μ-Opioids activate phospholipase C in SH-SY5Y human neuroblastoma cells via calcium-channel opening

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We have recently reported that, in SH-SY5Y cells, μ-opioid receptor occupancy activates phospholipase C via a pertussis toxin-sensitive G-protein. In the present study we have further characterized the mechanisms involved in this process. Fentanyl (0.1 μM) caused a monophasic increase in inositol 1,4,5-trisphosphate mass formation, with a peak (20.5 ± 3.6 pmol/mg of protein) at 15 s. Incubation in Ca^{2+}-free buffer abolished this response, while Ca^{2+} replacement 1 min later restored the stimulation of inositol 1,4,5-trisphosphate formation (20.1 ± 0.6 pmol/mg of protein). In addition, nifedipine (1 nM–0.1 mM), an L-type Ca^{2+}-channel antagonist, caused a dose-dependent inhibition of inositol 1,4,5-trisphosphate formation, with an IC_{50} of 60.3 ± 1.1 nM. Elevation of endogenous β/γ subunits by selective activation of δ-opioid and α_2 adrenoceptors failed to stimulate phospholipase C. Fentanyl also caused a dose-dependent (EC_{50} of 16.2 ± 1.0 nM), additive enhancement of carbachol-induced inositol 1,4,5-trisphosphate formation. In summary, we have demonstrated that in SH-SY5Y cells activation of the μ-opioid receptor allows Ca^{2+} influx to activate phospholipase C. However, the possible role of this mechanism in the process of analgesia remains to be elucidated.

INTRODUCTION

Activation of opioid receptors has generally been considered to have inhibitory effects on neuronal post-receptor events (North, 1989; Childers, 1991). However, opioids also have excitatory effects on neuronal activity (Crain and Shen, 1990). For example, it has been shown that opioids can stimulate 3′,5′-cyclic adenosine monophosphate (cAMP) formation via a pertussis toxin-sensitive G-protein in neuronal cells from the rat olfactory bulb (Olianas and Onali, 1993). Furthermore, we have recently reported that, in SH-SY5Y human neuroblastoma cells, μ-opioids cause a transient, monophasic increase in inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] formation, which involves a pertussis toxin-sensitive G-protein and is blocked by Ni^{2+} (Smart et al., 1994a).

One possible mechanism for this stimulation of Ins(1,4,5)P_3 formation by opioids is that β/γ subunits from G_i/G_o could activate a Ca^{2+}-sensitive isoform of phospholipase C (PLC) such as PLCβ2 or PLCβ3 (Camps et al., 1992; Carozzi et al., 1993). However, only activation of the μ-opioid receptor in SH-SY5Y cells stimulates Ins(1,4,5)P_3 formation (Smart et al., 1994a), yet both μ- and δ-opioid receptors couple to G_i. Since there are 2–5-fold more μ than δ (and no κ) receptors in these cells (Yu et al., 1986), δ-receptor activation may not liberate sufficent β/γ subunits to activate PLC. SH-SY5Y cells also express α_2 adrenoceptors, which couple via G_i to inhibit adenylate cyclase activity (Lambert and Nahorski, 1990a). Levels of α_2 expression are similar to those of the δ-opioid receptor.

Alternatively, the μ-opioid receptor could mediate the opening of a Ca^{2+} channel, allowing Ca^{2+}-influx-dependent activation of a Ca^{2+}-sensitive isoform of PLC. SH-SY5Y cells possess both L- and N-type voltage-sensitive Ca^{2+} channels (VSCC) (Toselli et al., 1991; Morton et al., 1992), and opioids have been shown to open VSCCs in NG108-15 cells and astrocytes (Jin et al., 1992; Eriksson et al., 1993), although it should be emphasized that opioids generally close VSCCs (Porzig, 1990).

In this study we have addressed both of these hypotheses. First, we increased the intracellular concentration of β/γ subunits from G_i by selective activation of μ-, δ- and α_2-receptors. Secondly, we further characterized the Ca^{2+} dependency of the μ-opioid stimulation of Ins(1,4,5)P_3 formation. We have shown that, in SH-SY5Y cells, activation of the μ-opioid receptor allows Ca^{2+} influx to activate PLC.

