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Endomorphin-1 induced desensitization and down-regulation of the recombinant μ-opioid receptor

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1 Endomorphin-1 (E1) is a peptide with high affinity and selectivity for the μ-opioid receptor. The aim of this study was to determine if endomorphin-1 caused desensitization and down-regulation of the μ-opioid receptor expressed in Chinese hamster ovary cells.

2 Following 10 μM E1 pre-treatment, desensitization was assessed by measuring cyclic AMP inhibition, down-regulation was assessed by [^{3}H]-diprenorphine ([^{3}H]-DPN) binding and immunoblotting.

3 Pre-treatment of CHOμ cells with 10 μM E1 for 11 and 18 h caused significant reduction in cyclic AMP inhibition. (11 h = 39.0 $\pm$ 16.7%, 18 h 47.0 $\pm$ 11.1% reduction).

4 At 18 h E1 pre-treatment there was an enhancement (4.5 fold) of cyclic AMP production under forskolin stimulated conditions accompanied by a small rightward shift in the concentration-response curve (pEC_{50} control = 7.8 $\pm$ 0.3, pEC_{50} E1 = 7.3 $\pm$ 0.2) when cells were re-challenged with E1.

5 In membranes prepared from untreated and 0.5 h E1 pre-treated cells, addition of GTPγS produced a significant rightward shift in the concentration response curves for E1 displacement of [^{3}H]-DPN (0 h K_i control = 7.86 $\pm$ 0.11, GTPγS = 7.37 $\pm$ 0.15; 0.5 h K_i control = 7.92 $\pm$ 0.12, GTPγS = 7.36 $\pm$ 0.08) This was not observed in membranes prepared from cells that had been treated with E1 for 18 h (18 h K_i control = 7.69 $\pm$ 0.11, GTPγS = 7.75 $\pm$ 0.08).

6 In whole cells E1 treatment caused a rapid loss of cell surface receptors such that at 0.5 h there was a 30.5 $\pm$ 1.5 reduction (this was unchanged for 18 h). In crude membranes a loss of receptors was also observed using radioligand binding or immuno-blotting protocols.

7 These data show that E1 causes desensitization and down-regulation of the rat μ-opioid receptor expressed in CHO cells. However, these two responses appear temporally distinct.

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Keywords: Endomorphin; opioid receptor; desensitization; down-regulation

Abbreviations: cyclic AMP, cyclic adenosine 3′5′-monophosphate; CHO, Chinese hamster ovary (cell); DPN, diprenorphine; GRK, G protein coupled receptor kinase; GTPγS, guanosine 5′-O-(3-thiotriphosphate); HEK, human embryonic kidney (cell); MAP, mitogen activated protein (kinase); PKC, protein kinase C

Introduction

Opioid receptors are typical of the G protein coupled receptor superfamily whose responses to both endogenous ligands and opioid analgesics are brought about by action of pertussis toxin sensitive $G_{i/o}$ G proteins. As is common with many other types of G protein coupled receptors, opioid receptors undergo agonist-induced desensitization and down-regulation following prolonged treatment (Zhang *et al*., 1998) Desensitization and/or down-regulation may be viewed as a mechanism whereby there is an attenuation of receptor signalling in response to the continued presence of agonist which may be accompanied by activation of another pathway, for example PKC or MAP kinase (Lefkowitz, 1998). Of particular interest in the opioid field is the μ-receptor, since it is this subtype that is the main analgesic target and is believed to be responsible for opioid tolerance. Perhaps the most extensively studied receptor with regard to desensitization/down-regulation is the β-adrenoreceptor, where there is a correlation between receptor-G protein coupling, phosphorylation and receptor internalization (Koenig & Edwardson, 1997). However it has become apparent that this may not be the case in certain opioid receptor signalling pathways. For example in transfected cell lines morphine activates the μ-opioid receptor, may cause receptor phosphorylation but is unable to cause receptor internalization (Chakrabarti *et al*., 1998, Kieth *et al*., 1998). In addition in HEK-293 cells expressing the δ-opioid receptor, phosphorylation is not required for internalization (Murray *et al*., 1998).

Endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) is a novel endogenous opioid peptide that has high affinity and selectivity for the μ-opioid receptor (Zadina *et al*., 1997). Endomorphin-1 has been shown to produce opioid-like effects, including analgesia (Stone *et al*., 1997; Zadina *et al*., 1997; Goldberg *et al*., 1998), and effects on second messenger systems (Harrison *et al*., 1999; Alt *et al*., 1998; Kakizawa *et al*., 1998; Kato *et al*., 1998). In addition, endomorphins produce a variety of cardiovascular effects consistent with opioids (Champion *et al*., 1997a,b,c; Czapla *et al*., 1998). To date, studies examining the effect of endomorphin-1 on μ-opioid receptor desensitization and down-regulation have been limited, pretreatment for 30 min with endomorphin results in a loss of cell surface receptors in transfected cell lines (Burford *et al*., 1998; McConalogue *et al*., 1999) and in guinea-pig ileum (McConalogue *et al*., 1999). Therefore in the present study we investigated the cellular responses to prolonged endomor-

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phin-1 treatment in Chinese hamster ovary cells expressing recombinant μ-opioid receptors. In this cell line endomorphin-1 causes a displacement of [^{3}H]-DPN binding, an inhibition of cyclic AMP formation and an increase in $[Ca^{2+}]_i$ (Harrison *et al*., 1999). In particular we have looked at loss of a functional response (cyclic AMP inhibition; desensitization) and loss of cell surface receptors (down-regulation).

Methods

Cell culture

CHO cells were transfected with the rat μ-opioid receptor using $CaPO_4$ precipitation (Bunzow *et al*., 1995) and were cultured in Hams F12 medium supplemented with foetal calf serum 10%, penicillin 100 u ml^{-1}, streptomycin 100 μg ml^{-1} and fungizone 2.5 μg ml^{-1} at 37°C in 5% carbon dioxide humidified air. Stock cultures, which also contained G418 250 μg ml^{-1} were sub-cultured twice weekly and used when confluent (3–5 days). Experimental cultures were G418 free for 3 days. Stock cultures were subcultured weekly and used when confluent (3–6 days).

Membrane preparation

Membranes were prepared by harvesting CHO cells as described above, and homogenizing with an Ultra Turrax for 30 s at 13,500 r.p.m. The membranes were centrifuged at 20,000 × *g*, 4°C for 10 min, washed with Tris buffer and homogenized and centrifuged twice more, as above.

Desensitization protocol

CHOμ cells (grown in 12 well plates, ∼438 μg protein well^{-1}) were incubated with 10 μM endomorphin-1 and 10 μM of each of the peptidase inhibitors amastatin, bestatin, captopril and phosphoramidon for various times in serum free media. Adherent cells were washed five times at 4°C in 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, HEPES 10 and BSA 0.5%, pH 7.4 with 10 M NaOH prior to all experiments.

For binding displacement studies, confluent cells were washed five times with 50 mM Tris buffer containing BSA 0.5%, pH7.4 with 10 M HCl then harvested by scraping. Membranes were prepared by homogenizing with an Ultra Turrax for 30 s at 13,500 r.p.m. at 4°C. The membranes were centrifuged at 20,400 × *g*, 4°C for 10 min, washed with Tris buffer and homogenized and centrifuged twice more, as above.

Measurement of cyclic AMP in adherent cells

Cyclic AMP experiments for desensitization studies were performed on adherent CHO cells in 12 well plates for 5 min at 37°C in 0.6 ml volumes of Krebs-HEPES buffer containing forskolin (10 μM), IBMX (1 mM) 0.5% BSA, 10 μM peptidase inhibitors and varying concentrations of E1 (10^{-5}–10^{-10} M). Reactions were terminated by the addition of 40 μl HCl (10 M). The pH was equilibrated with the addition of 40 μl NaOH (10 M) and 300 μl Tris-Cl buffer (pH 7.4). Following detachment of cells by scraping and centrifugation (12,000 × *g*) for 5 min, the concentration of cyclic AMP in the supernatant was measured using a specific protein-binding assay (Brown *et al*., 1971), with binding protein prepared from bovine adrenal cortex.

