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# The effect of C-terminal truncation of the recombinant δ-opioid receptor on $Ca_i^{2+}$ signaling

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**Abstract**

We have previously shown a stimulatory coupling of the recombinant δ-opioid receptor to phospholipase C leading to production of inositol (1,4,5) triphosphate [Ins(1,4,5)$P_3$] that is affected by truncation of the C-terminus of the receptor. Using a C-terminal mutant of the δ-opioid receptor lacking the final 37 amino acids (CHOδ37), we examined its coupling to intracellular calcium ion concentration ($[Ca^{2+}]_i$) compared to the full length wild type receptor (CHOδWT) in transfected Chinese hamster ovary (CHO) cells. D-[$Pen^{2,5}$]enkephalin (DPDPE) mediated increases in $[Ca^{2+}]_i$ were measured fluorimetrically in fura-2 loaded whole cell suspensions. DPDPE produced time- and concentration-dependent increases in $[Ca^{2+}]_i$ in CHOδWT and CHOδ37. In both cell types the DPDPE simulated increase in $[Ca^{2+}]_i$ was naloxone reversible and pertussis toxin and thapsigargin sensitive. Removal of the C-terminus resulted in a rightward shift of the $Ca^{2+}$ release concentration–response curve [$pEC_{50} = 8.43 \pm 0.13$ and $6.08 \pm 0.25$ for CHOδWT and CHOδ37, respectively]. These data indicate that the C-terminus of the recombinant δ-opioid receptor is important in $[Ca^{2+}]_i$ coupling and may be attributed to the effect of C-terminus truncation on phospholipase C coupling reported previously. 

*Keywords:* δ-Opioid receptor; $Ca^{2+}$, intracellular; CHO (Chinese hamster ovary) cell

## 1. Introduction

μ, δ and κ-opioid receptors belong to the family of seven trans-membrane spanning G protein coupled receptors and have all been cloned and sequenced (Evans et al., 1992; Kieffer et al., 1992; Thompson et al., 1993; Yasuda et al., 1993) Opioids produce their analgesic actions via an inhibition of excitatory neurotransmission (Leslie, 1987), thought to result via co-ordinated changes at a cellular level, such as closure of voltage-sensitive $Ca^{2+}$ channels (Porzig, 1990), activation of $K^+$ channels (North, 1989) and a reduction in cyclic AMP formation (Childers, 1991). However, there are now numerous reports of stimulatory effects of opioids, including stimulation of cAMP formation, phospholipase C activation and increases in intracellular calcium ion concentration ($[Ca^{2+}]_i$) (Sarne et al., 1996; Smart and Lambert, 1996; Crain and Shen, 1998; Harrison et al., 1998).

Cloning studies of opioid receptors have revealed repeated serine and threonine residues on the C-terminal tail of the δ-opioid receptor that are potential phosphorylation sites for kinases such as protein kinase C. Indeed it has been shown that these sites may be important in receptor regulation and desensitization (Cvejic et al., 1996; Trapaidze et al., 1996; Capeyrou et al., 1997; Pak et al., 1997). We have previously shown that truncation of the C-terminus of the δ-opioid receptor expressed in Chinese hamster ovary (CHO) cells by 37 amino acids alters the coupling of the receptor to phospholipase C (Hirst et al., 1998). In this study the time to peak inositol (1,4,5) triphosphate [Ins(1,4,5)$P_3$] formation increased with C-terminal truncation and the response rapidly desensitized. Therefore, the aim of this study was to examine the effects of C-terminal truncation of the cloned δ-opioid receptor on the subsequent intracellular $Ca^{2+}$ signal.

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## 2. Materials and methods

A deletion mutant of the full length mouse δ-opioid receptor with 37 C-terminal amino acids truncated was generated as described previously (Cvejic et al., 1996; Trapaidze et al., 1996) and transfected into CHO cells using Lipofectin.