MATERIALS AND METHODS

Cell culture and harvesting

SH-SY5Y human neuroblastoma cells (passages 70–90) were cultured in Minimum Essential Medium with Earle's salts supplemented with 2 mM L-glutamine, 100 i.u./ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, and 10% fetal calf serum (GIBCO, U.K.).

Cells were harvested with 10 mM Hepes-buffered saline/0.02% EDTA, pH 7.4, washed twice with, and then resuspended (final volume 0.3 ml) in, Krebs/Hepes buffer, pH 7.4, of the following composition (in mM), unless stated otherwise below: Na^+, 143.3; K^+, 4.7; Ca^{2+}, 2.5; Mg^{2+}, 1.2; Cl^-, 125.6; HCO_3^-, 25; $H_2PO_4^-$, 1.2; SO_4^{2-}, 1.2; glucose, 11.7; and Hepes, 10.

Measurement of Ins(1,4,5)P_3

Whole-cell suspensions (0.3 ml) were preincubated at 37 °C in Ca^{2+}-containing or Ca^{2+}-free buffer, with or without naloxone (1 μM), atropine (1 μM) or nifedipine (1 nM–0.1 mM) for

Abbreviations used: $[Ca^{2+}]_i$, intracellular concentration of free calcium; cAMP, 3′,5′-cyclic adenosine monophosphate; PLC, phospholipase C; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; DAMGO, [D-Ala^2, MePhe^4, Gly(ol)^5]enkephalin; DPDPE, [D-Pen^{2,5}]enkephalin; IBMX, isobutylmethylxanthine; VSCC, voltage-sensitive calcium channels; ROCC, receptor-operated calcium channels.

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15 min. The cell suspensions were then incubated with fentanyl (1 nM–10 μM), carbachol (0.1 μM–1 mM), [D-Ala^2, MePhe^4, Gly(ol)^5]enkephalin (DAMGO; 1 μM), [D-Pen^{2,5}]enkephalin (DPDPE; 10 μM), or clonidine (1 μM) in various combinations for 0–300 s. In some studies, cells were suspended in Ca^{2+}-free buffer 1–2 min before incubation with fentanyl (0.1 μM) and the Ca^{2+} (2.5 mM) subsequently replaced (1–11 min later). Reactions were terminated by the addition of 0.3 ml of 1 M trichloroacetic acid.

Ins(1,4,5)P_3 was extracted with Freon/octylamine (1:1, v/v) and neutralized with 25 mM $NaHCO_3$. Ins(1,4,5)P_3 was assayed using a bovine adrenocortical binding protein and [^{3}H]-Ins(1,4,5)P_3 (41 Ci/mol; Amersham, U.K.) at 4 °C. Authentic Ins(1,4,5)P_3 (0.036–12 pmol; Siemat, U.K.) in buffer, taken through an identical extraction process, was used as a standard. Non-specific binding was defined in the presence of excess Ins(1,4,5)P_3 (0.3 nmol). Bound [^{3}H]Ins(1,4,5)P_3 was separated by rapid vacuum filtration (Challiss et al., 1988).

Measurement of cAMP

Whole-cell suspensions (0.3 ml) were incubated in the presence of isobutylmethylxanthine (IBMX) (1 mM), with or without fentanyl (1 μM) at 37 °C for 0–300 s. Reactions were terminated by the addition of 20 μl of HCl (10 M), 20 μl of NaOH (10M), and 180 μl of Tris (1 M, pH 7.5). The concentration of cAMP was measured in the supernatants by a specific radiolabelled receptor assay, as described previously (Brown et al., 1971).

Data analysis

All data are given as mean ± S.E.M. unless otherwise stated, and are shown as primary data (pmol/mg of protein) throughout, although some variation between passages does occur. EC_{50} (half-maximal stimulation) and IC_{50} (half-maximal inhibition) values were obtained by computer-assisted curve fitting using GRAPHPAD. Statistical comparisons made where appropriate by Student's t-test (paired or unpaired) and/or ANOVA and were considered significant when $P < 0.05$.

