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δ- and μ-opioid receptor mobilization of intracellular calcium in SH-SY5Y human neuroblastoma cells

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1 In this study we have investigated δ and μ opioid receptor-mediated elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the human neuroblastoma cell line, SH-SY5Y.

2 The Ca^{2+}-sensitive dye, fura-2, was used to measure $[Ca^{2+}]_i$ in confluent monolayers of SH-SY5Y cells. Neither the δ-opioid agonist, DPDPE ([D-Pen^{2,5}]-enkephalin) nor the μ-opioid agonist, DAMGO (Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol enkephalin) elevated $[Ca^{2+}]_i$ when applied alone. However, when either DPDPE or DAMGO was applied in the presence of the cholinoceptor agonist, carbachol (100 nM – 1 mM) they evoked an elevation of $[Ca^{2+}]_i$ above that caused by carbachol alone.

3 In the presence of 1 μM or 100 μM carbachol, DPDPE elevated $[Ca^{2+}]_i$ with an EC_{50} of 10 nM. The elevation of $[Ca^{2+}]_i$ was independent of the concentration of carbachol. The EC_{50} for DAMGO elevating $[Ca^{2+}]_i$ in the presence of 1 μM and 100 μM carbachol was 270 nM and 145 nM respectively.

4 The δ-receptor antagonist, naltrindole (30 nM), blocked the elevations of $[Ca^{2+}]_i$ by DPDPE (100 nM) without affecting those caused by DAMGO while the μ-receptor antagonist, CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Pen-Thr-NH_2) (100 nM – 1 μM) blocked the elevations of $[Ca^{2+}]_i$ caused by DAMGO (1 μM) without affecting those caused by DPDPE.

5 Block of carbachol activation of muscarinic receptors with atropine (10 μM) abolished the elevation of $[Ca^{2+}]_i$ by the opioids. The nicotinic receptor antagonist, mecamylamine (10 μM), did not affect the elevations of $[Ca^{2+}]_i$ caused by opioids in the presence of carbachol.

6 Muscarinic receptor activation, not a rise in $[Ca^{2+}]_i$, was required to reveal the opioid response. The Ca^{2+} channel activator, maitotoxin (3 ng ml^{-1}), also elevated $[Ca^{2+}]_i$ but subsequent application of opioid in the presence of maitotoxin caused no further changes in $[Ca^{2+}]_i$.

7 The elevations of $[Ca^{2+}]_i$ by DPDPE and DAMGO were abolished by pretreatment of the cells with pertussis toxin (200 ng ml^{-1}, 16 h). This treatment did not significantly affect the response of the cells to carbachol.

8 The opioids appeared to elevate $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from intracellular stores. Both DPDPE and DAMGO continued to elevate $[Ca^{2+}]_i$ when applied in nominally Ca^{2+}-free external buffer or when applied in a buffer containing a cocktail of Ca^{2+} entry inhibitors. Thapsigargin (100 nM), an agent which discharges intracellular Ca^{2+} stores, also blocked the opioid elevations of $[Ca^{2+}]_i$.

9 δ and μ Opioids did not appear to mobilize intracellular Ca^{2+} by modulating the activity of protein kinases. The application of H-89 (10 μM), an inhibitor of protein kinase A, H-7 (100 μM), an inhibitor of protein kinase C, protein kinase A and cyclic GMP-dependent protein kinase, or Bis I, an inhibitor of protein kinase C, did not alter the opioid mobilization of $[Ca^{2+}]_i$.

10 Thus, in SH-SY5Y cells, opioids can mobilize Ca^{2+} from intracellular stores but they require ongoing muscarinic receptor activation. Opioids do not elevate $[Ca^{2+}]_i$ when applied alone.

Keywords: SH-SY5Y; opioids; intracellular calcium; muscarinic receptors; calcium mobilization

Introduction

Opioid receptors, members of the inhibitory, G_i/G_o coupled receptor superfamily, are coupled to multiple cellular effectors. The best characterized of these are inhibition of neuronal voltage-gated N-type Ca current (Seward *et al.*, 1991), activation of several types of potassium currents (North, 1993) and inhibition of adenylyl cyclase (Collier & Roy, 1974). In virtually every case these effects have been demonstrated to occur through activation of G-proteins of the G_i or G_o classes. Recently, opioid receptor activation has also been suggested to *increase* intracellular free Ca^{2+} levels in two ways; first by releasing Ca^{2+} from intracellular stores (Okajima *et al.*, 1993; Jin *et al.*, 1994) and second by activation of calcium entry across the plasma membrane (Eriksson *et al.*, 1993; Jin *et al.*, 1993; Smart *et al.*, 1994; 1995; Tang *et al.*, 1994).

Opioid-induced Ca release from intracellular stores has to date only been observed for δ receptor activation in the NG108-15 cell line (Okajima & Kondo, 1993; Okajima *et al.*, 1993; Jin *et al.*, 1994; Connor *et al.*, 1994). This opioid elevation of intracellular Ca^{2+} was observed with the concomitant activation of phospholipase C (PLC) by application of bradykinin or uridine triphosphate and was not generally seen when the δ-opioid agonist was applied alone (Okajima & Kondo 1992; Okajima *et al.*, 1992; 1993; Connor *et al.*, 1994, but see Jin *et al.*, 1994).

Opioid receptor stimulation of Ca^{2+} entry has been reported to occur in a number of neuronal cell types. δ-Opioid receptor activation resulted in Ca^{2+} entry in NG108-15 cells (Jin *et al.*, 1993) and the dorsal root ganglion neuroblastoma cell line, ND8-47 (Tang *et al.*, 1994) while in cultured astrocytes the Ca^{2+} entry resulted from κ opioid receptor activation (Eriksson *et al.*, 1993). In these cases the observed Ca^{2+} influx was demonstrated to occur through L-type Ca channels, which in some cases were thought to have been opened by membrane depolarization following opioid receptor activation (Jin *et al.*, 1993).

