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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

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1 In SH-SY5Y cells, μ-opioids cause a rapidly desensitizing activation of phospholipase C (PLC), that appears secondary to Ca^{2+} influx via L-type voltage-sensitive Ca^{2+} channels (VSCCs). The aim of the present study was to characterize the mechanisms of desensitization of the μ-opioid-induced inositol (1,4,5) triphosphate (Ins(1,4,5)P_3) response, by use of a stereospecific radioreceptor mass assay.

2 (R+)-Bay K 8644 (1 nM-10 μM) dose-dependently inhibited fentanyl-induced Ins(1,4,5)P_3 formation, with an IC_{50} of 28.5 nM, confirming our earlier observations that μ-opioids open L-type VSCCs, thus allowing Ca^{2+} influx to activate PLC.

3 Ro 31-8220 (0.1 nM-10 μM), a protein kinase C inhibitor, dose-dependently enhanced fentanyl-induced Ins(1,4,5)P_3 formation (EC_{50} = 20.0 nM), whilst acute phorbol 12,13-dibutrate (1 μM) abolished the response.

4 H-89 (1 nM-10 μM), a protein kinase A inhibitor, also dose-dependently enhanced fentanyl-induced Ins(1,4,5)P_3 formation (EC_{50} = 93 nM), whilst dibutryl cyclic AMP (0.5 mM) abolished the response.

5 Blockade of Ca^{2+}-activated K^+ currents with 4-aminopyridine (2 mM) or iberiotoxin (10 nM) had no effect on fentanyl-induced Ins(1,4,5)P_3 formation but further increased the Ro 31-8220-enhanced response.

6 All three mechanisms had additive, or even supra-additive, effects, but only at later (120-300 s) time points. In addition, fentanyl-induced Ins(1,4,5)P_3 formation, even if enhanced by H-89, Ro 31-8220 and/or 4-aminopyridine, was inhibited by nifedipine (1 nM-10 μM).

7 In conclusion, desensitization of the μ-opioid-induced activation of PLC is multifactorial, involving protein kinases C and A and Ca^{2+}-activated K^+ efflux, but the L-type VSCC is of critical importance and may be a possible common site of action.

Keywords: μ-Opioids; inositol(1,4,5)triphosphate; L-type voltage-sensitive Ca^{2+} channels; SH-SY5Y human neuroblastoma cells; protein kinase C; protein kinase A

Introduction

We have recently reported that in SH-SY5Y human neuroblastoma cells, μ-opioid receptor occupancy causes a Ca^{2+}-dependent activation of phospholipase C (PLC), leading to an increase in inositol(1,4, 5)triphosphate (Ins(1,4,5)P_3) formation (Smart *et al.*, 1994; 1995). There are several types (denoted L,N,T and P) of voltage-sensitive Ca^{2+} channels (VSCCs), each of which has different pharmacological and electrophysiological profiles (Spedding & Paoletti, 1992). However, SH-SY5Y cells possess only L- and N-type VSCCs (Morton *et al.*, 1992; Reeve *et al.*, 1994), and we have previously shown that nifedipine dose-dependently inhibits the fentanyl-induced Ins(1,4,5)P_3 response (Smart *et al.*, 1995), suggesting that, in SH-SY5Y cells, μ-opioid-induced Ca^{2+} influx occurs via L-type VSCCs. Opioids have also been reported to open L-type VSCCs in astrocytes (Eriksson *et al.*, 1993) and NG108-15 cells (Jin *et al.*, 1992), although it should be noted that opioids generally close VSCCs (Porzig, 1990).

The μ-opioid-induced activation of PLC in SH-SY5Y cells is transient, with Ins(1,4,5)P_3 formation peaking at 15 s and returning to basal levels within 1 to 2 min (Smart *et al.*, 1994; 1995), indicating that the response rapidly desensitizes. Throughout this paper we have used the term 'desensitization' in the context of the reduction of the (second messenger) response seen with increasing duration of the stimulus (i.e. receptor occupancy by agonist), as described by Lohse (1993), which probably reflects a type of autoinhibitory feedback. There are several possible mechanisms for such a rapid desensitization, including receptor phosphorylation, uncoupling of G-proteins from receptors, modulation of ion channel function, and combinations thereof (Lohse, 1993). However, all these mechanisms potentially involve increased protein kinase C (PKC) activity (Lohse, 1993). Indeed, PKC has been reported to uncouple G-proteins from opioid receptors in guinea-pig striatal membranes (Fukushima *et al.*, 1994) and SH-SY5Y cells (Lin *et al.*, 1994). Furthermore, PKC is known to modulate the activity of various ion channels (Shearman *et al.*, 1989; Petersen & Berridge, 1994; Tuominen *et al.*, 1994), including the L-type VSCC (Schuhmann & Groschner, 1994). As activation of PLC stimulates PKC activity, via diacylglycerol, in addition to stimulating Ins(1,4,5)P_3 formation (Berridge, 1993), μ-opioids would also be expected to increase PKC activity in SH-SY5Y cells. Indeed, a μ-opioid-induced activation of PKC has been reported for dorsal horn neurones (Chen & Huang, 1991).