Binding for desensitization studies

(i)–Whole cells Radioligand binding studies were performed at 4°C for 3 h on adherent cells in 12-well plates in 1 ml volumes of Krebs-HEPES buffer containing a saturating concentration of [^{3}H]-DPN (∼2.5 nM). Non-specific binding was defined using 10 μM naloxone. Reactions were terminated by removal of the buffer (and washed twice) and incubation with 0.4 M perchloric acid. Following detachment of cells by scraping, radioactivity was extracted for at least 6 h in Optiphase safe scintillant and bound radioactivity measured by liquid scintillation spectroscopy.

(ii)–Membranes CHOμ membranes were incubated in 1 ml volumes of Tris buffer at 4°C for 3 h in the presence of a saturating concentration [^{3}H]-DPN (∼2.5 nM). Non-specific binding was defined using 10 μM naloxone. Bound and free radioactivity were separated by rapid vacuum filtration using a Brandel cell harvester onto Whatman GF/B filters and washed with 3 × 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.

GTPγS displacement studies

CHOμ membranes were incubated in 1 ml volumes of Tris buffer at 4°C for 3 h in the presence of a low concentration [^{3}H]-DPN (∼0.3 nM), peptidase inhibitors and E1 (10^{-11}–10^{-5} M) in the presence or absence of GPTγS (50 μM). Non-specific binding was defined using 10 μM naloxone. Bound and free radioactivity were separated by rapid vacuum filtration using a Brandel cell harvester onto Whatman GF/B filters and washed with 3 × 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.

Immunoblotting

Electrophoresis and immunoblotting were carried out essentially as described in Maniatis *et al*. (1982) with modifications. Briefly, ∼50 μg lane^{-1} of CHOμ membranes from control and 0.5 h E1 pretreated cells were loaded (loading buffer; 50 mM Tris-Cl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) onto an 8% SDS–PAGE gel which was run (in 25 mM tris base, 250 mM glycine, pH 8.3, 0.1% SDS) at 175 V for 45 min. Proteins were then transferred onto nitrocellulose (in 39 mM glycine, 48 mM Tris-base, 0.037% SDS, 20% methanol, pH 8.3) using a semi-dry blotter 65 mA for 2.5 h. at 22°C. The filters were then blocked overnight in 5% low fat milk (in 20 mM tris-HCl, 0.5 M NaCl, 0.05% tween 20, 0.01% antifoam (A) and then incubated with primary antibody (diluted 1 in 2000 in blocking solution) directed against the rat μ-opioid receptor (Calbiochem, U.K.) for 2 h at 22°C. Filters were washed three times for 10 min (in 20 mM tris-HCl, 0.5 M NaCl) before incubation with goat anti rabbit IgG alkaline phosphatase (Sigma, U.K.) conjugate (diluted 1 : 10,000 in blocking solution) for 2 h at 22°C. Protein bands were visualized using BCIP/NBT Sigma-fast tablets (as directed by manufacture's instructions) for 5–10 min at 22°C until appearance of dark pink bands became apparent. Protein

markers were visualized by staining with Brilliant Blue (0.25 g in 45% H_2O, 45% methanol, 10% glacial acetic acid) at 4°C overnight followed by destaining (in 45% H_2O, 45% methanol, 10% glacial acetic acid for ~3 h at 22°C).

Data analysis

All data are mean ± s.e.mean of $n \geqslant 3$. pEC_{50} values (log half maximal inhibition of cyclic AMP formation) and E_{max} (per cent maximal inhibition of cyclic AMP formation) were obtained by computer assisted direct curve fitting of the individual curves using GRAPHPAD-PRISM. Displacement studies were analysed by computer assisted direct curve fitting of the individual curves and corrected for the amount of radiolabel used using the Cheng-Prussoff equation to yield pK_i values (where $K_d = 0.20$ nM, Harrison *et al.*, unpublished data). Statistical analysis was performed using ANOVA or Student's *t*-test where appropriate. Where data is given as pmol well^{-1}, the volume of a well = 980 μl. Optical density of immumo-blotted bands was performed using Image Master system (Amersham Parmacia Biotech).