RESULTS

Fentanyl (0.1 μM) caused a monophasic increase in Ins(1,4,5)P_3 formation, which peaked (26.5 ± 3.6 pmol/mg of protein) at 15 s and returned to basal levels (6.0 ± 0.5 pmol/mg of protein) between 1 and 2 min (Figure 1), as previously reported (Smart et al., 1994a). In contrast, fentanyl (1 μM) inhibited cAMP formation, with maximum inhibition (40%) occurring in 30 s and being maintained as long as the agonist remained on the receptor (Figure 1), although it should be noted that phosphodiesterase activity was inhibited with IBMX.

DAMGO (1 μM) stimulated Ins(1,4,5)P_3 formation at 15 s, while DPDPE (10 μM) and clonidine (1 μM), alone or in combination, did not (Table 1). Indeed, neither DPDPE nor clonidine had any effect on Ins(1,4,5)P_3 formation throughout the entire 0–300 s time course (results not shown). Furthermore, neither DPDPE (10 μM) nor clonidine (1 μM), alone or in combination, enhanced the stimulation of Ins(1,4,5)P_3 formation caused by a submaximal dose of DAMGO (1 μM) (Table 1).

Fentanyl (0.1 μM) had no effect on Ins(1,4,5)P_3 formation in Ca^{2+}-free buffer, as previously reported (Smart et al., 1994a). However, when Ca^{2+} (2.5 mM) was replaced 1 min after the fentanyl challenge, there was a subsequent substantial stimulation of Ins(1,4,5)P_3 formation (Figure 2). This response was time-dependent as, while Ca^{2+} replacement at 2 min caused a small but

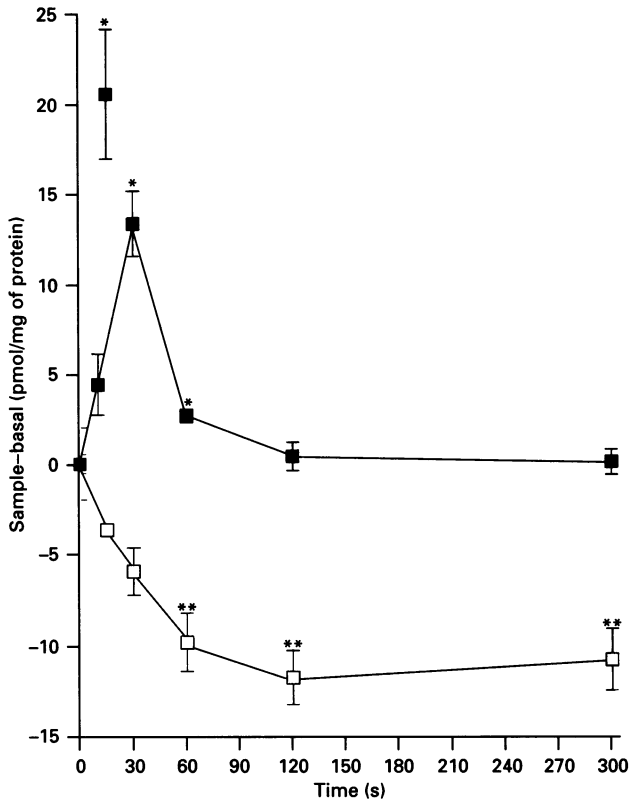

Figure 1 The effects of fentanyl on Ins(1,4,5)P_3 and cAMP formation in SH-SY5Y cells

Cell suspensions (0.3 ml) were incubated with fentanyl (0.1 μM) for 0–300 s. Ins(1,4,5)P_3 and cAMP were measured by specific radio-receptor mass assays. Data are mean ± S.E.M., where $n = 3$. Whole curves for Ins(1,4,5)P_3 formation (■) and cAMP formation (□) are $P < 0.05$ by ANOVA. * denotes $P < 0.05$ (t-test) increased compared with basal, and ** denotes $P < 0.05$ (t-test) decreased compared with basal.

Table 1 Ins(1,4,5)P_3 formation following G_i activation in SH-SY5Y cells

Whole-cell suspensions (0.3 ml) were preincubated at 37 °C for 15 min, and then incubated with various combinations of DAMGO (1 μM), DPDPE (10 μM) and clonidine (1 μM) for 15 s. Ins(1,4,5)P_3 was measured by a specific radio-receptor mass assay. All results are mean ± S.E.M. * denotes significantly ($P < 0.05$, t-test) increased compared with basal.