Protein kinase A (PKA) has also been implicated in the rapid desensitization of other responses (Lohse, 1993) and been shown to modulate the activity of both K^+ and Ca^{2+} ion channels (Levitan, 1985). Inhibition of PKA activity potentiated the δ-opioid-induced, Ca^{2+}-driven Cl^- current in *Xenopus* oocytes (Ueda *et al.*, 1994).

Alternatively, the μ-opioid-induced Ins(1,4,5)P_3 response could be desensitizating because K^+ currents, activated either directly by μ-opioid receptor occupancy (North, 1989) or indirectly via Ca^{2+} influx (De Peyer *et al.*, 1982), are hyperpo-

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larizing the cell, thus favouring the closed state of L-type VSCCs. However, this mechanism could still involve PKC and PKA, as both of these protein kinases modulate K^+ channel activity (Levitan, 1985; Shearman *et al.*, 1989), including that of the Ca^{2+}-activated K^+ channel (DePeyer *et al.*, 1982; Chen & Yu, 1994).

Therefore, the present study was undertaken to characterize the mechanisms responsible for the rapid desensitization of the μ-opioid-induced activation of PLC in SH-SY5Y cells. We have demonstrated that the rapid desensitization of the μ-opioid-induced Ins(1,4,5)P_3 response is multifactorial, involving increased PKC and PKA activity as well as cell hyperpolarization via the opening of Ca^{2+}-activated K^+ channels. Furthermore, the data suggest that the L-type VSCC represents a potential common site of action for all these mechanisms.

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 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2.5 μg ml^{-1} fungizone, and 10% foetal calf serum.

Cells were harvested with 10 mM HEPES-buffered saline/0.02% EDTA, pH 7.4, washed twice with and then 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_2^{2-} 1.2, glucose 11.7 and HEPES 10.

Measurement of Ins(1,4,5)P_3

Whole cell suspensions (final volume 0.3 ml) were preincubated at 37°C for 15 min with the following drugs in various combinations: (R+)-Bay K 8644 (1 nM–10 μM), an L-type VSCC antagonist (Van Amsterdam *et al.*, 1989); Ro 31-8220 (0.1 nM–10 μM), a PKC inhibitor (Davis *et al.*, 1989); phorbol 12,13-dibutyrate (PDBu, 1 μM); H-89 (1 nM –10 μM), a PKA inhibitor (Geilen *et al.*, 1992); dibutryl 12,13 cyclic AMP (db cyclic AMP, 0.5 mM); 4-aminopyridine (4-AP, 2 mM), a relatively non-selective K^+ channel antagonist (Forsythe *et al.*, 1992); iberiotoxin (IBTX, 10 nM), a selective Ca^{2+}-activated K^+ channel antagonist (Galvez *et al.*, 1990); and nifedipine (1 nM–10 μM), an L-type VSCC antagonist (Spedding & Paoletti, 1992). The cells were then incubated with fentanyl (0.1 μM) for 0–300 s. 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, vol:vol) 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 $mmol^{-1}$) at 4°C. Authentic Ins(1,4,5)P_3 (0.036–12 pmol) 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).

Source of reagents

All cell culture materials were supplied by GIBCO (UK). (R+)-Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) was obtained from RBI (U.S.A.), via SEMAT technical supplies (U.K.). Ro 31-8220 was donated by Roche (U.K.). H-89 (N-[2-bromocinnamyl(amino)ethyl]-5-isoquinoline sulphonamide) was obtained from Calbiochem (U.K.). [^{3}H]-Ins(1,4,5)P_3 was supplied by Amersham (U.K.). All other reagents were obtained from Sigma (U.K.).