Results

Pre-treatment of CHOμ cells with 10 μM endomorphin-1 for 0.5, 1, 2, 3, 4, 5 or 8 h failed to significantly ($P<0.05$) reduce forskolin stimulated cyclic AMP formation compared to control when there was a subsequent challenge with 10 μM E1. However, there was a significant reduction ($P<0.05$) after 11 h and 18 h E1 pre-treatment when compared to control values (Figure 1), suggesting that it takes between 8–11 h for the receptor to desensitize. The reduction observed at 18 h was reversed by the opioid antagonist naloxone (data not shown).

Table 1 shows the absolute level of cyclic AMP production (expressed in pmol well^{-1}) under basal and forskolin stimulated conditions in control, 0.5 h E1 pre-treatment (i.e. when there was no loss of cyclic AMP inhibition) and 18 h E1 pre-treatment (i.e. when there was loss of cyclic AMP inhibition). This data may be suggestive of receptor-G protein uncoupling since increased levels of cyclic AMP could be explained by the loss of an inhibitory response and/or constitutive activity of the μ-opioid receptor since the increase in cyclic AMP formation occurred in the absence of re-challenge with E1. To further probe this effect, we examined concentration response curves for the inhibition of cyclic AMP formation between untreated and cells pre-treated with 10 μM E1 for 18 h (Figure 2). Again, there was a reduction in the maximal levels of cyclic AMP inhibition when cells were re-challenged with high concentrations of E1. Moreover, there was a small rightward shift in the concentration-response curves between the two treatments (pEC_{50} control = 7.8 ± 0.03, pEC_{50} E1 = 7.3 ± 0.2) although this failed to reach statistical significance. In addition, at low concentrations of E1 there was an enhancement of cyclic AMP formation to levels above those of forskolin controls (depicted as a negative inhibition), and in agreement with mass measurements above (Table 1).

In order to determine if G protein–receptor uncoupling occurred, we performed E1 displacement studies in the presence and absence of GTPγS, a non-hydrolysable form of GTP (Figure 3 and Table 2). In membranes prepared from un-treated and 0.5 h cells, addition of GTPγS produced a significant rightward shift in the concentration response curves. This was not present in membranes prepared from cells that had been treated with E1 for 18 h.

The loss of a functional response may (Hashimoto *et al.*, 2000) or may not (Bot *et al.*, 1998) be correlated with loss of cell surface receptors. To examine if the loss of cyclic AMP inhibition correlated with loss of cell surface receptors we

Table 1 Absolute levels of cyclic AMP produced under basal and forskolin stimulated conditions in control (untreated), 0.5 h and 18 h E1 pre-treated CHOμ cells

	[cyclic AMP] pmol well^{-1}	
E1 pre-treatment	*Basal*	*Forskolin*
Control	UD	10.1 ± 1.3
0.5 h	UD	10.1 ± 1.9
18 h	UD	45.1 ± 8.1*

UD = undetectable. *Significantly different compared to control. $P<0.05$.

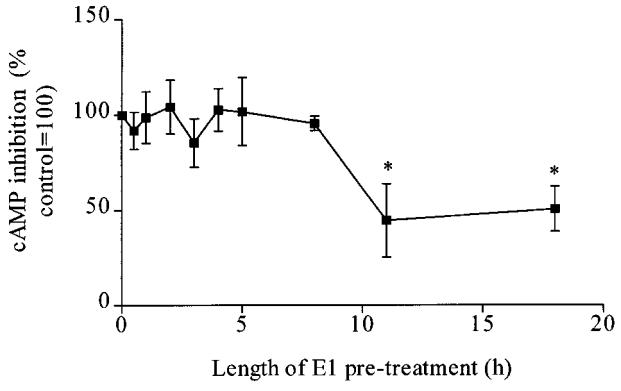

Figure 1 Pre-treatment for 11 or 18 h with 10 μM E1 causes a reduction in the level of cyclic AMP inhibition when cells were re-challenged with 10 μM E1. No reduction in cyclic AMP inhibition was seen for pre-treatments of up to 8 h. All data are expressed as a percentage of their own control. Studies were performed on adherent CHOμ cells for 5 min at 37°C. cyclic AMP was measured using a specific protein binding assay. Data are mean ± s.e.mean for $n \geqslant 3$ (where $n = 3$ experiments were performed in triplicate). *Indicates significance $P<0.05$ by paired Student's *t*-test.