### 2.1. Cell culture and harvesting

CHO cells expressing the entire mouse δ-opioid receptor (CHOδWT) or the δ-opioid receptor with the final 37 C-terminal amino acids deleted (CHOδ37) were cultured in Hams F12 medium supplemented with 10% fetal calf serum, penicillin 100 U/ml, streptomycin 100 μg/ml and fungizone 2.5 μg/ml at 37°C in 5% carbon dioxide humidified air. Stock cultures, which also contained 250 μg/ml of G418 were subcultured twice weekly and used when confluent (3–5 days). Experimental cultures were G418-free for 3 days. Confluent cells were harvested with 10 mM HEPES buffered saline/0.05% EDTA, pH 7.4, washed twice with and resuspended in either Krebs HEPES buffer of the following composition (mM): $Na^+$ (143.3), $K^+$ (4.7), $Ca^{2+}$ (2.5), $Mg^{2+}$ (1.2), $Cl^-$ (125.6), $H_2PO_4^{2-}$ (1.2), $SO_4^{2-}$ (1.2), Glucose (11.7) and HEPES (10), pH 7.4 with 10 M NaOH (whole cells) or 50 mM Tris buffer, pH 7.4 with 10 M HCl (membranes).

### 2.2. Membrane preparation

Membranes prepared by harvesting CHO cells as described above, these were homogenised with an Ultra Turrax for 30 s at 13 500 rpm. The membranes were centrifuged at $20374 \times g$, 4°C for 10 min, washed with Tris buffer, then homogenised and centrifuged twice more.

### 2.3. Measurement of [$^3$H]diprenorphine binding

This was performed as described previously (Lambert et al., 1993) to determine receptor density ($B_{max}$) and radioligand dissociation constant ($K_d$). Briefly, saturation studies were performed in 1 ml assay volumes of 50 mM Tris, pH 7.4 at 20°C for 60 min with [$^3$H]diprenorphine and CHOδWT or CHOδ37 membranes. Non-specific binding was defined at each concentration of ligand using 10 μM naloxone. Bound and free radioactivity were separated by rapid vacuum filtration using a Brandell cell harvester onto Whatman GF/B filters and washed with $3 \times 4$ ml aliquots of cold Tris buffer. Radioactivity was extracted for at least 6 h in Optiphase safe scintillant and bound radioactivity measured by liquid scintillation spectroscopy.

Table 1
Scatchard analysis of [$^3$H]-diprenorphine binding on membranes prepared from CHOδWT and CHOδ37 cells. Data are mean ± S.E.M. ($n \geq 5$)

| | $B_{max}$ | $K_d$ |
|---|---|---|
| CHOδWT | 234 ± 10.0 | 0.41 ± 0.04 |
| CHOδ37 | 85 ± 13.4 | 0.23 ± 0.03[a] |

[a] $P < 0.05$, decreased compared to CHOδWT.

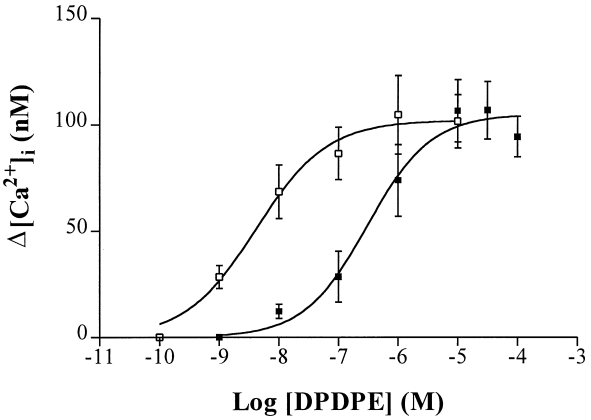



Fig. 1. Concentration-dependent increases in $[Ca^{2+}]_i$ with DPDPE in CHOδWT (□) and CHOδ37 (■) cells. Experiments were performed in fura-2 loaded whole cell suspensions as described in Section 2 and data are change in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$). Data are mean ± S.E.M. ($n \geq 5$), whole curves were $P < 0.05$ by ANOVA.

### 2.4. Measurement of intracellular calcium ($[Ca^{2+}]_i$)

Confluent cells were harvested and washed in Krebs HEPES buffer ($2 \times 20$ ml) as above. Cell suspensions were loaded with 3 μM Fura 2 acetoxymethylester for 30 min at 37°C, washed and then incubated at 20°C for 20 min. $[Ca^{2+}]_i$ were measured at 37°C, using a Perkin–Elmer LS50B fluorimeter at 340/380 nm excitation and 510 nm emission. $[Ca^{2+}]_i$ was calculated from the 340/380 ratio according to Grynkiewicz et al. (1985), with $R_{max}$ and $R_{min}$ being determined using 0.1% Triton-X100, and 4.5 mM EGTA (pH > 8), respectively.