Data analysis

All data are given as mean ± s.e.mean unless otherwise stated. EC_{50} (half maximal stimulation) and IC_{50} (half maximal inhibition) values were obtained by computer-assisted curve (non-linear regression model) fitting using GRAPHPAD, and are mean values from replicate analyses. Statistical comparisons were made where appropriate by ANOVA and/or Student's *t* test, and were considered to be significant when $P<0.05$.

Results

Fentanyl (0.1 μM) caused a transient, monophasic increase in Ins(1,4,5)P_3 formation (peaking at 15 s) in SH-SY5Y cells (e.g. Figure 2) as previously described (Smart *et al.*, 1994; 1995). The magnitude of the peak response varied considerably between batches of cells (17–32 pmol mg^{-1} protein), but was consistent within a batch. Basal Ins(1,4,5)P_3 formation also varied considerably between batches (6–16 pmol mg^{-1} protein), but once again was consistent within a batch. All time courses contain their own control.

(R+)-Bay K 8644 (1 nM–10 μM), an L-type VSCC antagonist (Van Amsterdam *et al.*, 1989), dose-dependently inhibited fentanyl-induced Ins(1,4,5)P_3 formation (Figure 1), with an IC_{50} of 28.5 nM, which, in agreement with the findings of our previous study using nifedipine (Smart *et al.*, 1995), indicates that μ-opioids open L-type VSCCs in SH-SY5Y cells and so allow Ca^{2+} influx to activate PLC.

Ro 31-8220 (1 μM), a PKC inhibitor (Davis *et al.*, 1989), enhanced the fentanyl-induced Ins(1,4,5)P_3 response (Figure 2a), shifting the peak from 15 to 30 s, whilst increasing its magnitude (from 18.2 ± 1.5 to 33.4 ± 2.4 pmol mg^{-1} protein), as well as prolonging the response (from 1 to 2 min). This enhancement of fentanyl-induced Ins(1,4,5)P_3 formation by Ro 31-8220 (0.1 nM–10 μM) was dose-dependent (Figure 3), with an EC_{50} (at 30 s) of 20.0 nM, consistent with the IC_{50} of 30 nM previously reported for the inhibition of PKC activity

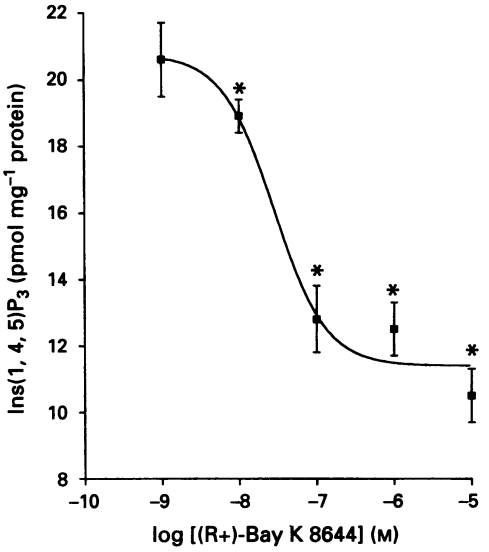

Figure 1 (R+)-Bay K 8644 dose-dependent inhibition of fentanyl-induced Ins(1,4,5)P_3 formation in SH-SY5Y cells. Whole cell suspensions (0.3 ml) were preincubated at 37°C for 15 min, with (R+)-Bay K 8644 (1 nM–10 μM). The cells were then incubated with fentanyl (0.1 μM) for 15 s. Ins(1,4,5)P_3 was measured by a specific radioreceptor mass assay. Data are mean ± s.e.mean, $n=5$. *$P<0.05$ (*t* test): decreased compared to fentanyl alone (21.1 ± 0.8 pmol mg^{-1} protein). Basal Ins(1,4,5)P_3 formation was 9.6 ± 0.3 pmol mg^{-1} protein (9.3 ± 0.8 pmol mg^{-1} protein in the presence of 1 μM (R+)-Bay K 8644). For abbreviations in this and subsequent figure legends see text.

by Ro 31-8220 in rat brain (Davis *et al.*, 1989). Furthermore, activation of PKC by acute exposure to PDBu (1 μM) abolished, whilst down-regulation of PKC by chronic (72 h) exposure to PDBu (1 μM) enhanced, the fentanyl-induced Ins(1,4,5)P_3 response (data not shown). Neither Ro 31-8220 (Figure 2a) nor PDBU (data not shown) affected basal Ins(1,4,5)P_3 formation. These data strongly suggest that increased PKC activity contributes to the desensitization of the μ-opioid-induced activation of PLC.

