *et al*., 1995). Both the coupling to AC and PLC are mediated by a PTX-sensitive G-protein in a naloxone-reversible manner, where μ-opioid receptor occupation inhibits cyclic AMP formation, stimulates Ins(1,4,5)P_3 formation and increases $[Ca^{2+}]_i$. Furthermore, activation of PLC by opioids in the CHOμ cells is dependent, at least in part, on Ca^{2+} influx, suggesting the cloned μ-opioid receptor also opens a Ca^{2+} channel. This second messenger coupling is identical to that displayed by the endogenous μ-opioid receptor in SH-SY5Y cells (Lambert *et al*., 1993; Smart *et al*., 1994).

When expressed in CHO cells the TS11 clone bound [^{3}H]-DPN in a dose-related and saturable manner, yielding a K_d (0.17 – 0.31 nM) consistent with that (0.23 nM) previously obtained for this clone (Bunzow *et al*., 1995), as well as those found for other μ-opioid receptor clones (Chen *et al*., 1993; Thompson *et al*., 1993; Knapp *et al*., 1995). Furthermore, both the K_d and the levels of expression of TS11 in CHOμ cells were comparable with those of the endogenous receptor in SH-SY5Y cells (Lambert *et al*., 1993). However, the expression levels varied (88 – 326 fmol mg^{-1} protein) between passages, although the K_d remained relatively constant. Despite the de-

Table 1 Pharmacology of the opioid-induced Ins(1,4,5)P_3 and cyclic AMP responses in CHOμ cells

	Ins(1,4,5)P_3 (pmol mg^{-1} protein)	*Inhibition of FSK stimulated cyclic AMP* (%)
Basal	28.2 ± 4.6	–
Fetanyl	87.4 ± 8.3*	46.5 ± 2.2#
+ PTX	32.7 ± 7.4	3.7 ± 3.7
Basal	15.3 ± 2.6	–
Morphine	47.2 ± 8.6*	30.9 ± 6.1#
DAMGO	78.6 ± 10.3*	41.4 ± 4.8#
DPDPE	16.4 ± 3.6	8.6 ± 3.6

Whole cell suspension (0.3 ml) of pertussis toxin (PTX, 100 ng ml^{-1} for 24 h)-treated or untreated cells were incubated with forskolin (FSK, 1μM), fentanyl (1 μM), morphine (1 μM), DAMGO (1 μM) or DPDPE (1 μM) in various combinations for 60 s. Data are mean ± s.e.mean, $n = 4-5$. *Denotes increased ($P < 0.05$) compared to paired basal Ins(1,4,5)P_3 formation (which varied between, but not within, the two experiments). #Denotes decreased ($P < 0.05$) compared to forskolin alone.

Table 2 Naloxone and Ni^{2+} inhibit the opioid-induced Ins(1,4,5)P_3 response

	Ins(1,4,5)P_3(pmol mg^{-1} protein)
Basal,	15.3±2.6
+naloxone (1 μM)	18.0±4.5
+Ni^{2+} (2.5mM)	7.0±1.9
Fentanyl (1 μM)	72.3±9.7*
+naloxone (1 μM)	19.5±3.7#
+Ni^{2+} (2.5 mM)	35.2±6.7*#

Whole cell suspensions (0.3 ml) were preincubated with or without naloxone (1 μM) or Ni^{2+} (2.5 mM) at 37°C for 15 min, and then incubated with fentanyl (1 μM) for 60 s. Data are mean±s.e.mean, $n=4$. *Denotes increased ($P<0.05$) compared to basal. #Denotes decreased ($P<0.05$) compared to fentanyl alone.