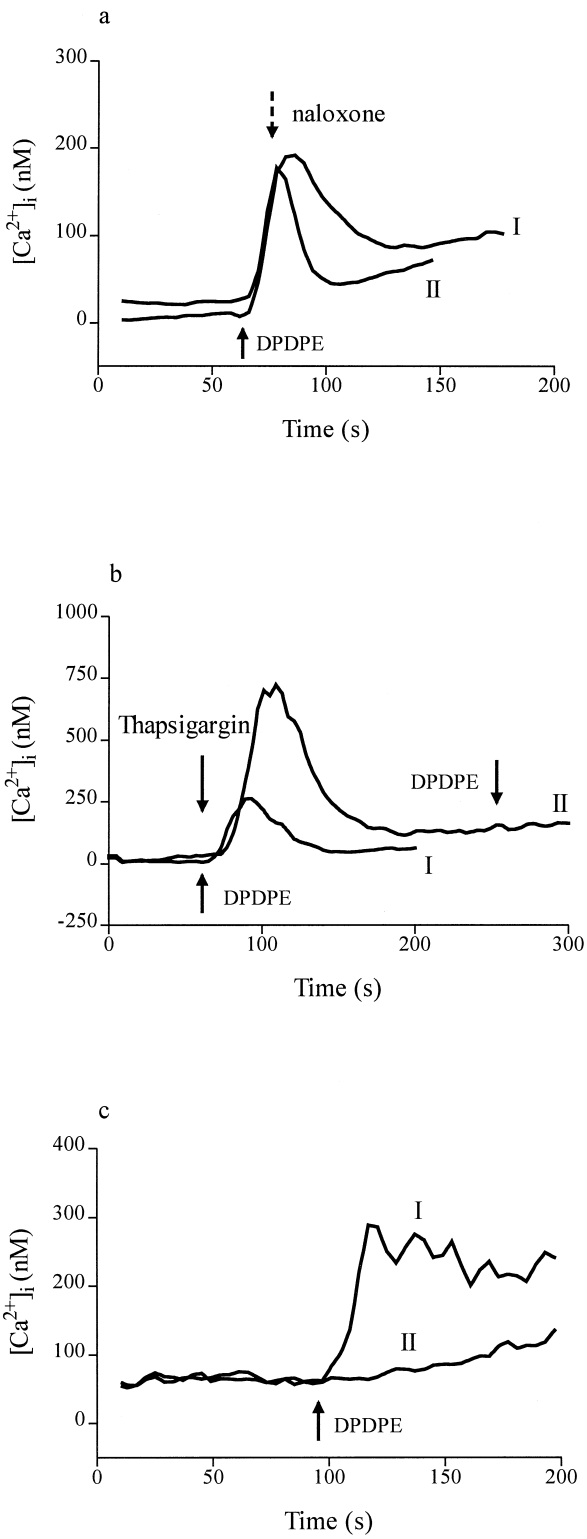
### 2.5. Data analysis

All data are represented as mean ± S.E.M. or a single representative trace of $n \geq 3$. Maximum binding and dissociation constant values from [$^3$H]diprenorphine binding studies were obtained from Scatchard analysis of the specific binding data. $pEC_{50}$ values (half log maximal stimulation) were obtained by computer assisted curve fitting of the individual curves using GRAPHPAD-PRISM. Statistical analysis was performed using Student's *t*-test or analysis of variance (ANOVA) where appropriate.

## 3. Results

The binding of [$^3$H]diprenorphine to membranes prepared from CHOδWT and CHOδ37 was concentration-de-

pendent and saturable with $K_d$ and $B_{max}$ values shown in Table 1. Deletion of the final 37 amino acids from the C-terminus produced a small but significant increase in $K_d$, consistent with our previous report (Hirst et al., 1998).




There was considerable differences between batches in the absolute change in $[Ca^{2+}]_i$ in response to DPDPE (e.g., 43 nM to 550 nM, 1 μM DPDPE CHOδWT), but within one batch (i.e., one subculture) the response was less variable (e.g., 52 nM to 78 nM 1 μM DPDPE CHOδWT). There was no loss of response with increasing passage. In some batches of cells (~60%) a distinct plateau was not evident.