SH-SY5Y cells are a human neuroblastoma cell line unique

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in expressing both μ and δ opioid receptors (Sonnenfeld *et al.*, 1985; Kazmi & Mishra 1986). Both μ and δ receptors inhibit N-type Ca channels (Seward *et al.*, 1990; 1991) and adenylyl cyclase (Kazmi & Mishra, 1987; Yu & Sadee, 1988) in SH-SY5Y cells. Recently, it has been observed that μ but not δ receptor activation leads to an elevation of inositol (1,4,5) trisphosphate (IP_3) in SH-SY5Y cells (Smart *et al.*, 1994; 1995). This elevation of IP_3 was blocked by removal of extracellular Ca^{2+} and preincubation of the cells with the L-type Ca channel blocker, nifedipine. The authors thus concluded that the elevation of intracellular IP_3 came about as a consequence of opioid-stimulated Ca^{2+} entry through L-type Ca channels. In the light of these intriguing observations we have examined the effects of both μ and δ receptor activation on intracellular Ca^{2+} levels in undifferentiated SH-SY5Y cells. We set out to determine if opioid agonists could mobilize intracellular Ca^{2+} alone or if they required concomitant PLC activation by another agonist and whether opioids could simultaneously promote Ca^{2+} entry in SH-SY5Y cells. The cellular mechanism(s) underlying opioid-induced elevations of intracellular Ca^{2+} are unknown and we have also examined the possible mechanisms that underlie such changes. We find that in SH-SY5Y cells, μ and δ opioid receptor activation concomitant with muscarinic receptor activation leads to a mobilization of intracellular Ca^{2+} from thapsigargin-sensitive stores. We found no evidence that exposure of the cells to either μ or δ agonists alone evoked release from intracellular stores or stimulated Ca^{2+} entry. The mobilization of intracellular Ca^{2+} by the opioids required ongoing muscarinic receptor activation but did not result from modulation of the activity of protein kinase A (PKA), protein kinase C (PKC) or guanosine 3':5'-cyclic monophosphate (cyclic GMP) dependent protein kinase. Preliminary accounts of these findings have been presented to the International Narcotics Research Conference (Connor *et al.*, 1994) and the British Pharmacological Society (Connor & Henderson, 1994).

Methods

Cell culture

The studies described here were carried out on SH-SY5Y cells obtained from the European Collection of Animal Cell Cultures. The cells were cultured in Dulbeccos Modified Eagles Medium (DMEM) supplemented with glutamine (4 mM), penicillin (100 iu ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$) and foetal bovine serum (12.5%) in a humidified incubator with 5% CO_2. Cells used for Ca^{2+} measurements were seeded onto plastic slides and cultured in Leighton tubes (Costar) until confluent. Cells were passaged every week; cells from passages 9–42 were used in these experiments.

Intracellular calcium measurements

Intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$ was measured in confluent monolayers of SH-SY5Y cells with the fluorescent Ca^{2+}-sensitive dye, fura 2. The cells were washed 3 times with buffer before loading and then incubated with the methoxy-ester of fura-2 (3 μM) for 1 h at 37°C. Unless stated otherwise, experiments were carried out in buffer containing (mM): NaCl 140, KCl 2.0, $CaCl_2$ 2.5, $MgCl_2$ 1, N-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulphonic acid] (HEPES) 10, glucose 10, sucrose 40 and bovine serum albumin 0.05%, pH 7.3. 'Ca^{2+}-free' buffer was the same as the above except $MgCl_2$ was substituted for $CaCl_2$ and EGTA (ethylene glycol-*bis*[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid) (100 μM) was added to the buffer. After fura-2 loading, the plastic slips were cut in half and half was placed on a specially constructed block fitted inside a quartz cuvette; the cuvette was then placed in an LS-5B Perkin-Elmer spectrofluorimeter and perfused with buffer (5 ml min^{-1} at 37°C). Drugs were added to the perfusion buffer in known concentrations. The spectrofluorimeter was controlled by a computer running a Perkin-Elmer software package. For more details of the recording set up see Pickles & Cuthbert (1991). Generally both halves of a slip were used for experiments, each half was considered to be the same population in statistical analysis. Data are reported as mean ± standard error of the mean (s.e.mean); statistical comparisons were made by using Student's unpaired *t* test; a *P* value <0.05 was considered significant.

The fura-2 loaded cells were alternately exposed to light at 340 nm and 380 nm and the emission of the cells at 510 nm was recorded. The autofluorescence of unloaded cells was determined, subtracted from the recorded values and the corrected ratio of 340/380 emissions was converted to $[Ca^{2+}]_i$ by use of the equation given in Grynkiewicz *et al.* (1985). Maximum and minimum values of fura-2 fluorescence were determined by lysing the cells with digitonin in the presence of 20 mM Ca^{2+} or 10 mM EGTA respectively.

Drugs and chemicals

The DMEM and foetal bovine serum were purchased from GIBCO, buffer salts from BDH. Atropine methylbromide, carbamylcholine chloride (carbachol), Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol acetate (DAMGO), [D-$Pen^{2,5}$]-enkephalin (DPDPE), fura 2-AM, mecamylamine hydrochloride, and pertussis toxin were obtained from Sigma, UK. ω-Conotoxin GVIA was from Penninsula Laboratories; nimodipine was from Research Biochemicals International; (2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl)maleimide hydrochloride (BisI, GF-109203X), *N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinolinesulphonamide (H-89), maitotoxin and thapsigargin were from Calbiochem; 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H7) was from Tocris Cookson. CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Pen-Thr-NH_2) was a kind gift from Dr Victor Hruby.

Results

μ and δ opioid agonists elevate $[Ca^{2+}]_i$ only in the presence of muscarinic agonists

Application of the cholinoceptor agonist, carbachol, to confluent monolayers of SH-SY5Y cells resulted in a rapid increase in $[Ca^{2+}]_i$ (Figure 1). In the continued presence of carbachol the $[Ca^{2+}]_i$ declined to a plateau of elevated $[Ca^{2+}]_i$ that persisted as long as agonist was present. As previously reported (Lambert & Nahorski, 1990), the plateau phase of elevated $[Ca^{2+}]_i$ was due to Ca^{2+} entry as it was abolished in the absence of external Ca^{2+} (see Figure 5a). The muscarinic receptor antagonist, atropine (1 μM) abolished the transient increase in $[Ca^{2+}]_i$ in response to carbachol and markedly reduced the plateau. There remained, however, a small, persistent elevation of $[Ca^{2+}]_i$ in the presence of atropine (Figure 2b). This elevation was abolished by the nicotinic receptor antagonist mecamylamine (10 μM).

Application of the selective δ-opioid receptor agonist, DPDPE (100 nM, $n=37$) (Figure 1) or the selective μ-opioid receptor agonist, DAMGO (1 μM, $n=25$) alone never altered the $[Ca^{2+}]_i$ of the SH-SY5Y cells. However, when DPDPE or DAMGO was applied to the cells during continued carbachol (100 nM – 1 mM) exposure, there was a further rapid elevation of $[Ca^{2+}]_i$ above that caused by carbachol alone (Figure 1). The responses of the SH-SY5Y cells to DPDPE did not change over the course of this study; however, as the cells were passaged the DAMGO responses became smaller. Nevertheless, every population of cells tested from a given passage responded in a consistent fashion to DPDPE or DAMGO. When the DPDPE or DAMGO applications were restricted to short periods (30–60 s) and spaced at least 10 min apart the increases in $[Ca^{2+}]_i$ evoked by either drug were reproducible on a given slide of cells for as long as carbachol was present (up to 90 min). We have not sought to investigate opioid receptor

desensitization in this study. Thus in all experiments test concentrations of opioids were applied for 60 s or less at intervals of at least 10 min to avoid desensitization.