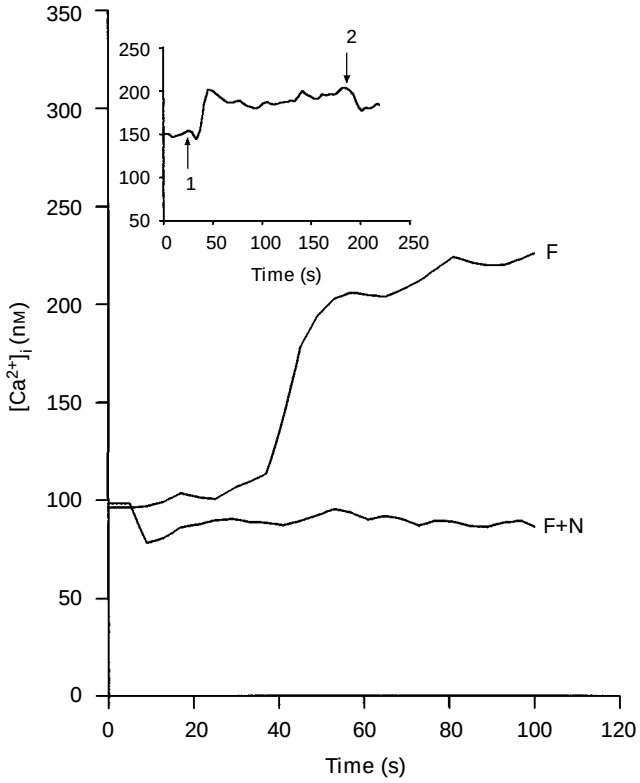

Figure 6 A typical trace (of $n=6$) depicting a rise in $[Ca^{2+}]_i$ with the addition of fentanyl (F, 1 μM). Preincubation with naloxone (1 μM) abolished the response (F+N, $n=6$). Inset shows that naloxone (arrow 2) reversed the fentanyl (arrow 1)-induced elevation of $[Ca^{2+}]_i$ at 200 s ($n=4$).

clining B_{max} the functional responses were unaffected. Similarly, in CHO cells expressing the cloned δ-opioid receptor, expression levels had little effect on functional coupling (Law *et al*., 1994). However, firm conclusions relating to expression-dependent second messenger coupling cannot be drawn based on a 3–4 fold variation in B_{max}.

Fentanyl, DAMGO and morphine inhibited forskolin-stimulated cyclic AMP formation in CHOμ cells, whilst DPDPE did not, in agreement with the previously demonstrated μ-opioid-mediated inhibition of AC in cells expressing this (Bunzow *et al*., 1995) and other (Chen *et al*., 1993; Thompson *et al*., 1993) μ-opioid receptor clones. The fentanyl-induced inhibition of AC in CHOμ cells was dose-dependent, with an IC_{50} (38 nM) comparable to that (27.1 nM) in SH-SY5Y cells (Lambert *et al*., 1993). This effect was reversed in a dose-dependent manner by naloxone, indicating an opioid receptor-mediated event. Similarly, DAMGO dose-dependently inhibited cyclic AMP formation in cells expressing this (Bunzow *et al*., 1995) and other (Chakrabarti *et al*., 1995a; Piros *et al*., 1995) μ-opioid receptor clones. It is worth noting that the μ-opioid-induced inhibition of cyclic AMP in CHOμ cells was PTX-sensitive. These findings are consistent with those from studies with other cloned (Reisine, 1995; Zimprich *et al*., 1995) or endogenous (Smart *et al*., 1994) μ-opioid receptors. Fentanyl had no effect on basal cyclic AMP formation in CHOμ cells, even though fentanyl inhibited basal cyclic AMP in SH-SY5Y cells (Smart *et al*., 1995). Moreover, the cloned δ-opioid receptor activated type II AC when expressed in kidney 293 cells (Tsu *et al*., 1995).

Fentanyl caused a naloxone-reversible stimulation of Ins(1,4,5)P_3 formation in CHOμ cells, which was dose-dependent, with an EC_{50} (11.2 nM) similar to that (16.0 nM) obtained from studies with cells expressing endogenous μ-opioid receptors (Smart *et al*., 1994). Furthermore, DAMGO and morphine, but not DPDPE, also stimulated Ins(1,4,5)P_3 formation in CHOμ cells. These data clearly indicate that the cloned μ-opioid receptor in CHOμ cells couples positively to PLC. Comparison of the displacement curve with the Ins(1,4,5)P_3 and cyclic AMP dose-response curves for fentanyl shows that at the concentration (~10 nM) where the high affinity site saturates there is a significant inhibition of cyclic-AMP and stimulation of Ins(1,4,5)P_3, indicating that the high affinity (i.e. G protein-coupled) site is functionally coupled to AC and PLC. Moreover, in displacement experiments, GppNHp (100 μM) produced a classical rightward shift in the curve and a loss of high affinity sites. There have been previous limited studies which suggested the cloned μ-opioid receptor coupled to PLC, but these were controversial as one group showed stimulation, but only at high doses (Zimprich *et al*., 1995), whilst another group found both inhibition (Johnson *et al*., 1994) and stimulation (Johnson *et al*., 1995). Moreover, the cloned δ-opioid receptor has been found to couple to PLC (Tsu *et al*., 1995).