Addition	n	Ins(1,4,5)P_3 (pmol/mg of protein)
None	5	8.2 ± 0.4
DPDPE	5	9.3 ± 0.6
Clonidine	5	7.3 ± 0.6
DPDPE + clonidine	5	8.9 ± 0.7
DAMGO	5	27.7 ± 1.0*
DAMGO + DPDPE	5	27.3 ± 1.1*
DAMGO + clonidine	5	27.4 ± 0.7*
DAMGO + DPDPE + clonidine	5	27.5 ± 1.0*

significant ($P < 0.05$) stimulation of Ins(1,4,5)P_3 formation, Ca^{2+} replacement at 4 min or later was ineffective (Figure 2 inset). In addition, nifedipine (1 nM–0.1 mM), an L-type Ca^{2+}-channel antagonist (Spedding and Paoletti, 1992), caused a dose-de-

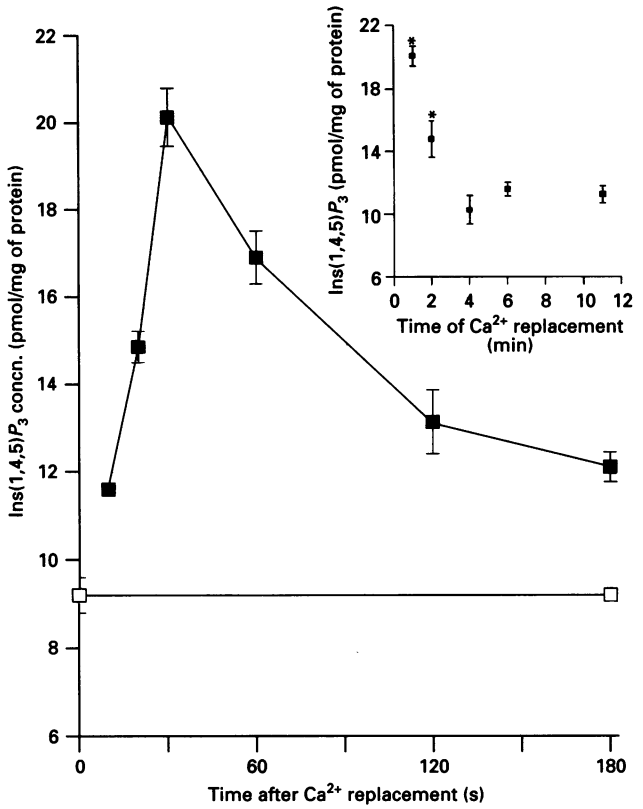

Figure 2 Effect of Ca^{2+} replacement on fentanyl-induced Ins(1,4,5)P_3 formation

Main panel depicts time course of fentanyl-induced Ins(1,4,5)P_3 formation following Ca^{2+} replacement at 1 min. Inset shows that the effect of Ca^{2+} replacement was time dependent. Whole-cell suspensions were preincubated in Ca^{2+}-free buffer at 37 °C for 15 min, and then incubated with fentanyl (0.1 μM) for 0–11 min before the Ca^{2+} (2.5 mM) was (■), or was not (□), replaced. Data are mean ± S.E.M., where $n = 5$. Whole time course $P < 0.05$ by ANOVA. * denotes $P < 0.05$ (*t*-test) increased compared with basal.