We further investigated opioid-induced elevations of $[Ca^{2+}]_i$ at two test concentrations of carbachol, a relatively low concentration of 1 μM and a relatively high concentration of 100 μM. The EC_{50} for carbachol-induced elevation of intracellular Ca^{2+} in SH-SY5Y cells is 40 μM (Lambert & Nahorski, 1990). Carbachol, 1 μM elevated $[Ca^{2+}]_i$ from a basal level of 57±4 nM to a peak of 120±10 nM ($n=19$); after 10 min in the continued presence of carbachol the $[Ca^{2+}]_i$ had declined to 65±3 nM. DPDPE (100 nM) applied in the presence of 1 μM carbachol elevated $[Ca^{2+}]_i$ by 45±4 nM ($n=11$), while DAMGO (1 μM) caused an increase of 23±4 nM ($n=7$).

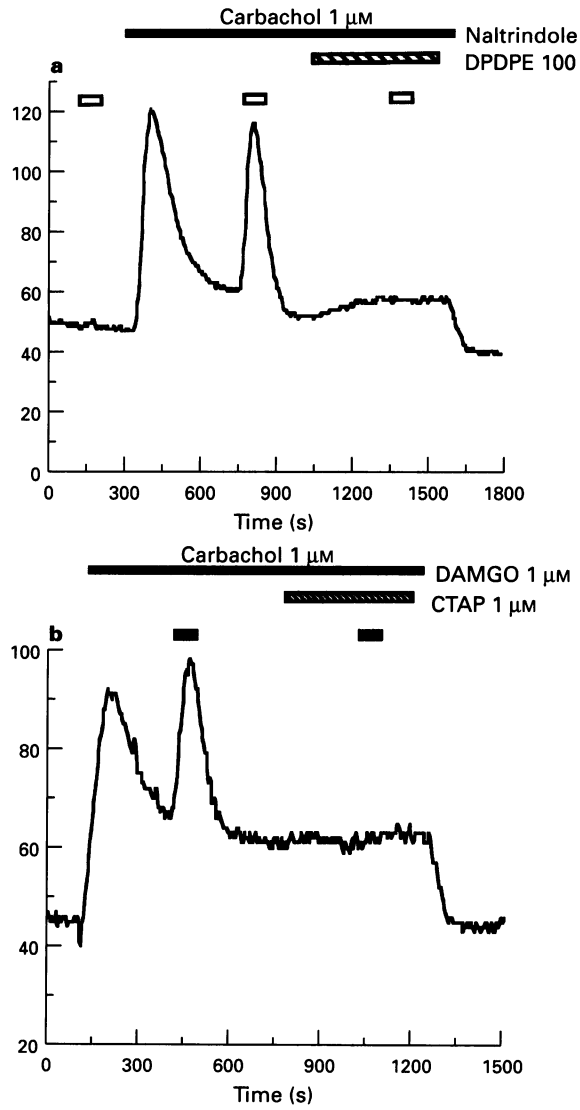

Figure 1 DPDPE and DAMGO elevate $[Ca^{2+}]_i$ in the presence of carbachol. The traces represent continuous records of $[Ca^{2+}]_i$ in single populations of cells, determined as described in Methods. Drugs were perfused for the duration of the bars. (a) Carbachol (1 μM) caused a rapid elevation of $[Ca^{2+}]_i$ followed by a sustained plateau of elevated $[Ca^{2+}]_i$. DPDPE (100 nM) alone did not elevate $[Ca^{2+}]_i$ but when it was applied in the presence of carbachol (1 μM) there was a robust elevation of $[Ca^{2+}]_i$. The ability of DPDPE to elevate $[Ca^{2+}]_i$ was blocked by the δ receptor antagonist, naltrindole. (b) DAMGO (1 μM) caused a transient increase in $[Ca^{2+}]_i$ when applied in the presence of carbachol (1 μM); this increase was blocked by the μ receptor antagonist, CTAP. For abbreviations in this and subsequent legends, see text.

Carbachol at a concentration of 100 μM elevated $[Ca^{2+}]_i$ from 50±2 nM to 390±20 nM ($n=54$); after 10 min in 100 μM carbachol, $[Ca^{2+}]_i$ had declined to 140±8 nM. DPDPE (100 nM) applied in the presence of 1 μM carbachol elevated $[Ca^{2+}]_i$ by 52±6 nM ($n=11$) while DAMGO (1 μM) elevated $[Ca^{2+}]_i$ by 38±5 nM ($n=12$). The increases in $[Ca^{2+}]_i$ brought about by both DPDPE and DAMGO in the presence of carbachol were blocked by selective opioid antagonists. The δ-receptor selective antagonist, naltrindole (30 nM), abolished the rise in $[Ca^{2+}]_i$ brought about by DPDPE (100 nM, $n=4$, Figure 1a) but did not affect the response to DAMGO while the μ-receptor selective antagonist, CTAP (100 nM– 1 μM) abolished the rise in $[Ca^{2+}]_i$ brought about by DAMGO (1 μM, $n=4$, Figure 1b) but did not affect the response to DPDPE.

We investigated the relationship between carbachol concentration and opioid-induced elevations of $[Ca^{2+}]_i$ by determining the potency and maximum effectiveness of DPDPE and DAMGO at the two concentrations of muscarinic agonist. Concentration-response curves for DPDPE and DAMGO in the presence of carbachol were generated by applying various concentrations of the opioids to individual monolayers for 60 s every 10 min (Figure 2). The order in which the different concentrations of opioids were applied was randomized and varied between each experiment. When DPDPE was applied in the continued presence of 1 μM carbachol the EC_{50} for elevation of $[Ca^{2+}]_i$ was 11 nM ($n=6$, 95% confidence limits 6 to 18 nM, Hill slope 1.1); when applied in the presence of 100 μM carbachol, the EC_{50} was 12 nM ($n=8$, 95% confidence limits 8 to 21 nM, Hill slope 1.0). The magnitude of the elevation of $[Ca^{2+}]_i$ by maximally effective concentrations of DPDPE was not significantly different at the two concentrations of carbachol. In the presence of 1 μM carbachol the elevation of $[Ca^{2+}]_i$ was 52±7 nM, the elevation of $[Ca^{2+}]_i$ in the presence of 100 μM carbachol was 45±4 nM ($P>0.37$). DAMGO was considerably less potent at elevating $[Ca^{2+}]_i$ than DPDPE. The EC_{50} for elevation of $[Ca^{2+}]_i$ by DAMGO in the presence of 1 μM carbachol was 270 nM ($n=7$, 95% confidence limits 90 to 830 nM, Hill slope 0.7) and in the presence of 100 μM carbachol the EC_{50} was 145 nM ($n=7$, 95% confidence limits 70 to 300 nM, Hill slope 0.7). Maximally effective concentrations of DAMGO appeared to elevate $[Ca^{2+}]_i$ more in the presence of 100 μM carbachol (45±7 nM, $n=8$) than in the presence of 1 μM carbachol (29±6 nM, $n=7$).

