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The effects of endomorphin-1 and endomorphin-2 in CHO cells expressing recombinant μ-opioid receptors and SH-SY5Y cells

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1 Endomorphin-1 and -2 (E-1/E-2) have been proposed as endogenous ligands for the μ-opioid receptor. The aims of this study are to characterize the binding of E-1/E-2 and the subsequent effects on cyclic AMP formation and $[Ca^{2+}]_i$ levels in SH-SY5Y and Chinese hamster ovary (CHO) cells expressing endogenous and recombinant μ-opioid receptors.

2 E-1 displaced [^{3}H]-diprenorphine ([^{3}H]-DPN) binding in CHOμ and SH-SY5Y membranes with pK_i values of 8.02 ± 0.09 and 8.54 ± 0.13 respectively. E-2 displaced [^{3}H]-DPN binding in CHOμ and SH-SY5Y cells with pK_i values of 7.82 ± 0.11 and 8.43 ± 0.13 respectively. E-1/E-2 bound weakly to CHOδ and CHOκ membranes, with IC_{50} values of greater than 10 μM.

3 In CHOμ cells, E-1/E-2 inhibited forskolin (1 μM) stimulated cyclic AMP formation with pIC_{50} values of 8.03 ± 0.16 ($I_{max} = 53.0 \pm 9.3\%$) and 8.15 ± 0.24 ($I_{max} = 56.3 \pm 3.8\%$) respectively. In SH-SY5Y cells E1/E2 inhibited forskolin stimulated cyclic AMP formation with pIC_{50} values of 7.72 ± 0.13 ($I_{max} = 46.9 \pm 5.6\%$) and 8.11 ± 0.31 ($I_{max} = 40.2 \pm 2.8\%$) respectively.

4 E-1/E-2 (1 μM) increased $[Ca^{2+}]_i$ in fura-2 loaded CHOμ cell suspensions in a thapsigargin sensitive and naloxone reversible manner. Mean increases observed were 106 ± 28 and 69 ± 6.7 nM respectively. In single adherent cells E-1/E-2 (1 μM) increased $[Ca^{2+}]_i$ with a mean 340/380 ratio change of 0.81 ± 0.09 and 0.40 ± 0.08 ratio units respectively. E-1/E-2 failed to increase intracellular calcium in CHOδ, CHOκ and SH-SY5Y cells.

5 These data show that E-1/E-2 bind with high affinity and selectivity to μ-opioid receptors and modulate signal transduction pathways typical of opioids. This provides further evidence that these two peptides may be endogenous ligands at the μ-opioid receptor.

Keywords: Endomorphin; opioid receptor; intracellular calcium; cyclic AMP

Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium ion concentration; cyclic AMP, cyclic adenosine 3′,5′-monophosphate; CHO, Chinese hamster ovary (cell); DAMGO, [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin; DPN, diprenorphine; Ins(1,4,5)P_3, Inositol(1,4,5)triphosphate; PLC, phospholipase C

Introduction

Opioid receptors exist as three principal types, μ, δ and κ. During the 1970s, endogenous ligands that act at these receptors were identified as enkephalins, endorphins, and dynorphins (Hughes *et al*., 1975; Bradbury *et al*., 1976; Goldstein *et al*., 1979). However, none of these peptides showed high selectivity for the μ-opioid receptor. Recently two novel peptides have been isolated from bovine brain (Zadina *et al*., 1997) that have high affinity and selectivity for this receptor, and have been termed endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2). Furthermore, both peptides have been isolated from human brain (Hackler *et al*., 1997) and immunoreactivity for these peptides has been shown to occur in brain regions where μ-opioid receptors are located (Zadina *et al*., 1997; Pierce *et al*., 1998; Schreff *et al*., 1998; Martin-Schild *et al*., 1997). Endomorphin-1 and endomorphin-2 have been shown to mediate analgesia (Zadina *et al*., 1997; Stone *et al*., 1997; Goldberg *et al*., 1998) in mice, and to have a variety of cardiovascular effects consistent with opioids (Champion *et al*., 1997a,b,c; Czapla *et al*., 1998). In addition, endomorphin-1 and -2 reduce the electrically evoked C-fibre response of spinal neurons, whilst only endomorphin-1 reduces Aβ fibre evoked responses (Chapman *et al*., 1997).