H-89 (10 μM), a PKA inhibitor (Geilen *et al.*, 1992), also enhanced fentanyl-induced Ins(1,4,5)P_3 formation (Figure 2b), again shifting the peak from 15 to 30 s, but without increasing its magnitude. However, H-89 also broadened the peak to 60 s, and prolonged the response to 5 min, when sampling ended (Figure 2b). This enhancement of the μ-opioid-induced activation of PLC by H-89 (1 nM – 10 μM) was dose-dependent (data not shown), with an EC_{50} of 93 nM, consistent with an inhibition of PKA, as H-89 has a K_i for PKA of 48 nM (Geilen *et al.*, 1992). Furthermore, activation of PKA with db cyclic-AMP (0.5 mM) abolished the fentanyl-induced Ins(1,4,5)P_3 response (data not shown). Neither H-89 (Figure 2b) nor db cyclic AMP (data not shown) affected basal Ins(1,4,5)P_3 formation. These data show that PKA also plays a role in the desensitization of μ-opioid-induced Ins(1,4,5)P_3 formation.

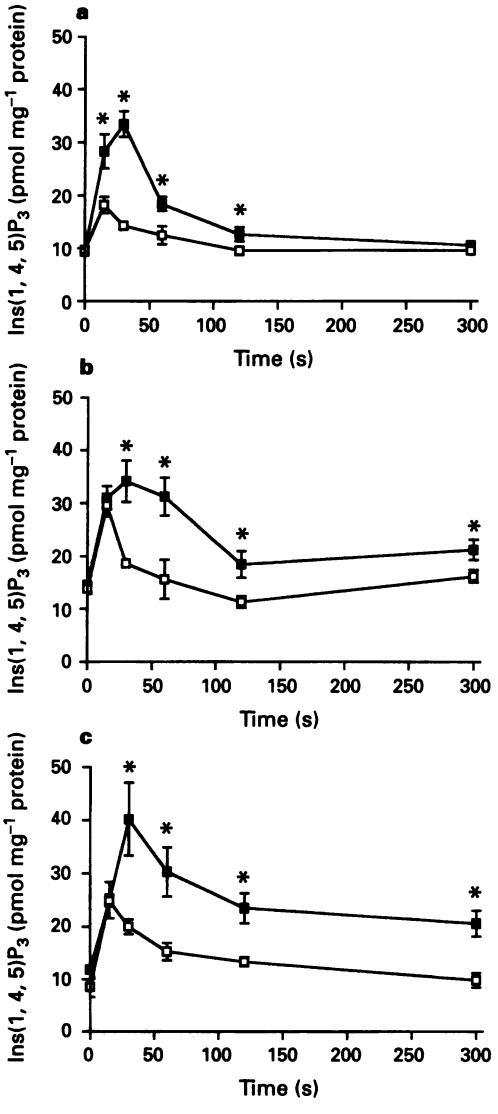

Figure 2 Inhibition of PKC and/or PKA enhances fentanyl-induced Ins(1,4,5)P_3 formation in SH-SY5Y cells. Whole cell suspensions (0.3 ml) were preincubated at 37°C for 15 min, with (■) or without (□) either Ro 31-8220 (1 μM), a PKC inhibitor (a); H-89 (10 μM), a PKA inhibitor (b); or Ro 31-8220 (1 μM) and H-89 (10 μM) together (c). The cells were then incubated with fentanyl (0.1 μM) for 0 – 300 s. Data are mean ± s.e.mean, $n=4$. $^*P<0.05$ (*t* test): increased compared to fentanyl alone.