DPDPE produced a concentration-dependent increase in $[Ca^{2+}]_i$ in CHOδWT and CHOδ37 cells (Fig. 1) with $pEC_{50}$ values of $8.43 \pm 0.13$ (3.68 nM) and $6.08 \pm 0.25$ (0.831 μM), respectively ($p \leq 0.05$). The κ-opioid receptor selective agonist, spiradoline (1 μM CHOδWT, 30 μM CHOδ37) and the μ-opioid receptor selective agonist [D-Ala$^2$, *N*-Me-Phe$^4$,Gly$^5$-ol]-enkephalin (DAMGO), (1 μM CHOδWT, 30 μM CHOδ37) had no effect on $[Ca^{2+}]_i$ (data not shown). The elevation of $[Ca^{2+}]_i$ produced by DPDPE in CHOδWT and CHOδ37 cells was time-dependent with maximal increases in $[Ca^{2+}]_i$ occurring $27.9 \pm 1.3$ s after DPDPE addition in CHOδWT and $23.9 \pm 1.2$ s in CHOδ37. In both cell types the response was naloxone reversible (Fig. 2a and Fig. 3b) and thapsigargin sensitive (Fig. 2b) The mean increase in $[Ca^{2+}]_i$ elicited by thapsigargin was $324 \pm 122$ nM and $233 \pm 24$ nM in CHOδWT and CHOδ37, respectively. This increase was greater than that produced by DPDPE, implying that DPDPE released only a fraction of the Ins(1,4,5)$P_3$ sensitive pool. Pre-treatment with pertussis toxin (100 ng ml$^{-1}$, 24 h) completely abolished the DPDPE-induced increase in $[Ca^{2+}]_i$ in both CHOδWT and CHOδ37 cells (Fig. 2c, Table 2).

In the absence of extracellular $Ca^{2+}$ (+0.1 mM EGTA) the response evoked by DPDPE was monophasic, whilst in the presence of extracellular calcium there appeared to be a component of the response that could be attributed to extracellular $Ca^{2+}$ (Fig. 3a). We probed this further in CHOδWT cells. In extracellular $Ca^{2+}$-free conditions (+0.1 mM EGTA), replacement of extracellular $Ca^{2+}$ (2.5 mM) resulted in an increase in $[Ca^{2+}]_i$ above the normal peak phase that was independent of opioid receptor activation. The majority of this increase was $Mn^{2+}$ quenchable, indicating leakage of fura-2 from the cell (Fig. 3c).

Fig. 2. All specimen traces are representative of CHOδWT, similar data was obtained for CHOδ37. (a) Specimen trace for CHOδWT depicting naloxone reversibility of the DPDPE-induced increase in $[Ca^{2+}]_i$. 1 μM DPDPE was added at 60 s. I = control, II = naloxone (10 μM) treated, added at peak response. Data is a representative trace from at least four other such experiments. (b) Specimen trace depicting thapsigargin sensitivity of the DPDPE-induced increase in $[Ca^{2+}]_i$. I = control, 1 μM DPDPE added at 60 s. II = thapsigargin (100 nM) treated, added at 60 s, 1 μM DPDPE added at arrow. Data is a representative trace from at least four other such experiments. (c) Specimen trace for CHOδWT depicting pertussis sensitivity of the DPDPE-induced increase in $[Ca^{2+}]_i$. I = control, 1 μM DPDPE added at 100 s. II = PTX (100 ng ml$^{-1}$, 24 h) pre-treated, 1 μM DPDPE added at 100 s. Data is a representative trace from at least two other such experiments.

## 4. Discussion

We have demonstrated that the affinity of [$^3$H]diprenorphine is increased by removal of the C-terminus of the δ-opioid receptor, consistent with our previous studies (Hirst et al., 1998). Although the $B_{max}$ values differ slightly in this study from those in our previous report, there is consistency in that CHOδWT had a slightly higher $B_{max}$ than CHOδ37. However, two other studies examining the effect of C terminal deletion of the μ-opioid receptor (Capeyrou et al., 1997) and the δ-opioid receptor (Zhu et al., 1997) found that truncation of the receptor did not affect [$^3$H]diprenorphine binding.