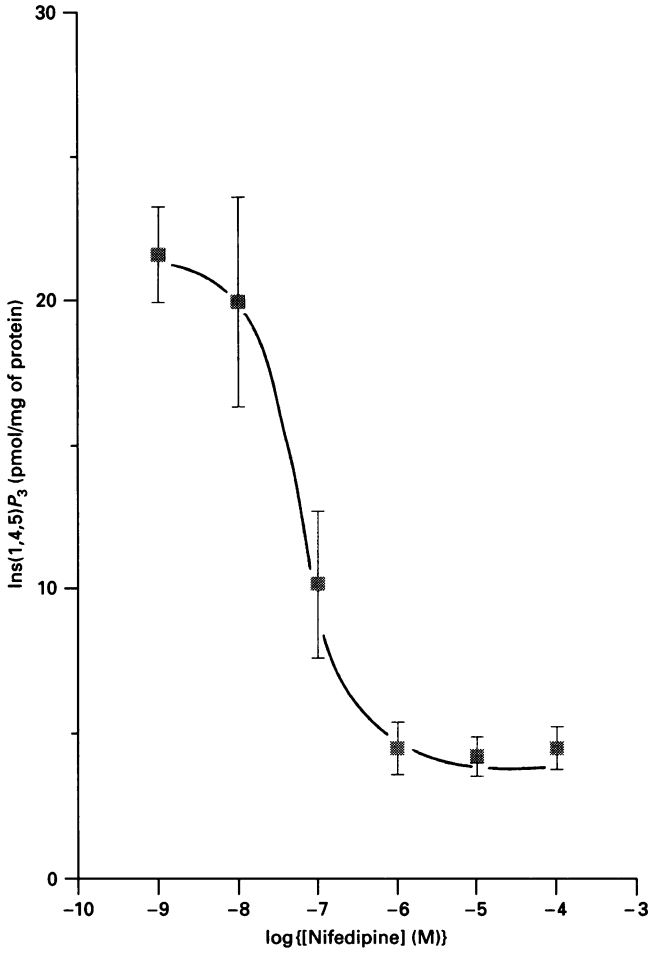

Figure 3 Dose-dependent inhibition of fentanyl-induced Ins(1,4,5)P_3 formation by nifedipine

Whole-cell suspensions were preincubated at 37 °C with nifedipine (1 nM–0.1 mM) for 15 min, and then incubated with fentanyl (0.1 μM) for 15 s. Data are mean ± S.E.M., where $n = 5$. Basal Ins(1,4,5)P_3 formation was 4.1 ± 0.4 pmol/mg of protein.

pendent inhibition of fentanyl-stimulated Ins(1,4,5)P_3 formation, with an IC_{50} of 60.3 ± 1.1 nM (Figure 3).

Carbachol (100 μM) caused a biphasic stimulation of Ins(1,4,5)P_3 formation (Figure 4), which rose from basal (12.8 ± 1.2 pmol/mg of protein) to a peak (8.2-fold basal) at 10 s and then declined until 60 s to a steady plateau phase (1.8-fold basal) which was maintained until sampling ended (300 s). Basal Ins(1,4,5)P_3 did not change over the 300 s time course (Figure 4 and data not shown). The peak phase, which releases Ca^{2+} from the intracellular stores, is Ca^{2+} independent, while the plateau phase, which may or may not release Ca^{2+}, is dependent on the influx of extracellular Ca^{2+} (Lambert and Nahorski, 1990b), via non-voltage-sensitive Ca^{2+} channels (Lambert et al., 1990).

Fentanyl (0.1 μM) enhanced carbachol-induced Ins(1,4,5)P_3 formation at both the peak (by 20.8 pmol/mg of protein) and plateau (by 16.0 pmol/mg of protein) phases (Figure 4). However, neither DPDPE (10 μM) nor clonidine (1 μM) enhanced carbachol-stimulated Ins(1,4,5)P_3 formation (results not shown). The enhancement of carbachol-stimulated Ins(1,4,5)P_3 formation by fentanyl was simply additive, rather than synergistic, with the carbachol component being totally reversed by atropine, as was the opioid component by naloxone (Table 2). Furthermore, this opioid–muscarinic interaction was dose-dependent for both fentanyl and carbachol (results not shown), with the EC_{50} values for the enhancement of carbachol-induced Ins(1,4,5)P_3 formation by fentanyl (16.2 ± 1.0 nM) and the enhancement of fentanyl-induced Ins(1,4,5)P_3 formation by carbachol (12.0 ± 1.9 μM) being similar to their previously reported EC_{50} values for the stimulation of basal Ins(1,4,5)P_3 formation (Smart et al., 1994a,b).