Carbachol will activate both muscarinic and nicotinic cholinoceptors on SH-SY5Y cells. Blockade of nicotinic receptors with mecamylamine (10 μM) reduced only slightly the carbachol-induced rise in $[Ca^{2+}]_i$ and did not significantly affect the increase in $[Ca^{2+}]_i$ caused by DPDPE or DAMGO. In the presence of mecamylamine (10 μM) the elevation of $[Ca^{2+}]_i$ by DAMGO (1 μM) was 101±12% of control ($n=4$) while the elevation caused by DPDPE was 87±9% ($n=4$, $P>0.4$) of the control elevations in 100 μM carbachol alone (Figure 3a). In contrast, blockade of muscarinic receptors with atropine (1 μM) prevented the DPDPE- and DAMGO-evoked increases in $[Ca^{2+}]_i$ in the presence of carbachol (1 μM or 100 μM) (Figure 3b). When, in the continued presence of carbachol, a high concentration of atropine (10 μM) was applied simultaneously with the opioid, there was also no elevation of $[Ca^{2+}]_i$. What was observed was a rapid fall in $[Ca^{2+}]_i$, identical to that seen when atropine alone was applied to cells in the presence of carbachol (Figure 3c). Thus, simultaneous muscarinic receptor activation is required for opioid-induced elevations of $[Ca^{2+}]_i$.

Pertussis toxin blocks the increase in $[Ca^{2+}]_i$ produced by opioids

The increases in $[Ca^{2+}]_i$ caused by DPDPE (1 μM) and DAMGO (10 μM) in the presence of carbachol (100 μM) were abolished by pretreatment of the cells with pertussis toxin (200 ng ml^{-1}) for 16 h ($n=4$) (Figure 4). The pertussis toxin treatment did not significantly alter the peak elevation of $[Ca^{2+}]_i$ of the cells to carbachol ($P>0.5$) when compared with cells of the same passage number tested on the same day.

Opioids mobilize intracellular Ca^{2+}

The increases in $[Ca^{2+}]_i$ caused by the opioids in the presence of carbachol reflected the mobilization of Ca^{2+} from internal stores, not Ca^{2+} entry across the plasma membrane. When the cells were bathed in nominally Ca^{2+}-free external medium, carbachol still caused a rapid elevation of $[Ca^{2+}]_i$ but the plateau phase of the response was abolished. If DPDPE ($n=5$) (Figure 5a) or DAMGO ($n=5$) was applied to the cells in the presence of carbachol but in the absence of external Ca^{2+}, an increase in $[Ca^{2+}]_i$ was still evoked. In another series of experiments the cells were maintained in normal buffer but Ca^{2+} entry processes were blocked. The cells were pretreated for 15 min with the irreversible N-type calcium channel blocker, ω-conotoxin GVIA (1 μM) and then the L-type calcium channel antagonist, nimodipine (3 μM), the nicotinic receptor antagonist mecamylamine (10 μM) and the non-specific Ca^{2+}-entry blocker, La^{3+} (10 μM) were added to the bathing medium. In the presence of this cocktail, carbachol (1–100 μM), still produced a rapid, transient increase in $[Ca^{2+}]_i$ but, as when external Ca^{2+} was removed, the sustained phase of increased $[Ca^{2+}]_i$ was abolished. Addition of DPDPE (100 nM, $n=4$) or DAMGO (3 μM, $n=4$) in the continued presence of carbachol and Ca^{2+} entry blockers still caused an increase in $[Ca^{2+}]_i$.

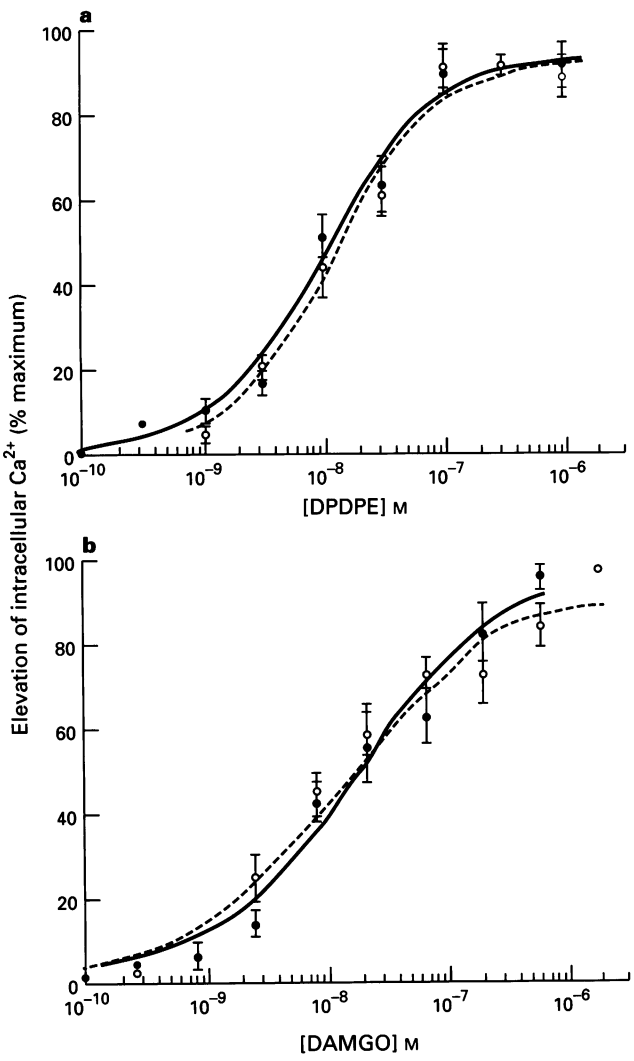

Figure 2 Concentration-response relationships for the elevation of $[Ca^{2+}]_i$ by DPDPE and DAMGO. (a) Concentration-response relationships for DPDPE elevation of $[Ca^{2+}]_i$ in the presence of two carbachol concentrations; 1 μM (○) and 100 μM (●). The curves represent pooled data obtained from 6 populations in the presence of 1 μM carbachol and 8 populations in 100 μM carbachol. The EC_{50} for DPDPE in the presence of 1 μM carbachol was 12 nM, in the presence of 100 μM carbachol it was 11 nM. (b) Concentration-response relationships for DAMGO elevation of $[Ca^{2+}]_i$ in the presence of two carbachol concentrations; 1 μM (○) and 100 μM (●). The curves represent pooled data obtained from 7 populations in the presence of 1 μM carbachol and 8 populations in 100 μM carbachol. The EC_{50} for DAMGO in the presence of 1 μM carbachol was 270 nM, in the presence of 100 μM carbachol it was 145 nM. For both (a) and (b), each point was normalized to maximum elevation of $[Ca^{2+}]_i$ that occurred within a given experiment and the data were then pooled.