Various other studies have shown that activation of (full length) recombinant opioid receptors causes a mobilization of $[Ca^{2+}]_i$ (Zimprich et al., 1995; Smart et al., 1997; Hirst et al., 1998; Spencer et al., 1998) and the results of this study are in agreement with these. We believe that the increase in $Ca_i^{2+}$ observed was due solely to release from intracellular stores, and that activation of the δ-opioid receptor did not induce $Ca^{2+}$ entry from extracellular sources. In some of our results it appears that a 'plateau' is present (e.g., Fig. 2c and Fig. 3a). We believe that this does not represent $Ca^{2+}$ entry into the cell, but is instead leakage of fura-2 from the cell. However, the possibility of $Ca^{2+}$ entry cannot be completely ruled out as it is possible that it is masked by fura-2 leakage from the cell. However, other studies have demonstrated that when transfected into host cells, recombinant opioid receptors cause an increase in $[Ca^{2+}]_i$ that is independent of extracellular $Ca^{2+}$, i.e., release is from stores (Zimprich et al., 1995; Spencer et al., 1998).

Our data show that deletion of the C-terminus of the δ-opioid receptor produces a marked rightward shift in the concentration–response for $Ca_i^{2+}$ release ($EC_{50}$ CHOδWT

Table 2
Effect of pertussis toxin (100 ng ml$^{-1}$, 24 h) on basal and peak $[Ca^{2+}]_i$ in CHOδWT and CHOδ37 cells. Data are mean ± S.E.M ($n \geq 3$)

| | CHOδWT | | CHOδ37 | |
|---|---|---|---|---|
| | PTX | Control | PTX | Control |
| Basal $[Ca^{2+}]_i$ (nM) | 54 ± 5 | 51 ± 8 | 57 ± 10 | 66 ± 12 |
| Peak $[Ca^{2+}]_i$ (nM) | 67 ± 11[a] | 425 ± 57 | 85 ± 16[a] | 465 ± 76 |

[a] $P < 0.05$, decreased compared to control.