DISCUSSION

We report here, for the first time in a neuronal preparation, that activation of the μ-opioid receptor allows Ca^{2+} influx to stimulate PLC activity, resulting in increased Ins(1,4,5)P_3 formation. Rises in Ins(1,4,5)P_3 concentrations have been implicated in a number of cellular responses, most notably the release of Ca^{2+} from intracellular stores and possibly Ca^{2+} entry (Berridge, 1993). Moreover, it has been proposed that Ins(1,4,5)P_3-induced rises in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) increase K^+ efflux, and thus cause hyperpolarization, and that this may be one of the mechanisms underlying morphine analgesia (Lipp, 1991).

In the current study, fentanyl stimulated Ins(1,4,5)P_3 formation and inhibited cAMP formation. While opioids are generally

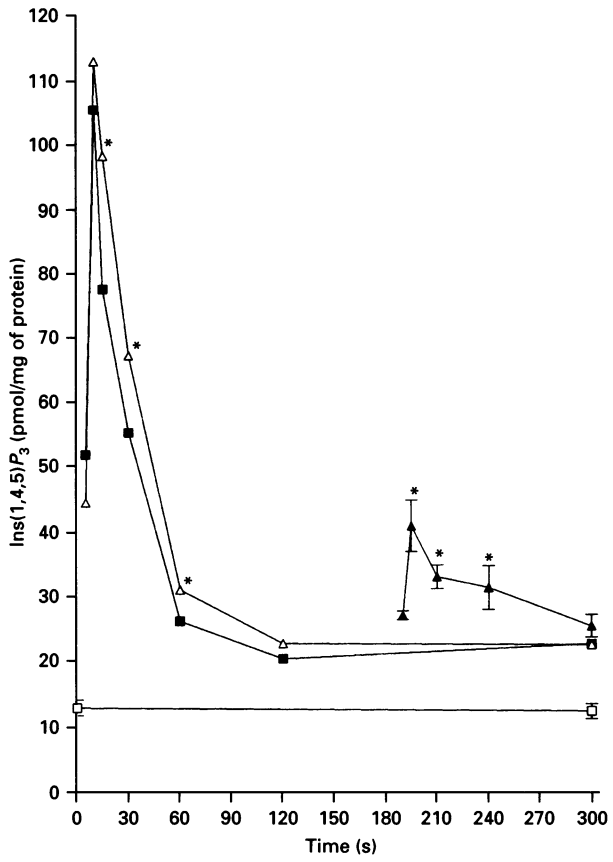

Figure 4 Fentanyl enhances carbachol-induced Ins(1,4,5)P_3 formation in SH-SY5Y cells

Cells were incubated with (■) or without (□) carbachol (CCh) (0.1 mM) for 0–300 s. Fentanyl (0.1 μM) was added to some of the cell suspensions either 0 s (△) or 180 s (▲) after CCh. Data are mean ± S.E.M., where $n = 5$. Error bars have been omitted from the CCh and CCh + Fentanyl (0 s) time courses for clarity (both $P < 0.05$ by ANOVA). * denotes $P < 0.05$ (paired *t*-test) increased compared with CCh.

Table 2 The enhancement of carbachol-induced Ins(1,4,5)P_3 by fentanyl is additive

Whole-cell suspensions (0.3 ml) were preincubated, with or without naloxone (1 μM) or atropine (1 μM), at 37 °C for 15 min. The cells were then incubated in the presence or absence of fentanyl (0.1 μM) and/or carbachol (CCh, 0.1 mM) for 15 s. All results are mean ± S.E.M. ($n = 4$–5). * denotes significantly ($P < 0.05$, *t*-test) increased compared with CCh alone. † denotes significantly ($P < 0.05$, *t*-test) decreased compared with CCh alone.