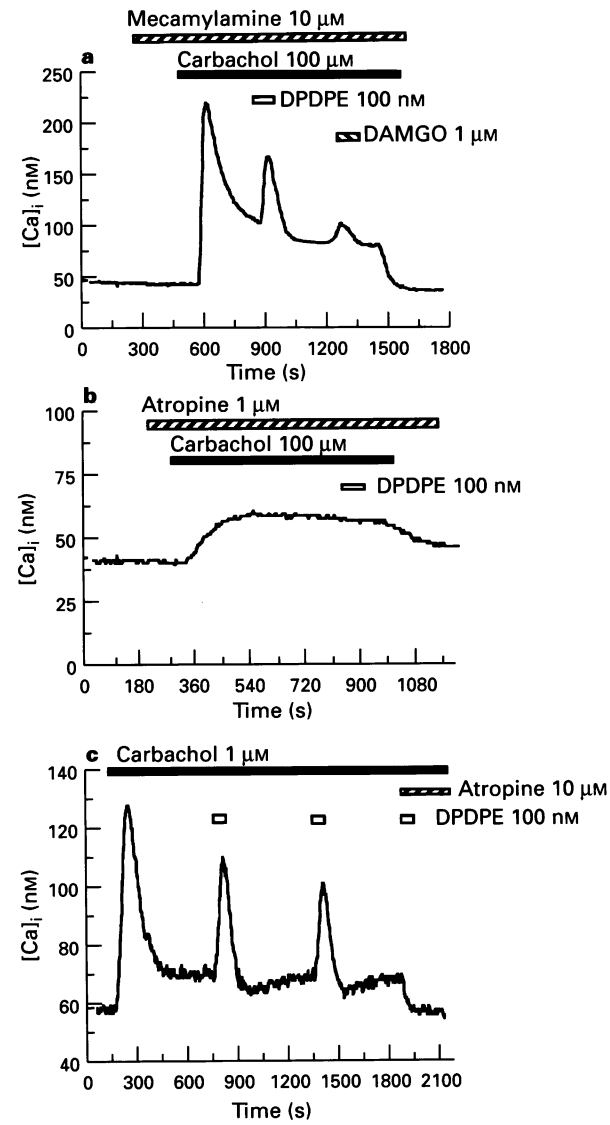

Figure 3 Muscarinic receptor activation required for elevation of $[Ca^{2+}]_i$ by DPDPE and DAMGO. The traces represent continuous records of $[Ca^{2+}]_i$ in single populations of cells, determined as described in Methods. Drugs were perfused for the duration of the bars. (a) In the presence of mecamylamine (10 μM), carbachol (100 μM) caused a large, transient, elevation in $[Ca^{2+}]_i$ followed by a sustained period of elevated $[Ca^{2+}]_i$ and both DPDPE (100 nM), and DAMGO (1 μM) elevated $[Ca^{2+}]_i$ when applied in the presence of carbachol. (b) Carbachol (100 μM) applied in the presence of atropine (1 μM) caused only a small, sustained, increase in $[Ca^{2+}]_i$. Subsequent co-application of DPDPE (100 nM) caused no further increase. (c) DPDPE (100 nM) was able to evoke repeated elevations of $[Ca^{2+}]_i$ when applied in the presence of carbachol (1 μM), however, when atropine (10 μM) was applied at the same time as DPDPE there was no elevation of $[Ca^{2+}]_i$.

DPDPE and DAMGO appeared to mobilize Ca^{2+} from IP_3-sensitive stores

Thapsigargin, an irreversible inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase (Thastrup *et al.*, 1990), occludes IP_3-induced Ca^{2+} release by promoting the emptying of the intracellular Ca^{2+} stores. When cells were exposed to thapsigargin (100 nM) there was a gradual increase of $[Ca^{2+}]_i$ from 61 ± 6 nM to 204 ± 6 nM ($n=7$). When carbachol (100 μM) was added after 10 min of thapsigargin exposure, there was a small, transient increase in $[Ca^{2+}]_i$ (by 20 ± 4 nM, $n=4$) but when DPDPE (100 nM, $n=4$) or DAMGO (3 μM, $n=3$) was then added in the presence of carbachol there was no further increase in $[Ca^{2+}]_i$. (Figure 5b).

Elevation of $[Ca^{2+}]_i$ alone was not sufficient to promote DPDPE mobilization of intracellular Ca^{2+}

We initially attempted to elevate $[Ca^{2+}]_i$ by depolarizing the cells with 50 mM K^+. This procedure, however, failed to alter $[Ca^{2+}]_i$. Under our culture conditions, undifferentiated SH-SY5Y cells appear to lack voltage-dependent Ca channels, a finding that we have confirmed electrophysiologically (Connor, unpublished observations). As an alternative to depolarization we used a potent activator of plasma membrane Ca channels, maitotoxin, to elevate $[Ca^{2+}]_i$. Maitotoxin has been shown to promote Ca^{2+} flow across the plasma membrane without directly affecting the intracellular Ca^{2+} stores (Soergel *et al.*, 1992). Application of maitotoxin (3 ng ml^{-1}) to SH-SY5Y cells elevated $[Ca^{2+}]_i$ from 53 ± 7 nM to 233 ± 71 nM ($n=7$), this elevation was dependent on the presence of extracellular Ca^{2+}. Application of DPDPE (100 nM, $n=4$) or DAMGO (3 μM, $n=3$) at any time during the period of elevated $[Ca^{2+}]_i$ caused by maitotoxin did not result in any further elevation of $[Ca^{2+}]_i$ (Figure 5c).