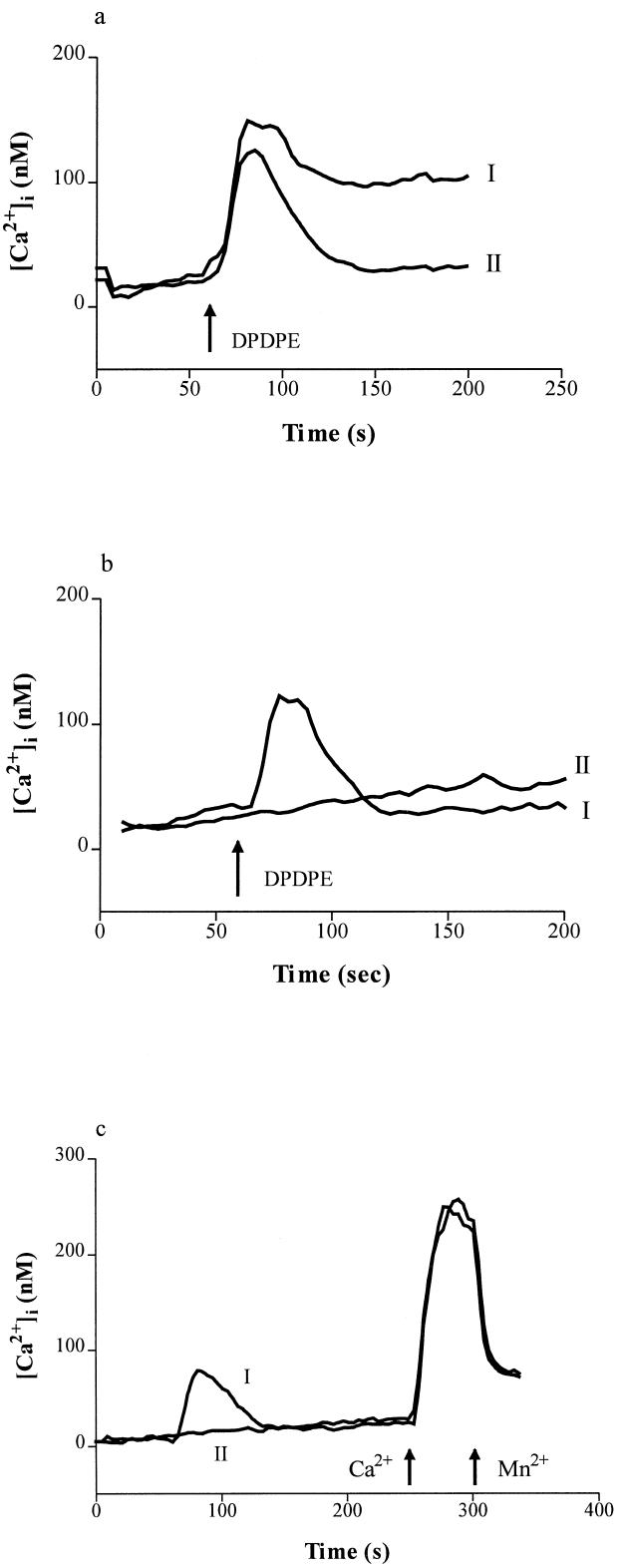



Fig. 3. (a) Specimen trace showing the effect of extracellular $Ca^{2+}$ on the DPDPE response for CHOδWT, 1 μM DPDPE added at 60 s, similar data was obtained for CHOδ37. I = extracellular $Ca^{2+}$ present, II = extracellular calcium absent. Data is a representative trace from at least four other such experiments. (b) Specimen trace from $n \geq 5$ others illustrating naloxone sensitivity of the DPDPE-induced increase in $[Ca^{2+}]_i$. I = control, II = pre-incubated with 10 μM naloxone. Experiments were conducted in the absence of extracellular $Ca^{2+}$. (c) Specimen trace from $n \geq 5$ others showing that $Ca^{2+}$ readdition produces a $Mn^{2+}$ quenchable increase in $[Ca^{2+}]_i$. 2.5 mM $Ca^{2+}$ was added at 250 s and 1 mM $Mn^{2+}$ was added at 325 s. Trace shows $Ca^{2+}$ increase with (I) and without (II) DPDPE stimulation.

= 3.68 nM, CHOδ37 = 0.831 μM, 225-fold difference). In a previous study, we examined the effect of the removal of the C-terminus of the δ-opioid receptor on phospholipase C coupling (Hirst et al., 1998). Whilst there was little effect on $EC_{50}$ values for phospholipase C coupling (CHOδWT = 55 nM, CHOδ37 = 14 nM), there were differences in the time course for $Ins(1,4,5)P_3$ accumulation between CHOδWT and CHOδ37. In CHOδWT, the response peaked at 15 s and remained elevated until sampling ended, whilst in CHOδ37 the response was monophasic, reaching a peak at 120 s. In CHOδWT cells there is a close temporal and concentration–response relationship between $Ins(1,4,5)P_3$ formation and increases in $[Ca^{2+}]_i$, suggesting that the induced increase in $Ins(1,4,5)P_3$ formation leads to a mobilization of intracellular $Ca^{2+}$. It is worth noting that in this clone the peak $Ca^{2+}$ declines (possibly masked by fura-2 leakage) despite elevated levels of $Ins(1,4,5)P_3$, a phenomenon also described in CHO cells transfected with M3 muscarinic receptors (Tobin et al., 1993). However, in the CHOδ37 clone the peak $Ca^{2+}$ response occurs at the same time as in the CHOδWT despite the $Ins(1,4,5)P_3$ response only reaching a peak at 120 s. There is a small increase in $Ins(1,4,5)P_3$ formation at 15 s in CHOδ37, and it may be possible that this is sufficient to cause mobilization of intracellular $Ca^{2+}$, even though the $EC_{50}$ values for $Ins(1,4,5)P_3$ formation and $Ca^{2+}$ release do not seem to suggest this. However, it should be noted that the $EC_{50}$ value for $Ins(1,4,5)P_3$ formation was taken at the maximal response time (120 s) whereas $EC_{50}$ values for $Ca^{2+}$ release were obtained at ~30 s following agonist stimulation. In cells expressing both the full length receptor and the truncated receptor the increase in $[Ca^{2+}]_i$ was thapsigargin sensitive, indicating release from intracellular stores, and pertussis sensitive, suggesting involvement of G proteins of the $G_{i/o}$ family. Therefore at present it remains unclear as to why truncation of the C-terminus of the δ-opioid receptor produces such a marked difference in the $Ca^{2+}$ concentration–response curves.