Addition(s)	Ins(1,4,5)P_3 (pmol/mg of protein)
None	6.5 ± 0.3
CCh	42.9 ± 4.8
Fentanyl	16.0 ± 1.0
CCh + fentanyl	55.9 ± 4.8*
CCh + fentanyl + atropine	16.3 ± 1.3†
CCh + fentanyl + naloxone	40.7 ± 6.7

considered to have inhibitory effects on neuronal activity, e.g. causing hyperpolarization and reducing neurotransmitter release (North, 1989), excitatory actions, such as depolarization and increased neurotransmitter release, have also been reported (Crain and Shen, 1990). Opioids usually inhibit cAMP formation (as confirmed here) and have little or no effect on Ins(1,4,5)P_3 formation (Childers, 1991), but opioid-induced stimulation of the production of both second messengers in neuronal tissues has recently been reported (Olianas and Onali, 1993; Smart et al., 1994a). Noteably, in this study fentanyl, over the same dose range, stimulated one second messenger while inhibiting the other. Although dual excitatory and inhibitory effects of opioids in neuronal tissue have been reported previously (Higashi et al., 1982; Jin et al., 1992), the type of effect seen depended on the dose of opioid used. For example, in NG108-15 cells nanomolar concentrations of δ-opioids increased $[Ca^{2+}]_i$, while at micromolar concentrations they decreased $[Ca^{2+}]_i$ (Jin et al., 1992). Taken at face value our data showing stimulation of Ins(1,4,5)P_3 formation and inhibition of cAMP turnover suggest that the μ-opioid receptor on SH-SY5Y cells is exhibiting G-protein promiscuity. In a recent report Johnson et al. (1994) described in COS cells transiently expressing the cloned opioid receptor μOR1, that promiscuity may occur to cAMP inhibition and inhibition of Ins(1,4,5)P_3 formation. However, our previous studies indicate that both events in SH-SY5Y cells are mediated by a pertussis toxin-sensitive G-protein (Smart et al., 1994a), implying G-protein fidelity. Clearly, further studies will be required to settle this issue, using titratable levels of cloned receptors at close to endogenous concentrations.

There are two possible mechanisms for the stimulation of Ins(1,4,5)P_3 formation caused by μ-opioids. First, β/γ subunits from G_i/G_o could activate PLC. Alternatively, μ-opioids could open a Ca^{2+} channel, allowing Ca^{2+}-influx-dependent activation of PLC.

Neither δ-opioid nor α_2 agonists stimulated Ins(1,4,5)P_3 formation, while the μ-opioid agonist DAMGO did (Table 1.). It is worth noting that SH-SY5Y cells do not express κ-opioid receptors (Yu et al., 1986). Furthermore, neither δ-opioid nor α_2 agonists enhanced the Ins(1,4,5)P_3 response to a submaximal dose of DAMGO, ruling out the possibility that insufficent β/γ subunits were liberated to have a stimulatory effect. Therefore, as all three of these receptors couple to G_i (Yu et al., 1986; Lambert and Nahorski, 1990a), this indicates that β/γ-subunit activation of PLC, as seen in other cell types (Birnbaumer, 1992), is unlikely to occur in SH-SY5Y cells. However, μ- and δ-opioid receptors couple to different subtypes of G_i in SH-SY5Y cells (Laugwitz et al., 1993), and they may also couple differentially to G_o, as in rat cortical membranes where the two receptor subtypes couple to different types of G_o (Moriaty et al., 1990; Georgoussi et al., 1993). Thus, as the type of β/γ subunit varies between G-protein subtypes (Pronin and Gautam, 1992), and some reports indicate that β/γ-determined signal transduction is selective (Kleuss et al., 1993), there remains the possibility that β/γ subunits are involved in the stimulatory effects of μ-opioids in SH-SY5Y cells.

Fentanyl did not increase Ins(1,4,5)P_3 formation in Ca^{2+}-free conditions, as previously reported (Smart et al., 1994a), but subsequent Ca^{2+} replacement at 1 min restored the stimulatory effect. This suggests that μ-opioids may open a Ca^{2+} channel, and that the subsequent Ca^{2+} influx activates PLC (Cockcroft and Thomas, 1992; Rhee and Choi, 1992). Indeed, it has recently been shown that Ca^{2+} activates the PLC isoform δ1 in transfected Chinese hamster ovary cells (Banno et al., 1994). While opioids generally close Ca^{2+} channels (Porzig, 1990), there is evidence that they can also open Ca^{2+} channels, as seen with δ-opioids in NG108-15 cells (Jin et al., 1992). Furthermore, κ-opioids open Ca^{2+} channels in both astrocytes and human B cells (Heagy et al., 1992; Eriksson et al., 1993). However, this putative opening of Ca^{2+} channels by μ-opioids was short-lived, with closing oc-

curring in 2 min, as indicated by the reduction in response to Ca^{2+} replacement (see Figure 2). Such rapid desensitization may result from receptor phosphorylation, although other mechanisms are possible (Lohse, 1993; Inglese et al., 1993).