Opioid receptors belong to the family of seven transmembrane spanning G protein coupled receptors. Binding of opioids to these receptors produces co-ordinated changes at a cellular level, namely closure of voltage sensitive calcium channels (Porzig, 1990), activation of potassium channels (North, 1989) and a reduction in cyclic AMP formation (Childers, 1991). Together these actions bring about a reduction in excitatory neurotransmission, which is the presumed mechanism by which opioids produce analgesia. In conjunction with this, opioids are also known to produce a variety of stimulatory effects, including stimulation of cyclic AMP formation, phospholipase C (PLC) activation and increases in $[Ca^{2+}]_i$ (Sarne *et al*., 1996; Smart & Lambert, 1996; Harrison *et al*., 1998a).

All three types of opioid receptors have been cloned (Kieffer *et al*., 1992; Evans *et al*., 1992; Thompson *et al*., 1993; Yasuda *et al*., 1993) and have pharmacological characteristics consistent with endogenous receptors (Knapp *et al*., 1995). Transfection of cloned opioid receptors into host cells allows a single subtype to be studied indepen-

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dently, an approach not always possible with clonal cell lines expressing multiple receptor subtypes (e.g. SH-SY5Y cells). The aim of the present study was to characterize the binding of endomorphin-1 and endomorphin-2 to membranes prepared from SH-SY5Y cells which express endogenous human μ-opioid receptors (Lambert *et al*., 1993) and CHO cells expressing recombinant rat μ-opioid receptors. In conjunction with this, we also aimed to determine the effect of endomorphin-1 and -2 on cyclic AMP formation and $[Ca^{2+}]_i$ in whole SH-SY5Y and CHOμ cells.

Methods

Cell culture and harvesting

CHO cells expressing the rat μ-opioid receptor 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 subcultured twice weekly and used when confluent (3–5 days). Experimental cultures were G418 free for 3 days. SH-SY5Y human neuroblastoma cells were cultured in minimal essential medium supplemented with foetal calf serum 10%, L-glutamine 2 mM, 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 were subcultured weekly and used when confluent (5–8 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, HEPES 10 and BSA 0.5%, pH 7.4 with 10 M NaOH (whole cells) or 50 mM Tris buffer containing BSA 0.5%, pH 7.4 with 10 M HCl (membranes). CHOμ cells for Ca^{2+} imaging studies were grown on glass coverslips (22 mm diameter) in six well plates seeded at a density of 200,000 cells per well in media as described above.

Membrane preparation

Membranes were prepared by harvesting CHO and SH-SY5Y cells as described above, and homogenizing with an Ultra Turrax for 30 s at 13,500 r.p.m. The membranes were centrifuged at 20,374 × *g*, 4°C for 10 min, washed with Tris buffer and homogenized and centrifuged twice more, as above.

[^{3}H]-diprenorphine (DPN) displacement studies

Displacement studies were performed as described previously (Lambert *et al*., 1993) in 1 ml volumes of 50 mM Tris, pH 7.4 at 20°C for 60 min with a fixed concentration of [^{3}H]-DPN (~0.4 nM), CHO or SH-SY5Y membranes and increasing concentrations of endomorphin-1 or endomorphin-2 (10^{-11}–10^{-5} M). Non specific binding was defined using 10 μM naloxone. The peptidase inhibitors amastatin, bestatin, captopril and phosphoramidon (30 μM of each) were included to prevent degradation of the peptides. Bound and free radioactivity were separated by rapid vacuum filtration using a Brandell 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.

Measurement of intracellular free calcium ($[Ca^{2+}]_i$) in cell suspensions

Confluent cells were harvested and washed in Krebs HEPES buffer (3 × 10 ml) as above. Cell suspensions were loaded with 3 μM Fura 2 acetoxy methylester (AM) for 30 min at 37°C, washed and then incubated at 20°C for 20 min. Intracellular calcium concentrations were measured at 37°C in the presence of 30 μM amastatin, bestatin, captopril and phosphoramidon, 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.