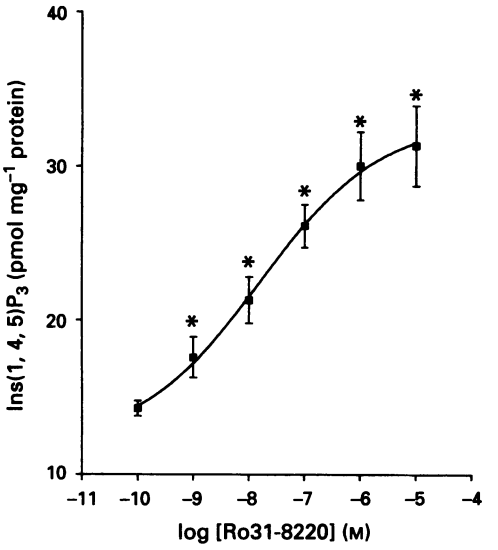

Figure 3 Dose-dependent enhancement by Ro 31-8220 of the fentanyl-induced Ins(1,4,5)P_3 response. Whole cell suspensions (0.3 ml) were preincubated at 37°C for 15 min, with Ro 31-8220 (0.1 nM – 10 μM), and then incubated with fentanyl (0.1 μM for 30 s. Data are mean ± s.e.mean, $n=5$. $^*P<0.05$ (*t* test): increased compared to fentanyl alone ($=14.5\pm0.9$ pmol mg^{-1} protein).

Simultaneous inhibition of PKC and PKA with a combination of Ro 31-8220 (1 μM) and H-89 (10 mM) also enhanced fentanyl-induced Ins(1,4,5)P_3 formation (Figure 2c), but at earlier time points (15 – 60 s) there was no additivity (Figure 2a,b,c). However, at 120 s there was an additive effect with H-89 and Ro 31-8220, whilst at 300 s there was a supra-additive enhancement (Table 1).

4-AP (2 mM), a relatively non-selective K^+ channel antagonist (Forsythe *et al.*, 1992), had no effect on fentanyl-induced Ins(1,4,5)P_3 formation, but did further increase the Ro 31-8220 (1 μM) enhanced, μ-opioid-induced Ins(1,4,5)P_3 response, although only at later (60 – 300 s) time points (Figure 4a), where the effect was supra-additive (Table 1). However, 4-AP (2 mM) had no effect on the H-89-enhanced response (data not shown). To identify the type of K^+ current involved, IBTX, a selective Ca^{2+}-activated K^+ channel antagonist (Galvez *et al.*, 1990), was used, IBTX (10 nM) also had no effect on fentanyl-induced Ins(1,4,5)P_3 formation, but further increased the Ro 31-8220-enhanced response at later (60 – 300 s) time points (Figure 4b), where, once again, the effect was supra-additive (Table 1). These data suggest that Ca^{2+}-activated K^+ currents, presumably by hyperpolarizing the cell, are also involved in the desensitization of the μ-opioid-induced activation of PLC, but only play a significant role if PKC is inhibited.

Nifedipine (1 nM – 10 μM), an L-type VSCC antagonist (Spedding & Paoletti, 1992), dose-dependently inhibited Ro 31-8220 (1 μM)-enhanced, fentanyl-induced Ins(1,4,5)P_3 formation (Figure 5), with an IC_{50} of 29.2 nM. We have previously reported that nifedipine inhibits (unenhanced) fentanyl-induced Ins(1,4,5)P_3 formation in SH-SY5Y cells, with an IC_{50} of 60.3 nM (Smart *et al.*, 1995). Furthermore, nifedipine (1 μM) also abolished the fentanyl-induced Ins(1,4,5)P_3 response in the presence of H-89 (10 μM) or 4-AP (2 mM) and Ro 31- 8220 (1 μM) (Table 2). This dose of nifedipine also completely inhibited (unenhanced) fentanyl-induced Ins(1,4,5)P_3 formation (Table 2 and see Smart *et al.*, 1995). These data suggest that all three mechanisms of desensitization involve the inactivation (i.e. closing) of the L-type VSCC.

Table 1 Additive and supra-additive enhancement of fentanyl-induced Ins(1,4,5)P_3 formation by PKC and PKA inhibitors, and K^+ channel blockade, in SH-SY5Y cells

	Ins(1,4,5)P_3 (sample-control; pmol mg^{-1} protein)	
	120s	*300s*
Ro 31-8220	3.6±1.1*	0.7±0.5
H-89	7.5±1.1*	5.0±3.1*
H-89+Ro 31-8220	10.1±2.5*[a]	10.7±3.0*[b]
4-AP	1.2±1.8	1.6±1.9
4-AP+Ro 31-8220	12.7±4.3*[b]	12.2±2.1*[b]
IBTX	−0.7±0.8	−0.2±0.7
IBTX+Ro 31-8220	13.2±5.6*[b]	10.3±4.9*[b]

Whole cell suspensions (0.3 ml) were preincubated at 37°C with Ro 31-8220 (1 μM), H-89 (10 μM), 4-AP (2 mM) and IBTX (10 nM) in various combinations for 15 min. The cells were then incubated with fentanyl (0.1 μM) for 120 or 300 s. Ins(1,4,5)P_3 was measured by a specific radioreceptor mass assay. Data are mean±s.e.mean, $n=4-6$. See text for abbreviations.
*$P<0.05$ (*t*-test) increased compared to fentanyl alone. [a] additive increase; [b] supra-additive increase.