There are several types of VSCCs (denoted L, N, T and P), each of which have different electrophysical and pharmacological properties, as well as receptor-operated Ca^{2+} channels (ROCC) (for review see Spedding and Paoletti, 1992). SH-SY5Y cells possess L- and N-type VSCCs (Toselli et al., 1991; Morton et al., 1992), as well as ROCCs mediating carbachol-induced Ca^{2+} entry, which is not sensitive to either dihydropyridines or conotoxins (Lambert et al., 1990). Nifedipine completely inhibited the fentanyl-induced Ins(1,4,5)P_3 response, indicating that μ-opioids may open an L-type VSCC (Spedding and Paoletti, 1992). This is in agreement with a recent study which showed κ-opioids opened L-type VSCCs in astrocytes (Eriksson et al., 1993). Furthermore, nifedipine antagonized morphine-induced analgesia in mice (Contreras et al., 1988), although this has been disputed (Omote et al., 1993; Wong et al., 1993). It is worth noting here that the μ-opioid stimulation of Ins(1,4,5)P_3 formation involves a pertussis toxin-sensitive G-protein (Smart et al., 1994a), and G-protein activation has been linked to Ca^{2+}-channel opening (Dolphin and Scott, 1989).

Fentanyl caused a dose-dependent enhancement of carbachol-induced Ins(1,4,5)P_3 formation. In addition, opioids enhanced Ins(1,4,5)P_3 formation induced by low doses (1–10 nM) of bradykinin in NG108-15 cells (Okajima et al., 1993). In contrast, in chromaffin cells opioids had no effect on muscarinic-induced total [^{3}H]inositol polyphosphate accumulation (Bunn et al., 1988), although it should be noted that this method was also unable to detect the opioid-induced increase in Ins(1,4,5)P_3 formation in SH-SY5Y cells (Smart et al., 1994a). This enhancement of muscarinic-induced Ins(1,4,5)P_3 formation by μ-opioids was additive (Table 2), unlike the synergistic enhancement of corticotrophin-releasing hormone-induced cAMP formation by opioids in the rat olfactory bulb (Olianas and Onali, 1993), suggesting that in SH-SY5Y cells the μ-opioid and the M_3 muscarinic receptor utilize the same pool of phosphoinositides. The enhancement of Ins(1,4,5)P_3 formation was not caused by changes in either opioid or muscarinic binding, as the affinities for both carbachol and fentanyl were unchanged (Smart et al., 1994a,b), even though fentanyl has been reported to be a very weak muscarinic antagonist (Hustveit and Setekleiv, 1993; Atcheson et al., 1994).

There are many studies showing an opioid receptor-mediated reduction in neurotransmitter release (see North, 1989), and this is generally believed to be the process underlying analgesia. However, we have previously shown in SH-SY5Y cells that, while μ-opioid receptor occupation reduced cAMP formation, there was no opioid-receptor-mediated inhibition of [^{3}H]noradrenaline release (Lambert et al., 1993; Atcheson et al., 1994). Moreover, in perfused SH-SY5Y cells in the present study, fentanyl (50 nM) failed to stimulate noradrenaline release (results not shown). This clearly indicates that the presumed increase in $[Ca^{2+}]_i$ associated with μ-opioid receptor occupancy may not be sufficient to activate the secretory mechanisms. The functional role of this brief Ca^{2+} influx is unclear, but may be an early warning of incoming noxious stimuli or could act as a trigger to hyperpolarization.

In conclusion, we report here that activation of the μ-opioid receptor briefly allows Ca^{2+} influx, posssibly via L-type Ca^{2+} channels, which stimulates PLC, leading to a transient increase in Ins(1,4,5)P_3 formation, in SH-SY5Y human neuroblastoma cells. Furthermore, activation of this system can enhance muscarinic-induced PLC activity by a post-receptor mechanism. However, the possible role of this mechanism in the process of analgesia still remains unclear.

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