The fentanyl-induced Ins(1,4,5)P_3 response in CHOμ cells was PTX-sensitive. The endogenous μ-opioid receptor-mediated activation of PLC is also PTX-sensitive (Smart *et al*., 1994), as is the cloned μ-opioid receptor-mediated activation of K^+ channels (Chen & Yu, 1994). Similarly, the cloned (Ueda *et al*., 1995a; Tsu *et al*., 1995) and endogenous (Jin *et al*., 1994; Smart & Lambert 1996a) δ-opioid receptor-mediated activation of PLC is PTX-sensitive. A recent study with cloned μ- and κ-opioid receptors in *Xenopus* oocytes has shown that these receptors only activate PLC-driven Cl^- currents if the oocytes were cotransfected with G_{i1} (Ueda *et al*., 1995b). However, in CHO cells the cloned μ-opioid receptor interacts with G_{i2}, G_{i3} & G_{o2}, but not G_{i1} (Chakrabarti *et al*., 1995b). Therefore, it appears that the type of $G_{i\alpha}$ subunit involved in the cloned μ-opioid receptor-mediated activation of PLC depends on the type of host cell used. In addition, it is likely that the differences in PLC coupling in different cell lines result from differing PLC isoform expression.

The PTX-sensitivity of the fentanyl-induced Ins(1,4,5)P_3 response in CHOμ cells suggests that the μ-opioid-induced activation of PLC may be caused by βγ subunits liberated from G_i, as proposed for the activation of PLC by the cloned δ-opioid receptor (Tsu *et al*., 1995). Alternatively, as the fentanyl-induced Ins(1,4,5)P_3 response was inhibited by Ni^{2+}, Ca^{2+} influx could be activating a calcium sensitive isoform of PLC in CHOμ cells. This is in agreement with studies of SH-SY5Y cells, where the endogenous μ-opioid receptor opens an L-type VSCC (Smart & Lambert, 1995; Smart *et al*., 1995a), thus allowing Ca^{2+} influx to activate PLC (Smart *et al*., 1994). Moreover, the δ-opioid receptor also opens L-type VSCCs in ND8-47 cells (Tang *et al*., 1995). The absence of [^{3}H]-PN200-110 binding sites on CHOμ membranes (data not shown) suggests a non L-channel-mediated effect. The cloned μ-opioid receptor closes VSCCs when expressed in NG108-15 (Morikawa *et al*., 1995) and GH_3 (Piros *et al*., 1995) cells. However, the TS11 clone expressed in CHO cells could open a 'receptor-

operated' Ca^{2+} channel, as shown for the M_3 muscarinic receptor (Lambert & Nahorski, 1990). Indeed, fentanyl increased $[Ca^{2+}]_i$ in CHOμ cells, as previously found for SH-SY5Y cells (Wandless *et al*., 1996). Other studies also suggest that activation of the cloned μ-opioid receptor increases $[Ca^{2+}]_i$ (Johnson *et al*., 1995; Zimprich *et al*., 1995).

In conclusion, the μ-opioid receptor clone TS11, expressed in CHO cells, couples positively to PLC, and negatively to AC via PTX-sensitive G-proteins. In addition, it is also coupled to an increase in $[Ca^{2+}]_i$, possibly via a receptor-operated Ca^{2+} channel. The stimulatory effects of opioids described here appear to be a widespread phenomenon (Smart & Lambert, 1996b) warranting further study.

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References

BROWN, B.L., ALBANO, J.D.M., EKINS, R.D. & SGHERZI, A.M. (1971). A simple and sensitive assay method for the measurement of adenosine 3′ : 5′-cyclic monophosphate. *Biochem. J*., **121,** 516 – 522.

BUNZOW, J.R., ZHANG, G., BOUVIER, C., SAEZ, C., RONNEKLEIV, O.K., KELLY, M.J. & GRANDY, D.K. (1995). Characterization and distribution of a cloned rat μ-opioid receptor. *J. Neurochem*., **64,** 14 – 24.

CHAKRABARTI, S., LAW, P.Y. & LOH, H.H. (1995a). Neuroblastoma $Neuro_{2A}$ cells stably expressing a cloned μ-opioid receptor: a specific cellular model to study acute and chronic effects of morphine. *Mol. Brain Res*., **30,** 269 – 278.

CHAKRABARTI, S., PRATHER, P.L., LAW, P.Y. & LOH, H.H. (1995b). Ability of μ-opioid ligands to promote α-azidoanilido [^{32}P]GTP labeling of multiple G protein α subunits. *J. Neurochem*., **64,** 2534 – 2543.