Measurement of $[Ca^{2+}]_i$ in single cells

Cells on coverslips were washed in Krebs-HEPES buffer of the following composition (mM): Na^+ 127.2, K^+ 6.0, Ca^{2+} 2.4, Mg^{2+} 1.2, Cl^- 134.4, $H_2PO_4^{2-}$ 1.2, Glucose 11.1, HEPES 5.0 and BSA 0.05%, pH 7.3 with 1 M NaOH then incubated with 2 μM fura-2 AM for 2 h at 20°C. Following loading, the coverslips were positioned onto the stage of a Nikon inverted microscope with a ×40 objective and perfused with Krebs-HEPES buffer to remove any free fura-2 AM and to allow hydrolysis of intracellular fura-2 AM. Cells were subjected to excitation at 340 and 380 nm using a spectral Wizard monochromater with a 700 ms exposure time, with emission measured at 510 nm. Cells were perfused at room temperature with 1 μM of endomorphin for 1 min and imaged using a cooled charge-coupled device camera and Merlin software (Life Science Resources). Data are expressed as 340/380 ratio values.

Measurement of cyclic AMP formation

Whole cell suspensions (0.3 ml) were incubated in the presence of isobutylmethylxanthine (1 mM), forskolin (1 μM), 30 μM amastatin, bestatin, captopril and phosphoramidon and either endomorphin-1 or endomorphin-2 (10^{-11}–10^{-5} M) and naloxone (10 μM) in various combinations for 15 min. Reactions were terminated as described previously (Hirst & Lambert, 1995), using 10 M HCl and neutralized with 10 M NaOH/1 mM Tris, pH 7.4. The concentration of cyclic AMP in the supernatant was measured using a specific protein binding assay (Brown *et al*., 1971).

Data analysis

All data are mean ± s.e.mean or a single representative trace of $n \geqslant 3$. pIC_{50} values (log half maximal inhibition) and I_{max} (maximal inhibition) 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 K_i values. K_d values entered in the Cheng-Prussoff equation were those obtained previously in this laboratory for SH-SY5Y (K_d = 0.17 nM, B_{max} = 167 fmol mg^{-1} protein, Lambert *et al*., 1993) and CHOμ cells (K_d = 0.19 nM, B_{max} = 266 fmol mg^{-1} protein, Smart *et al*., 1997). Statistical analysis was performed using ANOVA or Student's *t*-test where appropriate.

Results

Endomorphin-1 and endomorphin-2 concentration-dependently displaced [^{3}H]-DPN binding in CHOμ and SH-SY5Y cells (Figure 1a and b), which when corrected for the amount of radioligand used, yielded pK_i values shown in Table 1. In all cases the slope factor was less than unity (Table 1). There was a small but significant difference between pK_i values for the two cell types when analysed by Student's *t*-test (9.5 compared to 2.9 nM). Analysis of endomorphin-2 displacement of [^{3}H]-DPN between CHOμ and SH-SY5Y cells yielded a small but significant difference in pK_i values by *t*-test (15.1 compared to 3.7 nM). Endomorphin-1 and -2 bound weakly to membranes prepared from CHOδ and CHOκ cells, with IC_{50} values of greater than 10 μM (data not shown).

In CHOμ and SH-SY5Y cells endomorphin-1 and -2 produced a concentration-dependent inhibition of forskolin-stimulated cyclic AMP formation (Figure 2a and b), with pIC_{50} values and maximal inhibition shown in Table 2. This inhibition of forskolin-stimulated cyclic AMP formation was naloxone sensitive (data not shown). Analysis of pIC_{50} values by Student's *t*-test showed that there was no significant difference between CHOμ and SH-SY5Y cells for either endomorphin-1 or -2. There was also no significant difference between I_{max} values between CHOμ and SH-SY5Y cells for endomorphin-1, however there was a small but significant difference between I_{max} values between cell lines for endomorphin-2.