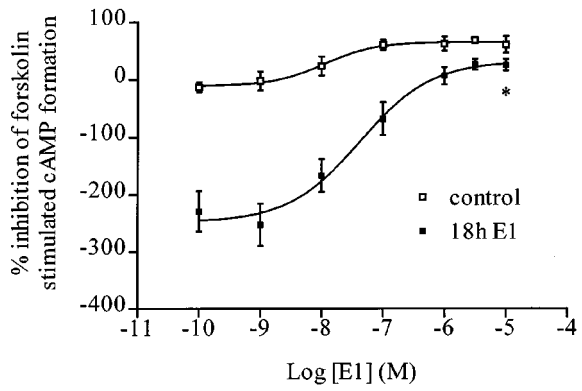

Figure 2 Endomorphin-1 produced a concentration-dependent inhibition of forskolin stimulated cyclic AMP formation in control and 18 h E1 treated cells. A negative inhibition is indicative of cyclic AMP levels above that of forskolin controls. Studies were performed on adherent CHOμ cells for 5 min at 37°C. cyclic AMP was measured using a specific protein binding assay. Data are mean ± s.e.mean for $n \geqslant 4$, whole curves are significantly different ($P<0.05$) by ANOVA. *Indicates significant ($P<0.05$, Student's *t*-test) reduction compared to control, consistent with Figure 1.