CHALLISS, R.A.J., BATTY, I.H. & NAHORSKI, S.R. (1988). Mass measurements of inositol(1,4,5)triphosphate in rat cerebral cortex slices using a radioreceptor assay: effects of neurotransmitters and depolarization. *Biochem. Biophys. Res. Commun*., **157,** 684 – 691.

CHEN, Y., MESTEK, A., LIU, J., HURLEY, J.A. & YU, L. (1993). Molecular cloning and functional expression of a μ-opioid receptor from rat brain. *Mol. Pharmacol*., **44,** 8 – 12.

CHEN, Y. & YU, L. (1994). Differential regulation by cAMP-dependent protein kinase and protein kinase C of the μ-opioid receptor coupling to a G protein-activated K^+ channel. *J. Biol. Chem*., **269,** 7839 – 7842.

EVANS, C.J., KEITH, D.E., MORRISON, H., MAGENDZO, K. & EDWARDS, R.H. (1992). Cloning of a delta opioid receptor by functional expression. *Science*, **258,** 1952 – 1955.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem*., **260,** 3440 – 3450.

HIRST, R.A. & LAMBERT, D.G. (1995). Adenylyl cyclase in SH-SY5Y human neuroblastoma cells is regulated by intra- and extracellular calcium. *Biochem. Pharmacol*., **49,** 1633 – 1640.

JIN, W., LEE, N.M., LOH, H.H. & THAYER, S.A. (1994). Opioids mobilize calcium from inositol 1,4,5-triphosphate-sensitive stores in NG108-15 cells. *J. Neurosci*., **14,** 1920 – 1929.

JOHNSON, P.S., WANG, J.B. & UHL, G.R. (1995). μ-Opioid receptor structure function studies II: effects on second messenger systems. *Regul. Pep*., **54,** 139 – 140.

JOHNSON, P.S., WANG, J.B., WANG, W.F. & UHL, G.R. (1994). Expressed mu opiate receptor couples to adenylyl cyclase and phosphatidylinositol turnover. *NeuroReport*, **5,** 507 – 509.

KIEFFER, B.L., BEFORT, K., GAVERIAUX-RUFF, C. & HIRTH, C.G. (1992). The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. *Proc. Natl. Acad. Sci. U.S.A*., **89,** 12048 – 12052.

KNAPP, R.J., MALATYNSKA, E., COLLINS, N., FANG, L., WANG, J.Y., HRUBY, V.J., ROESKE, W.R. & YAMAMURA, H.I. (1995). Molecular biology and pharmacology of cloned opioid receptors. *FASEB J*., **9,** 516 – 525.

KOVOOR, A., HENRY, D.J. & CHAVKIN, C. (1995). Agonist-induced desensitization of the mu opioid receptor coupled potassium channel (GIRK1). *J. Biol. Chem*., **270,** 589 – 595.

LAMBERT, D.G., ATCHESON, R., HIRST, R.A. & ROWBOTHAM, D.J. (1993). Effects of morphine and its metabolites on opiate receptor binding, cAMP formation and [^{3}H]noradrenaline release from SH-SY5Y cells. *Biochem. Pharmacol*., **46,** 1145 – 1150.

LAMBERT, D.G., HIRST, R.A., SMART, D. & GRANDY, D.K. (1996). Characterization of CHO cells expressing the cloned rat μ-opioid receptor. *Br. J. Pharmacol. Proc. Suppl*., **117,** 6P.

LAMBERT, D.G. & NAHORSKI, S.R. (1990). Muscarinic-receptor-mediated changes in intracellular Ca^{2+} and inositol 1,4,5-trisphosphate mass in a human neuroblastoma cell line, SH-SY5Y. *Biochem. J*., **265,** 555 – 562.

LAW, P.Y., MCGINN, T.M., WICK, M.J., ERIKSON, L.J., EVANS, C. & LOH, H.H. (1994). Analysis of delta-opioid receptor activities stably expressed in CHO cell lines: function of receptor density? *J. Pharmacol. Exp. Ther*., **271,** 1686 – 1694.

MESTEK, A., HURLEY, J.H., BYE, L.S., CAMPBELL, A.D., CHEN, Y., TIAN, M., LIU, J., SCHULMAN, H. & YU, L. (1995). The human μ-opioid receptor: modulation of functional desensitization by calcium/calmodulin-dependent protein kinase and protein kinase C. *J. Neurosci*., **15,** 2396 – 2406.

MORIKAWA, H., FUKUDA, K., KATO, S., MORI, K. & HIGASHIDA, H. (1995). Coupling of the cloned μ-opioid receptor with the ω-conotoxin-sensitive Ca^{2+} current in NG108-15 cells. *J. Neurochem*., **65,** 1403 – 1406.