Endomorphin-1 and -2 increased intracellular calcium in CHOμ cell suspensions. The mean increases observed with 1 μM of peptide were 106 ± 28 nM and 69 ± 7 nM for

Table 1 Endomorphin-1 and -2 displace [^{3}H]-DPN binding from CHOμ and SH-SY5Y cells

	Endomorphin-1		*Endomorphin-2*	
	CHOμ	*SH-SY5Y*	*CHOμ*	*SH-SY5Y*
pK_i value	8.02 ± 0.09	8.54 ± 0.13	7.82 ± 0.11	8.43 ± 0.13
(nM)	(9.5)	(2.9)*	(15.1)	(3.7)*
Slope factor	0.72 ± 0.04	0.61 ± 0.10	0.59 ± 0.05	0.69 ± 0.11

*Indicates significant difference ($P<0.05$) between SH-SY5Y and CHOμ cells. Slope factor describes the steepness of the displacement curve.

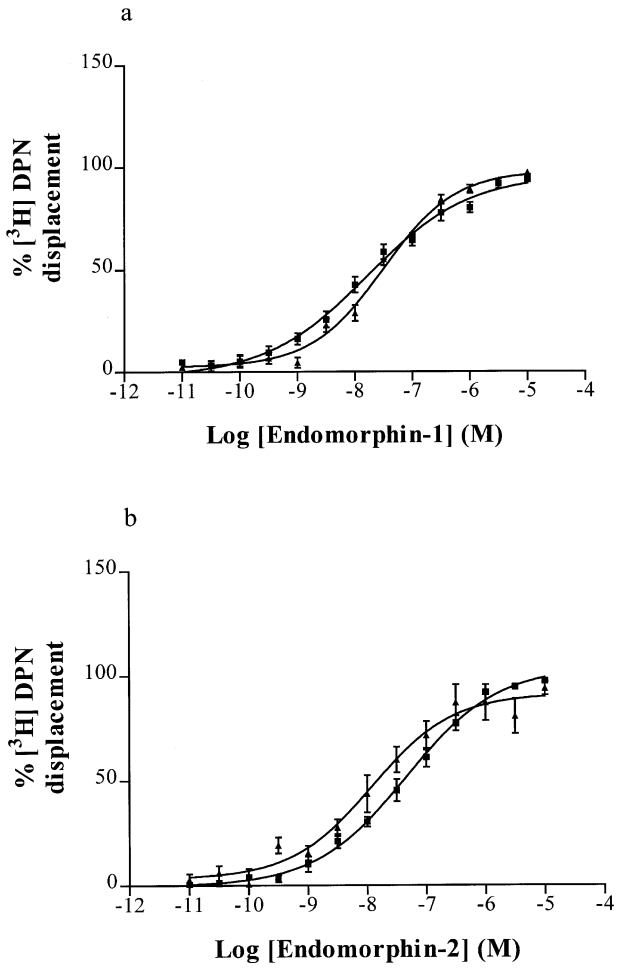

Figure 1 (a) Endomorphin-1 concentration-dependently displaced [^{3}H]-DPN in CHOμ (▲) and SH-SY5Y (■) membranes. Studies were performed at room temperature in 1 ml volumes for 60 min with a fixed concentration of [^{3}H]-DPN and endomorphin-1 as the displacer. Non-specific binding was defined in the presence of 10 μM naloxone. Data are mean ± s.e.mean for $n \geq 4$, displacement curves are $P<0.05$ by ANOVA. (b) Endomorphin-2 concentration-dependently displaced [^{3}H]-DPN in CHOμ (▲) and SH-SY5Y (■) membranes. Data are mean ± s.e.mean for $n \geq 4$, displacement curves are $P<0.05$ by ANOVA.