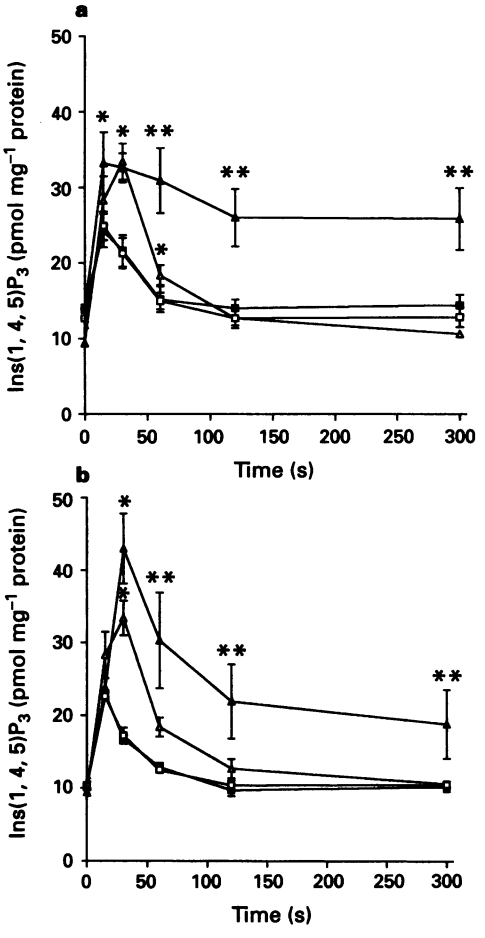

Figure 4 Blockade of K^+ efflux enhances fentanyl-induced Ins(1,4,5)P_3 formation only when PKC activity is inhibited. (a) Blockade of K^+ efflux with 4-AP (2 mM); (b) blockade of K^+ efflux with IBTX (10 nM). Whole cell suspensions (0.3 ml) were preincubated at 37°C for 15 min, with either buffer alone (□), 4-AP/IBTX (■), 1 μM Ro 31-8220 (△), or 1 μM Ro 31-8220 and 4-AP/IBTX (▲). The cells were then incubated with fentanyl (0.1 μM) for 0–300 s. Data are mean±s.e.mean, $n=4-6$. *$P<0.05$, increased compared to fentanyl alone. **$P<0.05$: increased compared to Ro 31-8220-enhanced response.

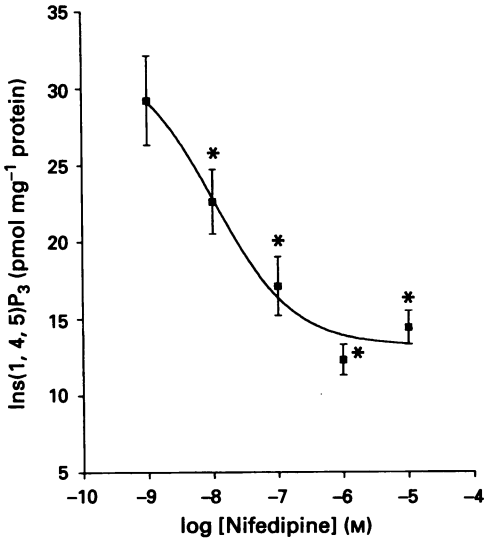

Figure 5 Ro 31-8220-enhanced fentanyl-induced Ins(1,4,5)P_3 formation is dose-dependently inhibited by nifedipine. Whole cell suspensions (0.3 ml) were preincubated at 37°C for 15 min, with Ro 31-8220 (1 μM) and nifedipine (1 nM–10 μM), and then incubated with fentanyl (0.1 μM) for 15 s. Data are mean±s.e.mean, $n=5$ *$P<0.05$: decreased compared to Ro 31-8220-enhanced response alone (=28.6±1.7 pmol mg^{-1} protein).