PIROS, E.T., PRATHER, P.L., LOH, H.H., LAW, P.Y., EVANS, C.J. & HALES, T.G. (1995). Ca^{2+} channel and adenylyl cyclase modulation by cloned μ-opioid receptors in GH_3 cells. *Mol. Pharmacol*., **47,** 1041 – 1049.

REISINE, T. (1995). Opiate receptors. *Neuropharmacology*, **34,** 463 – 472.

SEWARD, E., HAMMOND, C. & HENDERSON, G. (1991). μ-Opioid receptor mediated inhibition of N-type calcium-channel current. *Proc. R. Soc. Lond. (B.)*, **244,** 129 – 135.

SMART, D. & LAMBERT, D.G. (1995). Desensitization of the μ-opioid activation of phospholipase C in SH-SY5Y cells: the role of protein kinases C and A and Ca^{2+} -activated K^+ currents. *Br. J. Pharmacol*., **116,** 2655 – 2660.

SMART, D. & LAMBERT, D.G. (1996a). δ-Opioids stimulate inositiol(1,4,5)triphosphate formation, and so mobilize Ca^{2+} from intracellular stores, in undifferentiated NG108-15 cells. *J. Neurochem*., **66,** 1462 – 1467.

SMART, D. & LAMBERT, D.G. (1996b). The stimulatory effects of opioids and their possible role in the development of tolerance. *Trends Pharmacol. Sci*., **17,** 264 – 269.

SMART, D., SMITH, G. & LAMBERT, D.G. (1994). μ-Opioid receptor stimulation of inositol(1,4,5)trisphosphate formation via a pertussis toxin-sensitive G protein. *J. Neurochem*., **62,** 1009 – 1014.

SMART, D., SMITH, G. & LAMBERT, D.G. (1995a). μ-opioids activate phospholipase C in SH-SY5Y human neuroblastoma cells via calcium channel opening. *Biochem. J*., **305,** 577 – 582.

SMART, D., WANDLESS, A. & LAMBERT, D.G. (1995b). Activation of phospholipase C in SH-SY5Y neuroblastoma cells by potassium-induced calcium entry. *Br. J. Pharmacol*., **116,** 1797 – 1800.

TANG, T., KIANG, J.G., COTE, T.E. & COX, B.M. (1995). Antisense oligodeoxynucleotide to the G_{i2} protein α subunit sequence inhibits an opioid-induced increase in the intracellular free calcium concentration in ND8-47 neuroblastoma X dorsal root ganglion hybrid cells. *Mol. Pharmacol*., **48,** 189 – 193.

THOMPSON, R.C., MANSOUR, A., AKIL, H. & WATSON, S.J. (1993). Cloning and pharmacological characterization of a rat μ-opioid receptor. *Neuron*, **11,** 903 – 913.

TSU, R.C., CHAN, J.S.C. & WONG, Y.H. (1995). Regulation of multiple effectors by the cloned δ-opioid receptor: stimulation of phospholipase C and Type II adenylyl cyclase. *J. Neurochem*., **64,** 2700 – 2707.

UEDA, H., MIYAMAE, T., FUKUSHIMA, N., TAKESHIMA, H., FUKUDA, K., SASAKI, Y. & MISU, Y. (1995b). Opioid μ- and κ-receptor mediate phospholipase C activation through Gi1 in Xenopus oocytes. *Mol. Brain Res*., **32,** 166 – 170.

UEDA, H., MIYAMAE, T., HAYASHI, C., WATANABE, S., FUKUSHIMA, N., SASAKI, Y., IWAMURA, T. & MISU, Y. (1995a). Protein kinase C involvement in homologous desensitization of δ-opioid receptor coupled to G_{i1}-phospholipase C activation in Xenopus oocytes. *J. Neurosci.*, **15,** 7485–7499.

UHL, G.R., CHILDERS, S. & PASTERNAK, G. (1994). An opiate receptor gene family reunion. *Trends Neurosci.*, **17,** 89–93.

WANDLESS, A.L., SMART, D. & LAMBERT, D.G. (1996). Fentanyl increases intracellular Ca^{2+} concentrations in SH-SY5Y cells. *Br. J. Anaesth.*, **76,** 461–463.

ZIMPRICH, A., SIMON, T. & HOLLT, V. (1995). rMOR1 and rMOR1B: comparison of both rat μ-opioid receptor variants with respect to second messenger coupling. *Analgesia*, **1,** 886–889.

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