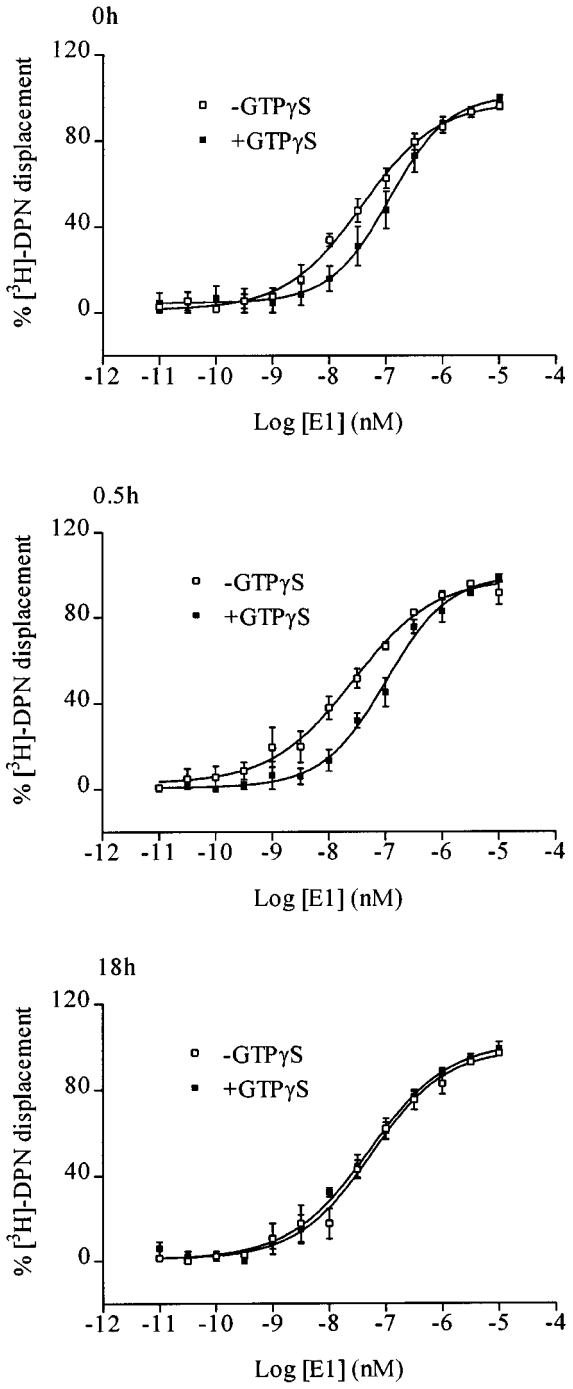

Figure 3 Endomorphin-1 produced a concentration-dependent displacement of [^{3}H]-DPN binding to CHOμ membranes in the presence or absence of 50 μM GTPγS. Studies were performed at 4°C in 1 ml volumes for 3 h with a fixed concentration of [^{3}H]-DPN and endomorphin-1 as the displacer. Non-specific binding was defined in the presence of 10 μM naloxone. Data are mean$\pm$s.e.mean for $n=5$. Whole curves and pK_i values were significantly different ($P<0.05$, ANOVA, Student's t-test respectively) in the presence and absence of GTPγS for membranes prepared from untreated (0 h) and 0.5 h E1 pre-treated cells. No significant difference was observed in membranes prepared from 18 h E1 pretreated cells.

examined the effect of varying lengths of E1 pretreatment on number of cell surface receptors (Figure 4). Cells were pre-treated with 10 μM E1 for various times, and then the B_{max} was determined using a saturating concentration of [^{3}H]-DPN (as protein was not determined in each experiment, absolute values for B_{max} in fmol mg protein^{-1} cannot be calculated).

Table 2 pK_i values for E1 displacement of [^{3}H]-DPN in the presence and absence of 50 μM GTPγS

Length of E1 pretreatment (h)	*Control*	*GTPγS*
	pK_i [nM]	
0	7.86$\pm$0.11 [13.8]	7.37$\pm$0.15 [42.7]*
0.5	7.92$\pm$0.12 [12.0]	7.36$\pm$0.08 [43.7]*
18	7.69$\pm$0.11 [20.4]	7.75$\pm$0.08 [17.8]

*Significantly different compared to control. $P<0.05$.

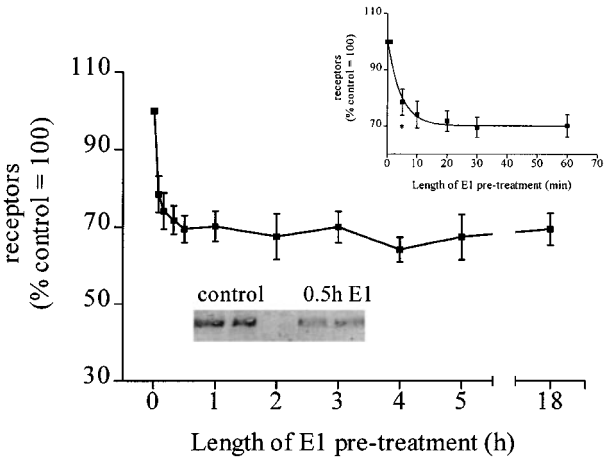

Figure 4 Pre-treatment with 10 μM E1 caused a rapid loss of cell surface receptors. Radioligand binding studies were performed at 4°C (to prevent receptor recycling and lipophilic ligands crossing the cell membrane) for 3 h on adherent CHOμ cells in 12-well plates in 1 ml volumes of Krebs-HEPES buffer containing a saturating concentration of [^{3}H]-DPN ($\sim$2.5 nM). Non-specific binding was defined using 10 μM naloxone. Top inset shows time course for up to 1 h E1 pre-treatment. *Indicates significant difference ($P<0.05$, student's t-test) compared to paired control. Bottom insert is representative immuno-blot showing four lanes – two control and two 0.5 h E1.