Discussion

We show here that μ-opioids open L-type VSCCs, allowing Ca^{2+} influx to activate PLC and so stimulate Ins(1,4,5)P_3 formation, in SH-SY5Y cells. We have also shown that the rapid desensitization (see Introduction for definition) of this response is multifactorial, involving increased PKC and PKA

Table 2 Inhibition by nifedipine of H-89 and 4-AP plus Ro 31-8220-enhanced fentanyl-induced Ins(1,4,5)P_3 formation in SH-SY5Y cells

Addition to preincubation	*Ins(1,4,5)P_3 (pmol mg^{-1} protein)*
None	25.0±0.8
+Nifedipine	17.1±1.7*
+H-89	25.5±1.0
+H-89 and nifedipine	17.5±0.6*
+4-AP and Ro 31-8220	33.7±2.7
+4-AP, Ro 31-8220 and nifedipine	15.9±1.3*

Whole cell suspensions (0.3 ml) were preincubated at 37°C for 15 min, with H-89 (10 μM), Ro 31-8220 (1 μM), 4-AP (2 mM), and nifedipine (1 μM) in various combinations. The cells were then incubated with fentanyl (0.1 μM) for 15 s. Data are mean±s.e.mean, $n=5$. (Basal Ins(1,4,5)P_3 formation=15.9±0.4 pmol mg^{-1} protein). For abbreviations see text.
*$P<0.05$ (*t* test) reduced compared to paired, non-nifedipine-treated response.

activity, and possibly hyperpolarization by Ca^{2+}-activated K^+ efflux. Moreover, the data demonstrate the vital role of the L-type VSCC and suggest that it may be a potential common site of action for desensitization. Hyperpolarization, due to increased Ca^{2+}-activated K^+ channel activity, has been proposed as one of the possible mechanisms underlying morphine analgesia (Lipp, 1991). Alternatively, increases in both PKC and PKA activity have recently been linked to the development of opioid tolerance (Narita *et al.*, 1994a,b,c; Aloyo, 1995).

In the current investigation, fentanyl stimulated Ins(1,4,5)P_3 formation, as previously reported (Smart *et al.*, 1994; 1995), and this response was dose-dependently inhibited by (R+)-Bay K 8644, an L-type VSCC antagonist (Van Amsterdam *et al.*, 1989). These data, in conjunction with those showing nifedipine also inhibited the μ-opioid-induced Ins(1,4,5)P_3 response (Smart *et al.*, 1995), indicate that μ-opioids open L-type VSCCs, allowing Ca^{2+} influx to stimulate PLC activity (Cockcroft & Thomas, 1992) in SH-SY5Y cells. Whilst opioids generally close VSCCs (Porzig, 1990), there is evidence that they can also open L-type VSCCs, as seen with δ-opioids in NG108-15 cells (Jin *et al.*, 1992) and κ-opioids in astrocytes (Eriksson *et al.*, 1993).

Fentanyl-induced Ins(1,4,5)P_3 formation rapidly desensitizes in SH-SY5Y cells. There are several possible mechanisms by which such a rapid desensitization could occur (see Introduction and Lohse, 1993), but all potentially involve phosphorylation by effector protein kinases (Lohse, 1993). Activation of PLC increases PKC activity via diacylglycerol, as well as stimulating Ins(1,4,5)P_3 formation (Berridge, 1993), and μ-opioids have been shown to increase PKC activity in dorsal horn neurones from rats (Chen & Huang, 1991). Acute activation of PKC with phorbol esters abolished, whilst inhibition of PKC with Ro 31-8220 (Davis *et al.*, 1989) or down-regulation of endogenous PKC activity by chronic (72 h) exposure to phorbol esters enhanced, fentanyl-induced Ins(1,4,5)P_3 formation. These data suggest that μ-opioid receptor occupancy also increases PKC activity, which in turn desensitizes the Ins(1,4,5)P_3 response.