Protein kinases A or C are not involved in the opioid induced mobilization of $[Ca^{2+}]_i$

Cells were first stimulated with carbachol and then a control elevation of $[Ca^{2+}]_i$ by DPDPE (100 nM) or DAMGO (3 μM) in the presence of carbachol was obtained. The cells were then exposed to the protein kinase inhibitors for 10 min in the

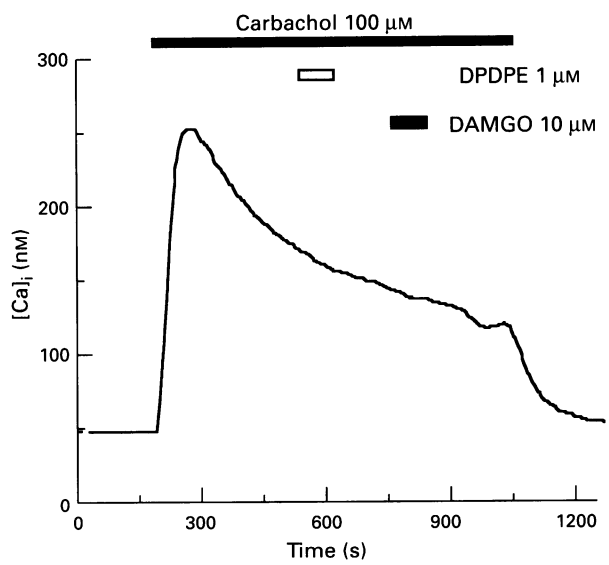

Figure 4 Opioid-induced elevations in $[Ca^{2+}]_i$ were pertussis toxin-sensitive. The trace represents a continuous record of $[Ca^{2+}]_i$ in a single population of cells, determined as described in Methods. Drugs were perfused for the duration of the bars. The cells were pretreated for 16 h with 200 ng ml^{-1} pertussis toxin. In the PTX-treated cells, neither DPDPE (1 μM) nor DAMGO (10 μM) elevated $[Ca^{2+}]_i$ in the presence of carbachol (100 μM).

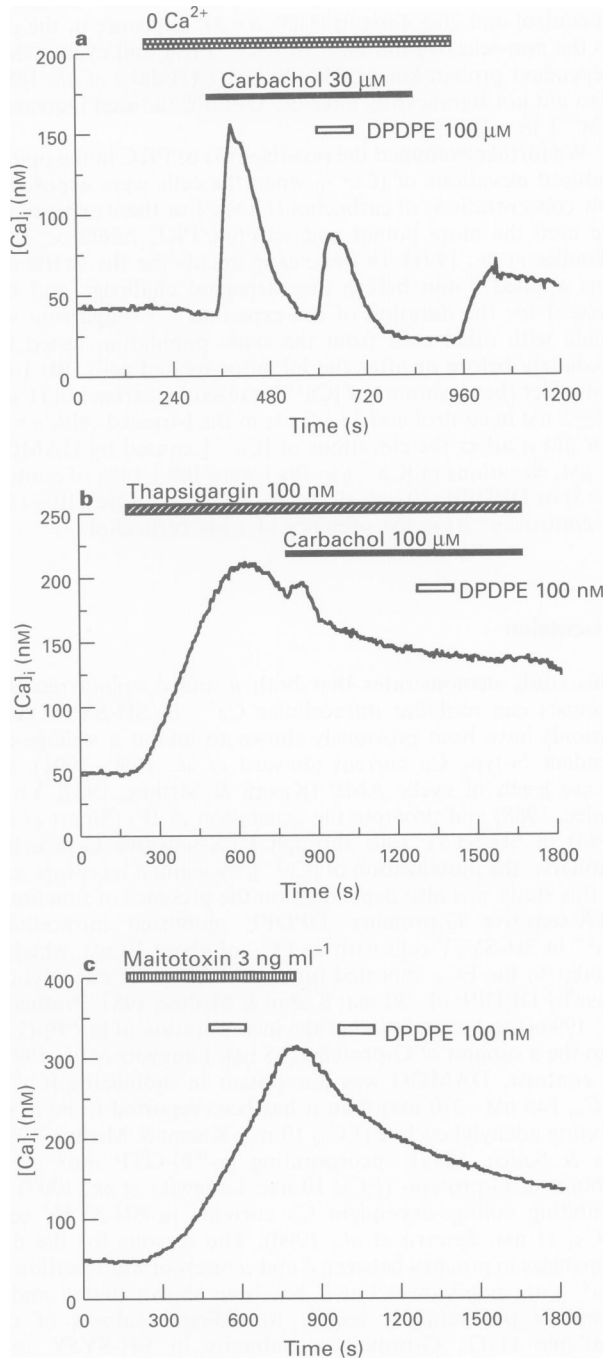

Figure 5 In the presence of carbachol, DPDPE and DAMGO mobilize Ca^{2+} from intracellular stores. The traces represent continuous records of $[Ca^{2+}]_i$ in single populations of cells, determined as described in Methods. Drugs were perfused for the duration of the bars. (a) In cells exposed to Ca^{2+}-free buffer (containing 100 μM EGTA) for 2 min prior to drug addition, carbachol (30 μM) elevated $[Ca^{2+}]_i$ briefly, as did subsequent co-application of DPDPE (100 μM). Note the absence of any plateau of elevated $[Ca^{2+}]_i$ following the initial carbachol-induced spike. This experiment is typical of 8 carried out with 30 μM or 100 μM carbachol and DPDPE (100 nM) or DAMGO (1 μM). (b) Thapsigargin elevated $[Ca^{2+}]_i$ and blocked subsequent elevation of $[Ca^{2+}]_i$ by carbachol (100 μM) or DPDPE (100 nM) in the presence of carbachol ($n=4$). (c) Maitotoxin (3 ng ml^{-1}) elevated $[Ca^{2+}]_i$, DPDPE (100 nM) added during the period of elevated $[Ca^{2+}]_i$ did not elevate $[Ca^{2+}]_i$ any further.

continued presence of carbachol and then retested with DPDPE or DAMGO. Incubation with a membrane-permeable inhibitor of PKA, H-89 (10 μM) (Chijiwa *et al.*, 1990), did not alter the elevation of $[Ca^{2+}]_i$ caused by DPDPE (44 ± 8 nM in control and 40 ± 6 nM in H-89, $n=5$) or DAMGO (22 ± 2 nM in control and 26 ± 4 nM in H-89, $n=3$). Exposure of the cells to the non-selective inhibitor of PKC, PKA and cyclic GMP-dependent protein kinase, H7 (100 μM), (Hidaka *et al.*, 1984), also did not significantly alter the DPDPE-induced increase in $[Ca^{2+}]_i$ ($n=4$).