E1 pretreatment produced a rapid, time dependent loss of cell surface receptors, with all time points after 1 min being significantly reduced ($P<0.05$) compared to control. Using crude membranes prepared from untreated and 0.5 h E1 pretreated cells there was a 63.5$\pm$3.8% reduction in cell surface receptors (B_{max} control = 767.1$\pm$77.8, 0.5 h E1 = 271.9$\pm$24.7 fmol mg protein^{-1}). This was greater than the reduction seen in whole cells ($\sim$30%) but was accompanied by a 63.5$\pm$3.8% reduction in optical density of μ-opioid receptor-specific stained bands determined by immuno-blotting.

Discussion

The data presented here show that endomorphin-1 causes desensitization and down-regulation of the rat μ-opioid receptor. However it takes prolonged agonist pre-treatments (between 8 – 11 h) to cause the receptor to desensitize. This desensitization is characterized by a reduction in maximal cyclic AMP inhibition and an up-regulation of forskolin stimulated cyclic AMP formation, which is probably due to receptor-G protein uncoupling. E1 also produces down-regulation of the μ-opioid receptor; this loss of cell surface receptors is rapid and the time course does not mirror that for loss of a functional response. In addition there is a discrepancy between studies using whole cells and crude membrane preparations.

Other studies showing endomorphin induced receptor down-regulation have demonstrated loss of cell surface receptors. Burford *et al.* (1998) reported that 30 min pretreatment with E1 in HEK 293 cells resulted in μ-opioid receptor internalization whilst McConalogue *et al.* (1999) also showed that a 30 min treatment of KNRK (rat kidney) cells with either E1 or E2 resulted in a loss (~30–40%) of cell surface receptors as measured by fluorescence microscopy. A loss of cell surface receptors also occurred in guinea-pig ileum. Therefore in terms of receptor loss the results of the temporal relationship in this study is in general agreement with previous studies. It is worth noting that in the two previously mentioned studies, receptor expression levels were not reported. A rapid loss of cell surface opioid receptors is also seen with opioid agonists other than E1 (Pei *et al.*, 1995; Hasbi *et al.*, 1998; Keith *et al.*, 1998; Murray *et al.*, 1998). In addition, it has been shown that loss of cell surface opioid receptors may be independent of G protein coupling (Yabaluri & Medzihradsky, 1997, Kato *et al.*, 1998, Li *et al.*, 1999, Pak *et al.*, 1999). In CHOμ cells over-expressing ~3 pmol of μ receptors, Pak *et al.* (1999) showed that down-regulation of opioid receptors occurred by two distinct pathways when cells were treated with 1 μM DAMGO for 1 h. The first of these was G protein dependent, involved G protein coupled receptor kinase (GRK) and was blocked by mutation of Thr^{294} on the C terminal tail of the receptor. The second pathway was G protein independent, involved a tyrosine kinase and was not affected by mutation of Thr^{394}. In CHOμ cells (B_{max} = 8 pmol mg protein^{-1}) loss of cell surface receptors was also independent of G protein coupling but the reduction in the level of cyclic AMP inhibition was G protein dependent (Kato *et al.*, 1998). Therefore it is feasible that in the present study loss of cell surface receptors may be independent of G protein coupling. The transfection of a receptor reserve (B_{max} = 600 fmol mg protein^{-1}) may allow functional coupling to adenylyl cyclase and be able to cause a 'GTP shift' even though 30% of cell surface receptors (measured in whole cells) have been lost. It then takes longer time periods (between 8 and 11 h) to cause loss of functional coupling. Indeed in C6 glial cells 12 h agonist pretreatment was required to induce uncoupling (Yabaluri & Medzihradsky, 1997). In contrast to this, other studies have indicated that G protein coupling is required in order for opioid receptors to down regulate (Yu *et al.*, 1997, Chakrabati *et al.*, 1997), or receptor loss may be partially G protein dependent (Zaki *et al.*, 2000).

The loss of cell surface receptors in membrane preparations measured in radioligand binding and immuno-blotting protocols is interesting. We postulate that receptors may be removed from the cell surface in vesicles (Lohse, 1993) which in the crude membrane preparations used here would be sedimented during the centrifugation process. The effect of this would be to mask any loss of surface receptors (as the total pool should remain constant). The fact that we have observed receptor loss may suggest that receptors have been degraded, even after 0.5 h of agonist pretreatment. However, it should be emphasized that this cannot be conclusively concluded from this data alone and further experimentation is required.