Protein kinase A is also involved in the rapid desensitization of μ-opioid-induced PLC activity, as H-89, a PKA inhibitor (Geilen *et al.*, 1992), enhanced, whilst activation of PKA with db cyclicAMP inhibited, the fentanyl-induced Ins(1,4,5)P_3 response. PKA has also been reported to be involved in the rapid desensitization of other responses (Lohse, 1993), including the desensitization of the δ-opioid induced Cl^- current in *Xenopus* oocytes (Ueda *et al.*, 1994). Concomitant inhibition of PKC and PKA, with Ro 31-8220 and H-89, also enhanced the fentanyl-induced Ins(1,4,5)P_3 response, but at earlier time points (15–60 s) this enhancement was only of similar magnitude to that seen with either inhibitor alone, suggesting that the effects of both PKC and PKA are necessary for the early phase of desensitization. However, at later time points (120 300 s) there was an additive, or even supra-additive, effect with these inhibitors (see Table 1), indicating that in the later phase of desensitization both protein kinases exert their own, separate inhibitory effects. One possible explanation for this interaction is that PKC could be activating adenylyl cyclase, as recently demonstrated (Kawabe *et al.*, 1994), leading to enhanced PKA activity, although this is unlikely as μ-opioids inhibit adenylyl cyclase in SH-SY5Y cells (Smart *et al.*, 1995). However, basal PKA tone could still be inhibiting L-type VSCC activity in SH-SY5Y cells, as seen in other cell types (Levitan, 1985). A more likely explanation for this interaction is that PKC and PKA are acting at the same site. It is also worth noting here that increases in the activity of PKC and PKA have recently been linked to the development of tolerance towards opioids in rats (Narita *et al.*, 1994a,b,c) and rabbits (Aloyo, 1995), although the exact mechanisms involved remain unclear.

Another possible mechanism for the desensitization of the μ-opioid-induced activation of PLC is that K^+ currents, activated either directly by μ-opioid receptor occupancy (North, 1989) or via Ca^{2+} influx (Chen & Yu, 1994), could be hyperpolarizing the cell and thus preventing further Ca^{2+} entry via the L-type VSCCs. Indeed, it has recently been reported that Ca^{2+}-activated K^+ channels are colocalized with, and preferentially couple to, L-type VSCCs (Wisgirda & Dryer, 1994). However, in the present study blockade of the Ca^{2+}-activated K^+ channels with either 4-AP or IBTX had no effect on fentanyl-induced Ins(1,4,5)P_3 formation, although both further increased the Ro 31-8220 (but not the H-89)-enhanced response, albeit only in the later phase of desensitization (60–300 s), where the effect was supra-additive (see Table 1). This suggests that PKC inhibits the opening of Ca^{2+}-activated K^+ channels, so preventing hyperpolarization. Indeed, PKC is known to inhibit a variety of K^+ channels (Shearman *et al.*, 1989), including the Ca^{2+}-activated K^+ channel (De Peyer *et al.*, 1982; Chen & Yu, 1994). It is worth noting here that activation of PKC with phorbol esters inhibited opioid-induced spinal analgesia in rats (Zhang *et al.*, 1990), and that SH-SY5Y cells express the same type of μ-opioid receptors as those activated in spinal analgesia (Elliott *et al.*, 1994).

Nifedipine, an L-type VSCC antagonist (Spedding & Paoletti, 1992), dose-dependently inhibited Ro 31-8220-enhanced, fentanyl-induced Ins(1,4,5)P_3 formation, with an IC_{50} similar to that previously reported for nifedipine on the (unenhanced) fentanyl-induced Ins(1,4,5)P_3 response (Smart *et al.*, 1995), indicating that PKC desensitizes the μ-opioid-induced activation of PLC by closing the L-type VSCC. In agreement with this, PKC has been reported to close L-type VSCCs in human vascular smooth muscle (Schuhmann & Groschner, 1994) and other tissues (Shearman *et al.*, 1989). Nifedipine also inhibited the H-89 and the combined 4-AP/Ro 31-8220-enhanced responses, suggesting a possible common site of action for these mechanisms of desensitization at the level of the L-type VSCC. The phosphorylation of ion channels by effector protein kinases has previously been proposed as one of the mechanisms underlying the rapid desensitization of a number of responses (Lohse, 1993). It is worth noting here that the μ-opioid-mediated opening of the L-type VSCC in SH-SY5Y cells involves a pertussis toxin-sensitive G-protein (Smart *et al.*, 1994; 1995), and that PKC has been shown to phosphorylate, and thus inactivate, such G-proteins in SH-SY5Y cells (Lin *et al.*, 1994). In addition, it has been reported that PKC uncouples δ-opioids from their G-proteins in guinea-pig striatal membranes (Fukushima *et al.*, 1994).

In conclusion, we have shown that the rapid desensitization of the μ-opioid-induced activation of PLC is multifactorial, involving increased PKC and PKA activity, as well as an underlying hyperpolarization of the cell by Ca^{2+}-activated K^+ currents that is normally masked by the actions of PKC. In addition, the data suggest that the common site of action of all these mechanisms of desensitization may be the L-type VSCC.

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