We further examined the possible role of PKC in the opioid-induced elevations of $[Ca^{2+}]_i$ when the cells were exposed to low concentrations of carbachol (1 μM). For these experiments we used the more potent and selective PKC inhibitor, Bis I (Toullec *et al.*, 1991). In these experiments the Bis I (100 nM) was applied 5 min before the carbachol challenge and was present for the duration of the experiment. Comparison was made with other cells from the same population tested immediately before or after the inhibitor-treated cells. Bis I did not affect the elevations of $[Ca^{2+}]_i$ caused by carbachol (1 μM, 32 ± 2 nM in control and 33 ± 2 nM in Bis I-treated cells, $n=9$), nor did it affect the elevations of $[Ca^{2+}]_i$ caused by DAMGO (1 μM, elevations of $[Ca^{2+}]_i$ in Bis I were 108 ± 14% of control, $n=5$) or DPDPE (30 nM, elevations of $[Ca^{2+}]_i$ were 110 ± 15% of control, $n=4$) in the presence of 1 μM carbachol.

Discussion

This study demonstrates that both μ and δ opioid receptor agonists can mobilize intracellular Ca^{2+} in SH-SY5Y cells. Opioids have been previously shown to inhibit a voltage-dependent N-type Ca current (Seward *et al.*, 1990; 1991), decrease levels of cyclic AMP (Kazmi & Mishra, 1987; Yu & Sadee, 1988) and promote the generation of IP_3 (Smart *et al.*, 1994) in SH-SY5Y cells through PTX-sensitive G-proteins. Similarly, the mobilization of $[Ca^{2+}]_i$ by μ and δ receptors seen in this study was also dependent on the presence of functional PTX-sensitive G-proteins. DPDPE mobilized intracellular Ca^{2+} in SH-SY5Y cells with an EC_{50} of about 10 nM, which is similar to the EC_{50} reported for the inhibition of adenylyl cyclase by DPDPE (4–30 nM; Kazmi & Mishra, 1987; Prather *et al.*, 1994a) and stimulation of the incorporation of [α-^{32}P]-GTP into the α subunit of G-proteins (65 nM; Laugwitz *et al.*, 1993). In contrast, DAMGO was less potent in mobilizing $[Ca^{2+}]_i$ (EC_{50} 145 nM–270 nM) than it has been reported to be in inhibiting adenylyl cyclase (EC_{50} 10 nM; Kazmi & Mishra, 1987; Yu & Sadee, 1988), incorporating [α-^{32}P]-GTP into the α subunit of G-proteins (EC_{50} 10 nM; Laugwitz *et al.*, 1993) or inhibiting voltage-dependent Ca currents in SH-SY5Y cells (EC_{50} 11 nM; Seward *et al.*, 1990). The reasons for the discrepancies in potency between δ and μ receptor mobilization of $[Ca^{2+}]_i$ are not known but it has been shown that μ and δ receptors preferentially couple to different subsets of the available G_i/G_o G-protein α subunits in SH-SY5Y cells (Laugwitz *et al.*, 1993). Thus it is possible that the Ca^{2+} mobilization brought about by opioids in SH-SY5Y cells is more efficiently supported by those G_i/G_o protein subtypes that are preferentially activated by δ receptor stimulation. The δ receptor mediating the mobilization of intracellular Ca^{2+} in SH-SY5Y cells does not appear to be of the δ_1 or δ_2 subtype (Connor *et al.*, 1995). This is consistent with the properties of the δ receptor cloned from SH-SY5Y cells, which exhibits a mixed δ_1/δ_2 pharmacological profile (Simonin *et al.*, 1994). It is clear that the μ and δ receptor-mediated mobilization of intracellular Ca^{2+} occurs over a similar agonist concentration-range as other opioid receptor-mediated processes in SH-SY5Y cells. Some authors have claimed that opioid receptor-mediated mobilization of intracellular Ca^{2+} occurs with much lower agonist concentrations than opioid receptor-mediated inhibition of Ca^{2+} entry through voltage-dependent Ca channels (Jin *et al.*, 1993; 1994; Prather *et al.*, 1994b), thus giving rise to the so-called 'excitatory' effects of opioids. A careful reading of the literature reveals that, where determined, the potencies for the two effects of opioid receptor activation are very similar, and the results of this study confirm that view.

We could detect μ or δ opioid-induced elevations of $[Ca^{2+}]_i$ only when the opioids were applied in the presence of muscarinic receptor agonists. We never observed an elevation of $[Ca^{2+}]_i$ in the presence of DPDPE or DAMGO alone. Muscarinic receptor activation was required for the opioid-induced elevations of $[Ca^{2+}]_i$ but the extent to which the muscarinic receptors were activated did not seem to be critical for the opioid-induced Ca^{2+} mobilizations. DPDPE elevated $[Ca^{2+}]_i$ by the same amount and with identical potency in the presence of either 1 μM or 100 μM carbachol. Although the μ receptor agonist, DAMGO, seemed to be less potent and caused a smaller elevation of $[Ca^{2+}]_i$ when applied in the presence of the lower concentration of carbachol, overall there did not seem to be a strong correlation between the concentrations of carbachol and the subsequent opioid response. Despite this apparent absence of any direct correlation between the extent of muscarinic receptor activation and opioid-induced elevations of $[Ca^{2+}]_i$, we were unable to separate the events temporally. Simultaneous application of a muscarinic antagonist with the opioid agonist was sufficient to block the opioid-induced rise in $[Ca^{2+}]_i$, regardless of whether the carbachol concentration was 1 μM or 100 μM. It has been suggested that the activation of PLC by bradykinin receptor or purinoceptor agonists in NG108-15 cells 'primes' the cells to respond to subsequent opioid application with a rise in $[Ca^{2+}]_i$ (Okajima & Kondo, 1992; Tomura *et al.*, 1992; Okajima *et al.*, 1993). If muscarinic receptor occupancy leads to the generation of some 'priming' agent within SH-SY5Y cells, then the lifetime of this agent is very short. Our results suggest that muscarinic receptor activation is permissive for opioid mobilization of intracellular Ca^{2+} but that the extent of muscarinic activation is not *per se* a crucial determinant of the magnitude of the subsequent opioid response. The nature of the link between muscarinic receptor occupancy and opioid-induced mobilization of $[Ca^{2+}]_i$ is at present unclear.

The mechanism by which opioids mobilize Ca^{2+} in SH-SY5Y cells is not clear. The simplest explanation would be that opioids are generating an intracellular messenger such as IP_3, which subsequently induces the release of Ca^{2+} from thapsigargin-sensitive intracellular stores. There is, however, no evidence that opioid receptors can couple to G proteins of the G_q family, in which α subunits directly activate PLC (Exton, 1994). Additionally, it is clear that the opioid mobilization of Ca^{2+} described here is being mediated by pertussis toxin-sensitive G-proteins of the G_i or G_o class, the α subunits of which do not stimulate PLC.