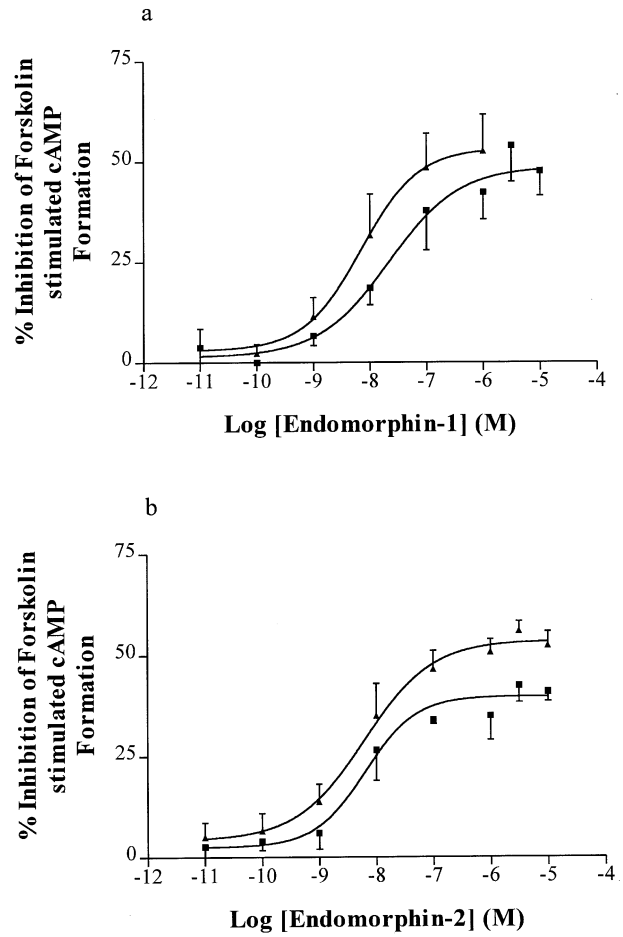

Figure 2 (a) Endomorphin-1 concentration-dependently inhibited forskolin stimulated cyclic AMP formation in CHOμ (▲) and SH-SY5Y (■) cells. Concentration-dependence curves are $P<0.05$ by ANOVA, data are mean ± s.e.mean for $n=4-5$. Cyclic AMP was measured by protein binding assay. (b) Endomorphin-2 concentration-dependently inhibited forskolin stimulated cyclic AMP formation in CHOμ (▲) and SH-SY5Y (■) cells. Concentration-dependence curves are $P<0.05$ by ANOVA, data are mean ± s.e.mean for $n=4-5$. Cyclic AMP was measured by protein binding assay.