It is apparent that there are marked differences in opioid receptor desensitization/down-regulation depending on the parameters studied, for example cell differences exist; in HEK 293 cells phosphorylation is not required for dynamin dependent endocytosis of the δ-opioid receptor, whereas in CHO cells expressing the same receptor this was not the case. In addition, some groups have found the involvement of GRKs in down-regulation (Hasbi *et al.*, 1998; Zhang *et al.*, 1998; Li *et al.*, 1999) but not PKC. However in SH-SY5Y cells, PKC appears to be involved, in particular α, ε, ζ isoforms (Kramer & Simon, 1999a,b). MAP kinase has also been implicated in μ-opioid receptor desensitization in HEK 239 cells (Schmidt *et al.*, 2000). It therefore appears that certain cell lines possess different second messenger systems and these in turn affect down regulation of receptors in that particular line. It is also interesting to note that whilst in clonal cells lines there seldom appears to be more than 60% receptor internalization, in *ex vivo* preparations, internalization is nearer 100% (McConalogue *et al.*, 1999; Marvizon *et al.*, 1999). Since transfected cell lines usually express higher levels of receptors than found endogenously, it may be that in transfected cell lines the endocytotic machinery of the cell becomes saturated, only allowing up to 60% internalization. It has also become apparent that there may be receptor subtype differences, for example at the μ-opioid receptor, morphine induced receptor internalization can be induced by GRK over-expression, but this is not the case with the δ receptor (Zhang *et al.*, 1998; 1999). In addition in $nuero_{2A}$ cells, δ-opioid receptor down regulation is independent of G protein coupling whereas the μ-opioid receptor requires the formation of a high affinity G protein complex (Chakrabarti *et al.*, 1997). Species differences have also been noted, as in CHO cells expressing the recombinant human κ receptor, U50488 causes internalization, but the same agonist does not induce internalization of the rat κ-opioid receptor when both are expressed at similar levels (Li *et al.*, 1999). Partial agonists, e.g. morphine (Lambert *et al.*, 1993) may also regulate receptor function differently (Yabaluri & Medzihradsky, 1997), perhaps due to their ability to induce activation of β-arrestin (Shultz *et al.*, 1999). However, inability to internalize receptors may not be due to their potency since levorphanol which has similar potency to fentanyl does not desensitize or internalize the μ-opioid receptor (Bot *et al.*, 1998). In addition, the δ-opioid receptor agonist SNC80 may down-regulate the δ-opioid receptor differently to DPDPE by utilizing receptor domains other than the C terminal tail (Okura *et al.*, 2000).

In the present study it could be argued that if receptor/G protein coupling occurs, then there should be a marked shift to the right on the concentration response curve for inhibition of cyclic AMP formation. We did notice a small shift to the right but this did not reach statistical significance. In a study by Kato *et al.* (1998), there was an enhancement of the forskolin response following 4 h DAMGO pretreatment, but in agreement with our study, no change in the EC_{50} values for cyclic AMP inhibition between control and DAMGO treated cells. An enhancement of forskolin stimulated cyclic AMP formation following chronic agonist treatment has been demonstrated for the μ-opioid receptor (Bot *et al.*, 1998; Kato *et al.*, 1998) and the human nociceptin receptor expressed in CHO cells (Hashimoto *et al.*, 2000). However, in the latter study the maximal response was not affected. The enhancement of cyclic AMP may be due to constitutive activity (i.e. is not affected by the presence of agonist) of the opioid receptor under investigation.

In conclusion we have demonstrated that endomorphin-1, causes a rapid loss of cell surface μ-opioid receptors. In addition, prolonged treatment causes an uncoupling of receptor from G-protein. The physiological role of receptor desensitization and down-regulation may serve to rapidly remove receptors from the cell surface, were they may either be recycled to the cell surface or subjected to degradation. Future studies involving different cell lines, cell lines with

differing levels of receptor expression will provide further valuable information.

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