It could be inferred that the data reported here resulted from differing levels of receptor expression. Indeed, it is possible that a receptor reserve could exist for $G_i\alpha$ mediated responses but not for $G_i\beta\gamma$ mediated responses. Whilst we accept that we cannot completely exclude expression differences as the underlying explanation for our data, we believe that this explanation is extremely unlikely for the following reasons; firstly we have carefully chosen from a range of clones (Cvejic et al., 1996) with similar levels of receptor expression. Indeed, in the cells used in this study there is only a 2.7-fold differences in receptor number. Moreover, in our previous study (Hirst et al., 1998) we failed to see any modulation of either $pIC_{50}$ for cAMP ($G_i\alpha$) or $pEC_{50}$ for $Ins(1,4,5)P_3$ (presumably $G_i\beta\gamma$) in either cell line. Secondly, the maximal increases in $[Ca^{2+}]_i$ are similar between the two clones. In a study examining the effects of expression-dependent coupling of the recombinant κ-opioid receptor to cAMP formation, it was found that a 7.5-fold variation in expression levels halved the agonist inhibition of cAMP formation but did not alter $EC_{50}$ values (Hirst et al., 1997). Spencer et al. (1998) observed that when expression levels of the κ-opioid receptor were ~10-fold lower than μ and δ expression, maximal increases in $[Ca^{2+}]_i$ were reduced. It is known that phosphorylation of the $Ins(1,4,5)P_3$ receptor by protein kinase C enhances $Ca^{2+}$ release (Furuichi and Mikoshiba, 1995) If in the CHOδ37 cell line $Ca^{2+}$ release occurred when there was less $Ins(1,4,5)P_3$ mass than there was in CHOδWT, then it may be possible that there would also be less di-acylglycerol cleaved from phosphatidylinositol 4,5-bisphosphate, resulting in a lower level of protein kinase C activation. This would result in less phosphorylation of the $Ins(1,4,5)P_3$ receptor in the CHOδ37 cell line and, therefore, possibly shifting the CHOδ37 concentration–response curve to the right.

In a study utilizing two spice variants of the rat μ-opioid receptor, $\mu_1$-opioid receptor (MOR1) and $\mu_1$-opioid receptor B (MOR1B), where $\mu_1$-opioid receptor B is seven residues shorter than $\mu_1$-opioid receptor, Zimprich et al. (1995) demonstrated that when expressed in CHO cells both receptors mobilized $Ca^{2+}$ with similar $EC_{50}$ values ($EC_{50}$ $\mu_1$-opioid receptor = 1.5 μM, $EC_{50}$ $\mu_1$-opioid receptor B = 1.7 μM). However, Koch et al. (1997) found that $\mu_1$-opioid receptor B desensitized more slowly and resensitized faster compared to $\mu_1$-opioid receptor when measured at the level of cAMP. Other studies examining the role of the C-terminal tail of the δ-opioid receptor have shown that deletion of 37 C-terminal amino acids had no effect on coupling to adenylyl cyclase (Cvejic et al., 1996; Hirst et al., 1998), neither did deletion of all Ser and Thr residues from the C-terminus and the third intracellular loop (Capeyrou et al., 1997). Several groups have reported that specific serine and threonine residues on the C-terminus of the δ-opioid receptor expressed in CHO cells are important for down regulation/desensitization (Cvejic et al., 1996; Trapaidze et al., 1996; Murray et al., 1998). However, Afify et al. (1998) concluded that the C-terminus of the μ-opioid receptor but not the δ-opioid receptor is involved in down regulation in neuro-$_{2A}$ cells, whilst in human embryonic kidney cells, phosphorylation sites on the C-terminus do not appear to be important in down regulation (Murray et al., 1998). Whether there are distinct cell type differences remains to be resolved.

In summary, we report that when expressed in CHO cells the recombinant δ-opioid receptor couples to $Ca^{2+}$, and that a deletion of the C-terminus of this receptor produces marked differences in its coupling. The underlying molecular mechanisms behind this difference clearly merits further investigation and an examination of $Ins(1,4,5)P_3$ receptor phosphorylation states would be warranted. In addition, further studies using a number of clones with differing levels of expression are also indicated.

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