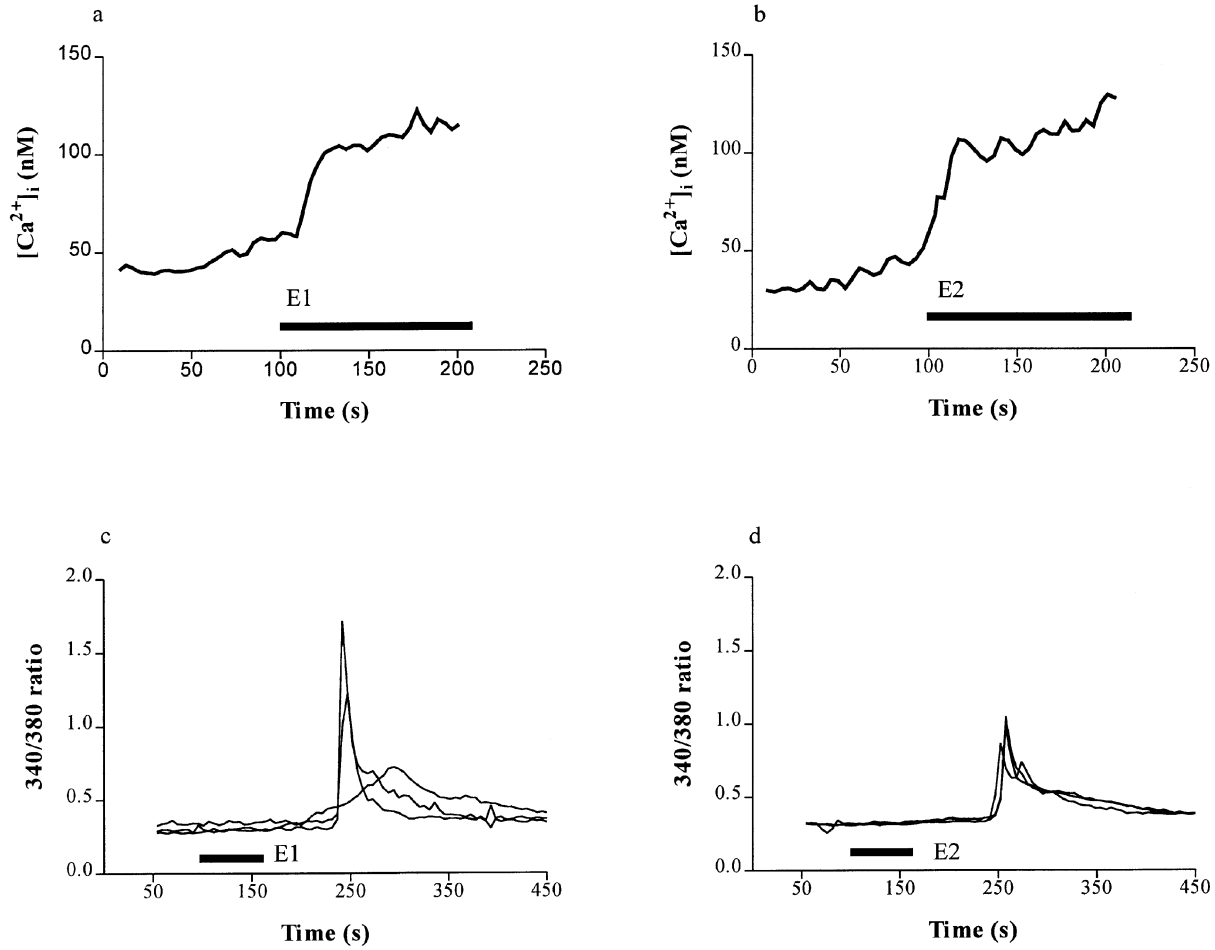

Figure 3 (a) A typical trace (of at least three others) depicting a rise in $[Ca^{2+}]_i$ with the addition of endomorphin-1 in CHOμ cells. $[Ca^{2+}]_i$ was measured in fura-2 loaded whole cell suspensions. (b) A typical trace (of at least three others) depicting a rise in $[Ca^{2+}]_i$ with the addition of endomorphin-2 in CHOμ cells. $[Ca^{2+}]_i$ was measured in fura-2 loaded whole cell suspensions. (c) Three typical traces of an individual single adherent CHOμ cell depicting a rise in $[Ca^{2+}]_i$ with the addition of endomorphin-1. $[Ca^{2+}]_i$ was measured in single adherent fura-2 loaded cells. (d) Three typical traces of an individual single adherent CHOμ cell depicting a rise in $[Ca^{2+}]_i$ with the addition of endomorphin-2. $[Ca^{2+}]_i$ was measured in single adherent fura-2 loaded cells.

Table 2 Endomorphin-1 and -2 inhibit forskolin stimulated cyclic AMP formation in CHOμ and SH-SY5Y cells

	Endomorphin-1		*Endomorphin-2*	
	CHOμ	*SH-SY5Y*	*CHOμ*	*SH-SY5Y*
pIC_{50} (nM)	8.03±0.16 (9.3)	7.72±0.13 (19.1)	8.15±0.24 (7.1)	8.11±0.31 (7.4)
% I_{max}	53.0±9.3	46.9±5.6	56.3±3.8	40.2±2.8*

*Indicates significant difference ($P<0.05$) between SH-SY5Y and CHOμ cells.

endomorphin-1 and endomorphin-2 respectively (Figure 3). This increase was thapsigargin sensitive with the increase in $[Ca^{2+}]_i$ observed to 100 nM thapsigargin being 337±83 nM. This increase was greater than that produced by endomorphin-1 and -2, implying that endomorphin released only a fraction of the Ins(1,4,5)P_3 sensitive pool. The increase in $[Ca^{2+}]_i$ was also naloxone (10 μM) reversible (data not shown). In CHO cell suspensions expressing recombinant δ- or recombinant κ-opioid receptors, endomorphin-1 and -2 failed to produce an increase in $[Ca^{2+}]_i$. In single adherent CHOμ cells endomorphin-1 and -2 increased intracellular calcium in a proportion of cells; endomorphin-1 = 7.2%, endomorphin-2 = 7.4%. In cells that responded to endomorphin-1 or endomorphin-2, the mean 340/380 ratio increase was 0.81±0.09 and 0.40±0.08 ratio units respectively when 1 μM of peptide was applied. CHOμ cells that failed to respond to endomorphin responded to 3 μM UTP, presumably acting *via* endogenous purinergic receptors. In SH-SY5Y cells endomorphin-1 and -2 failed to produce an increase in $[Ca^{2+}]_i$ in adherent or in suspended cells. Control experiments revealed that SH-SY5Y cells produced an increase in $[Ca^{2+}]_i$ in response to the muscarinic receptor agonist carbachol (1 mM). These data are consistent with our previous findings (Wandless *et al.*, 1996).