Recent experiments by Smart *et al.* (1994, 1995) have addressed the issue of whether opioids elevate IP_3 levels in SH-SY5Y cells. These experiments differ from ours primarily in that they measure IP_3 levels in cells at a defined moment, whilst we determine the $[Ca^{2+}]_i$ moment to moment over a prolonged period. Smart *et al.* reported a modest elevation of IP_3 levels following μ receptor activation alone, the stimulation of PLC activity observed in their experiments was thought to occur by a μ receptor-induced influx of extracellular Ca^{2+} through L-type Ca channels. The elevation of $[Ca^{2+}]_i$ by opioids described here does not result from IP_3 generated subsequent to an influx of extracellular Ca^{2+} because the elevation of $[Ca^{2+}]_i$ is not dependent on extracellular Ca^{2+}. Importantly, Smart *et al.*, also observed that the elevation of IP_3 by μ agonists was no greater in the presence of 100 μM carbachol, which suggests that in SH-SY5Y cells opioid and muscarinic receptors do not act synergistically on PLC. In our experiments, opioids alone did not cause the elevations of $[Ca^{2+}]_i$ that might be expected to follow the generation of IP_3 by the scheme of Smart *et al.* (1994, 1995). This is almost certainly due to the fact that under our culture conditions, undifferentiated SH-SY5Y cells appear to lack voltage-dependent Ca channels. Our evidence for this is two fold; firstly, our SH-SY5Y cells do not respond to depolariza-

tion with 50 mM KCl with an increase in $[Ca^{2+}]_i$; secondly, we have been unable to detect any Ca channels in electrophysiological experiments carried out in parallel with the studies described here (Connor, unpublished observations). It is clear that the phenomena described by us and by Smart *et al.* are different in a number of important respects and probably represent completely separate pathways by which opioids can modulate levels of $[Ca^{2+}]_i$. Nevertheless, their experiments provide evidence that opioids do not stimulate PLC directly in SH-SY5Y cells, either alone or in the presence of muscarinic agonists.

While opioids do not appear to increase IP_3 directly they could, in theory, be acting at any of a number of sites in the muscarinic receptor/PLC cascade to (re)sensitize the Ca^{2+} mobilization process. The opioids could be acting to reverse transiently the acute desensitization of the muscarinic receptor that follows carbachol exposure; they could alter the regulation of PLC, the IP_3 receptor itself or any of the enzymes involved in the resynthesis of PIP_2 from inositol polyphosphates (Wojcikiewicz *et al.*, 1994). Of the major second messenger pathways implicated in the control of such processes, only that involving cyclic AMP is known to be directly regulated by opioids. One of the primary cellular consequences of the generation of cyclic AMP is activation of PKA, which has been shown to phosphorylate the IP_3 receptor directly (Supattapone *et al.*, 1988). Opioids have been shown to inhibit adenylyl cyclase in SH-SY5Y cells (Kazmi & Mishra, 1987; Yu & Sadee, 1988). Incubation of the SH-SY5Y cells with a high concentration of the selective PKA inhibitor, H89 (Chijiwa *et al.*, 1990) neither mimicked nor occluded the opioid-induced mobilization of Ca^{2+}, suggesting that opioid receptor modulation of intracellular cyclic AMP levels was not involved in the observed elevation of $[Ca^{2+}]_i$. Another protein kinase, PKC, has also been shown to phosphorylate the IP_3 receptor. There is some evidence that opioids can modulate PKC activity (Chen & Huang, 1991); however, incubation of the SH-SY5Y cells with the non-selective protein kinase inhibitor H7, which inhibits PKC as well as PKA and cyclic GMP-dependent protein kinase (Hidaka *et al.*, 1984), or the selective PKC inhibitor, Bis I, did not mimic or block the opioid elevation of $[Ca^{2+}]_i$. Thus modulation of PKC- or PKA-dependent processes by opioid receptor activation is unlikely to be responsible for the observed elevation of $[Ca^{2+}]_i$. This report demonstrates that in SH-SY5Y cells, opioids elevate $[Ca^{2+}]_i$ by mobilizing it from intracellular stores, but the mechanism(s) underlying this phenomenon remains unclear.

There are a number of possible physiological consequences of opioid-induced elevations of $[Ca^{2+}]_i$. For example, many forms of the ubiquitous cytosolic enzyme, PKC, are activated by elevations in intracellular Ca^{2+} and/or diacylglycerol. Activation of PKC has been implicated in the regulation of cellular responses by opioids in spinal trigeminal neurones. In these cells, μ opioid agonists were shown to potentiate NMDA receptor currents by a PKC-dependent mechanism (Chen & Huang, 1991). A simple explanation for this observation would be that PKC was being activated secondary to local opioid-induced elevations of $[Ca^{2+}]_i$, mediated by either Ca^{2+} influx or Ca^{2+} mobilization. Additionally, all opioid receptors cloned thus far have a consensus phosphorylation site for PKC on their third intracellular loop, a region of the receptors implicated in coupling to G-proteins and receptor down-regulation and desensitization. In NG 108-15 cells, activation of PKC can selectively enhance the agonist mediated down-regulation of δ opioid receptors (Gucker & Bidlack, 1992); in that study activators of PKC did not alter δ receptor binding in the absence of δ receptor agonists.

This study demonstrates that in the same cells both δ and μ opioid receptors can interact with muscarinic cholinergic systems. It is intriguing that, in the presence of an agonist for an unrelated type of receptor, opioid receptor activation can have novel effects on second messenger systems that are not normally thought to be modulated by opioids. The interactions at the level of PLC between δ and μ opioids and agonists at purinoceptors, muscarinic and bradykinin receptors are reminiscent of the interactions between opioid receptors and other receptors that alter the activity of adenylyl cyclase in the rat olfactory bulb. In that system opioid or muscarinic receptor activation can act synergistically with activation of receptors for vasoactive intestinal peptide or corticotrophin releasing hormone to stimulate the production of cyclic AMP (Olianas & Onali, 1993). It is thought that the α subunits of the Gs heterotrimers act in concert with the $\beta\gamma$ subunits from the opioid or muscarinic receptors to stimulate the type II or type IV adenylyl cyclases, which are not stimulated appreciably by $\beta\gamma$ subunits alone (Tang & Gilman, 1991). Such interactions may be common in an *in vivo* situation where cells are exposed to many neurotransmitters and neuromodulatory substances. Careful investigation of the interactions between opioid receptors and other plasma membrane receptors may lead to new insights into the cellular consequences of opioid receptor activation.

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