Discussion

We show here that endomorphin-1 and -2 bind to the recombinant μ-opioid receptor expressed in CHO cells and the endogenous μ-opioid receptor in SH-SY5Y cells with high affinity and selectivity. In addition to this both peptides produced a concentration-dependent inhibition of forskolin stimulated cyclic AMP formation in each cell line. Further-

more, endomorphin-1 and -2 increased intracellular calcium in CHOμ cells, but failed to do so in SH-SY5Y cells.

Endomorphin-1 and -2 produced a concentration-dependent displacement of [^{3}H]-DPN binding in membranes prepared from CHOμ and SH-SY5Y cells, which when corrected for the amount of radiolabel used yielded K_i values as shown in Table 1. Although these values are ~10 fold lower than the original paper (Zadina *et al*., 1997), they are consistent with those proposed by Hososhata *et al*. (1998) (K_i values of 7.7 and 5.9 nM for endomorphin-1 and -2 respectively) for B82 fibroblasts transfected with the μ-opioid receptor. In CHO cells expressing μ-opioid receptors, K_i values of 0.66 and 0.58 nM were obtained for endomorphin-1 and -2 displacement of the respective iodinated endomorphin, whilst in mouse brain, displacement of [^{3}H]-DPN gave K_i values of 0.03 (Endomorphin-1) and 0.12 nM (Endomorphin-2) (Goldberg *et al*., 1998). Therefore there are marked variations in reported K_i values between studies and the reasons behind these discrepancies warrants further investigation. It is also unclear as to the reasons behind the small but significant difference in pK_i values for endomorphin-1 and -2 binding to CHOμ and SH-SY5Y membranes. Since this difference is very small, we believe that both endomorphins display essentially similar affinity in both cell types.

Both endomorphin-1 and -2 had similar effects on cyclic AMP formation in CHOμ cells, and this was comparable to that seen previously with fentanyl (IC_{50} = 38 nM), as well as morphine and DAMGO (Smart *et al*., 1997). In SH-SY5Y cells, endomorphin-1 and -2 also cause a concentration-dependent inhibition of forskolin-stimulated cyclic AMP formation, similar to that seen in CHO cells expressing the recombinant receptors, although the maximum inhibition was slightly lower, possibly reflecting absolute levels of receptor expression. We have previously shown that fentanyl also produces an inhibition of forskolin-stimulated cyclic AMP formation in these cells, with an IC_{50} value of 27 nM (Lambert *et al*., 1993). These inhibitory actions of opioids on cyclic AMP formation are widely observed and may be one of the mechanisms by which opioids produce analgesia, *via* modulation of the hyperpolarization activated cation current, I_h (Ingram & Williams, 1994).

At present there is some debate as to whether endomorphin-1 and -2 act as partial or full agonists. Various studies have demonstrated partial agonist activity of endomorphins, as measured by [^{35}S]-GTPγs binding (Sim *et al*., 1998; Hososata *et al*., 1998; Alt *et al*., 1998). However other studies found that the endomorphins were as efficacious as other μ agonists (Zadina *et al*., 1997; Higashida *et al*., 1998; Mima *et al*., 1997; Kakizawa *et al*., 1998; Harrison *et al*., 1998b). This apparent discrepancy could be explained in terms of the level of receptor expression. When a tissue expresses a large number of receptors, endomorphins may act as full agonists, whereas in tissue expressing a lower level of receptors, partial agonism may be observed. At the level of cyclic AMP inhibition, endomorphin-1 and -2 displayed similar efficacy (maximal inhibition) in CHOμ cells expressing 266 fmol mg^{-1} protein (Smart *et al*., 1997), and SH-SY5Y cells expressing 167 fmol mg^{-1} protein (Lambert *et al*., 1993). Indeed this was similar to that produced by the well described full μ-opioid agonist fentanyl, indicating the endomorphins were probably acting as full agonists in these tissues. Studies utilizing cells expressing recombinant receptors at different levels would help address this question.

The endomorphin-1 and -2 induced increase in $[Ca^{2+}]_i$ observed in CHOμ cells in suspension is consistent with our previous report showing that the μ-opioid fentanyl causes mobilization of $[Ca^{2+}]_i$ in CHOμ cells (Smart *et al*., 1997), as do morphine and DAMGO (Harrison & Lambert, unpublished observations). An increase in $[Ca^{2+}]_i$ was also observed in single adherent cells treated with endomorphin-1 and -2. Activation of recombinant μ-opioid receptors in adherent neuro-$_{2A}$ cells by μ-opioids has also been shown to increase $[Ca^{2+}]_i$ (Spencer *et al*., 1998). It is interesting to note that single adherent CHOμ cells appear to have a low response rate to endomorphins. However, this may be the underlying reason why, when $[Ca^{2+}]_i$ is measured in the same cell line in whole cell suspensions, the increase in $[Ca^{2+}]_i$ is relatively small and unpredictable (Harrison *et al*., 1997; Smart *et al*., 1997). A low response rate to opioids in adherent cells has also been demonstrated in another study using cells expressing endogenous opioid receptors (Sarne & Gafni, 1996), in which synchronization was found to increase the probability of detecting a response. Spencer *et al*. (1998) also noted a moderately low response rate to opioid agonist in neuro-$_{2A}$ cells expressing recombinant opioid receptors. The low response in adherent cells was not due to the fact that experiments were conducted at room temperature. Preliminary experiments have suggested that increasing the temperature of single adherent cells did not affect the response rate and conversely, cells in suspension still respond to agonist when at room temperature (data not shown).

In CHO cells expressing recombinant δ- and κ-opioid receptors, endomorphin-1 and -2 did not increase $[Ca^{2+}]_i$. In addition, endomorphin-1 and -2 bound only very weakly to membranes prepared from CHOδ and CHOκ cells (IC_{50} > 10 μM), confirming that the endomorphins are indeed highly selective for the μ-opioid receptor, as shown by Zadina *et al*. (1997).

In the present study, endomorphin-1 produced a greater increase in $[Ca^{2+}]_i$ than endomorphin-2. The reasons behind this remain unclear, since at the level of inhibition cyclic AMP formation, both peptides appear to be full agonists. Clearly more detailed studies of endomorphin induced mobilization of $[Ca^{2+}]_i$ are needed to resolve this question.

Interestingly, both endomorphins failed to mobilize Ca^{2+} from SH-SY5Y cells. However we have previously shown that in SH-SY5Y cells PLC activation is dependent upon Ca^{2+} entry (Smart *et al*., 1995). The subsequent Ins(1,4,5)P_3 formation does not appear to be sufficient to cause an increase in Ca^{2+}, although μ-opioid receptor activation in the same cells causes a small and unpredictable increase in $[Ca^{2+}]_i$ with very high concentrations of fentanyl (Wandless *et al*., 1996), which may be due to calcium entry. Other studies have found that 'priming' of SH-SY5Y cells with the muscarinic agonist carbachol is necessary to produce an opioid mediated increase in $[Ca^{2+}]_i$ (Connor & Henderson, 1996). The same was found in NG108-15 cells, where opioid elevation of intracellular calcium only occurred in the presence of bradykinin (Okajima *et al*., 1993). Two other studies examining the effects of endomorphin-1 and -2 on $[Ca^{2+}]_i$ have demonstrated that both peptides cause Ca^{2+} channel inhibition in NG108-15 cells (Mima *et al*., 1997; Higashida *et al*., 1998), an action that is typical of opioids, and may be related to the ability of opioids to produce analgesia. Pre-treatment of cells with thapsigargin directly releases Ca^{2+} from intracellular stores, therefore the finding that the endomorphin induced mobilization of intracellular calcium was abolished by pre-treatment with thapsigargin indicates that release was from intracellular stores.

In conclusion, endomorphin-1 and -2 bind with high selectivity and affinity at the μ-opioid receptor and activate

the μ-opioid receptor to drive signal transduction pathways characteristic of opioids. This provides further evidence that these two peptides may be endogenous μ-opioid ligands, and the physiological role of these peptides clearly warrants further study.

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