Activation of multiple signaling pathways in cells expressing recombinant opioid receptors

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester by

Charlotte Harrison
Department of Anaesthesia and Pain Management

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Charlotte Harrison

Although opioids have been used for centuries in the management of pain, the opioid receptor family has only recently been cloned, thus allowing detailed studies on opioid receptor mediated signal transduction pathways to be performed. In addition, two novel opioid peptides, endomorphin-1 and -2, were identified in 1997, and it is believed that these represent new endogenous μ-opioid receptor ligands. This thesis represents a study into opioid receptor mediated increases in $[\text{Ca}^{2+}]_{i}$, and provides an investigation into the cellular signaling pathways of endomorphin-1 and -2.

Activation of the recombinant δ-opioid receptor expressed in CHO cells (CHOδ) by [D-Pen$^{2,5}$]-enkephalin produced a concentration dependent, pertussis toxin and thapsigargin sensitive increase in $[\text{Ca}^{2+}]_{i}$ in whole cell suspensions. Truncation of this receptor by the 37 C-terminal amino acids produced a rightward shift in the concentration response curve for $[\text{Ca}^{2+}]_{i}$ release. In single adherent CHO$\mu$, CHO$\kappa$ (CHO cells expressing recombinant μ- or κ-opioid receptors respectively) or CHOδ cells, application of fentanyl, spiradoline or [D-Pen$^{2,5}$]-enkephalin respectively produced an increase in $[\text{Ca}^{2+}]_{i}$ in some cells. The putative μ-opioid receptor endogenous ligands endomorphin-1 and endomorphin-2 bound with high affinity and selectivity to μ-opioid receptors from CHO$\mu$ and SH-SY5Y cells. Endomorphin-1 and -2 concentration-dependently inhibited forskolin stimulated cAMP formation in CHO$\mu$ and SH-SY5Y cells and endomorphin-1 and -2 produced an increase in $[\text{Ca}^{2+}]_{i}$ in CHO$\mu$ cells. Prolonged endomorphin-1 pretreatment desensitized the μ-opioid receptor in CHO cells, characterized by a reduction in maximal endomorphin-1 mediated cAMP inhibition, an up-regulation of cAMP formation and was due to receptor - G protein uncoupling. Endomorphin-1 pre-treatment produced a rapid loss of cell surface receptors from CHO$\mu$ cells, which was possibly accompanied by receptor degradation.

Collectively these data add to our understanding of opioid receptor-mediated signal transduction pathways.
Acknowledgements

Thanks to my family for always being there, to all my friends, especially Jo for encouragement in the form of gin and tonic! To Adam, John and Steve for help with bits of data. Darren and Shaun from Parke-Davis for help with calcium imaging experiments.

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To mum, I know how much this would have meant to you.
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List of abbreviations

12HETE hydroxyeicosatetraenoic acid
12HPETE hydroperoxyeicosatetraenoic acid
5HT 5-hydroxytryptamine
AC adenylyl cyclase
ATCH adrenocorticotropic hormone
ATP adenosine triphosphate
BCP/NBT 5-bromo-4-chloro-3-indolylphosphate nitro blue tetrazolium
BSA bovine serum albumin
CaM calmodulin
cAMP cyclic adenosine monophosphate
cGMP cyclic guanosine monophosphate
CHO Chinese hamster ovary
CGRP calcitonin gene related peptide
CLIP corticotropic-like intermediary hormone
CNS central nervous system
CREB cAMP response element binding protein
CTAP D-Phe-Sys-Tyr-D-Trp-Arg-Thr-Pen-Thr amide
CTOP D-Phe-Sys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide
CTX cholera toxin
DADLE [D-Ala²,D-Ala⁵]-enkephalin
DAG di-acylglycerol
DAMGO [D-Ala²,Me-Phe⁴Gly-ol]-enkephalin
DMSO dimethylsulphoxide
DOR δ-opioid receptor (cloned)
DPDPE [D-Pen²,⁵]-enkephalin
DSLET [D-Ser⁵,Leu⁵]-enkephalyl-Thr
E1 endomorphin-1
E2 endomorphin-2
EDTA ethylenediaminetetraacetic acid
EGTA ethyleneglycol-di-(aminoethyl)-N,N',N'-tetraacetic acid
ERK extracellular regulated signal-kinase
FITC fluorescein isothiocyanate
GABA γ-amino butyric acid
GAP GTPase activating protein
GDP guanosine diphosphate
GPCR G protein coupled receptor
GTP guanosine triphosphate
GTPase guanosine triphosphate-ase
GTPγS guanosine 5'-O-(3-thiotri-phosphate)
HEK human embryonic kidney
HEPES N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IBMX 4-isobutyl-1-methylxanthine
Icrac calcium release activated calcium (current)
Ins(1,4,5)P₃ inositol(1,4,5)triphosphate
Kir Inwardly rectifying potassium channel
KOR κ-opioid receptor (cloned)
LPH lipotropin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6G</td>
<td>morphine-6-glucuronide</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MOR</td>
<td>μ-opioid receptor (cloned)</td>
</tr>
<tr>
<td>MSH</td>
<td>melanocyte-stimulating hormone</td>
</tr>
<tr>
<td>NA</td>
<td>noradrenaline</td>
</tr>
<tr>
<td>NAADP</td>
<td>nicotinic acid di-nucleotide phosphate</td>
</tr>
<tr>
<td>NCR</td>
<td>nociceptin receptor</td>
</tr>
<tr>
<td>NKA</td>
<td>neurokinin A</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NorBNI</td>
<td>nor-binaltorphimine</td>
</tr>
<tr>
<td>NRM</td>
<td>nucleus raphe magnus</td>
</tr>
<tr>
<td>NRPG</td>
<td>nucleus reticularis paragigantocellularis</td>
</tr>
<tr>
<td>NSB</td>
<td>non-specific binding</td>
</tr>
<tr>
<td>NST</td>
<td>nucleus of solitary tract</td>
</tr>
<tr>
<td>ORL-1</td>
<td>opioid receptor-like (receptor)</td>
</tr>
<tr>
<td>PAG</td>
<td>periaqueductal grey</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethylenimine</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositol-3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphoinositol(4,5)bis-phosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLA2</td>
<td>phospholipase A2</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>phospholipase D</td>
</tr>
<tr>
<td>POMC</td>
<td>pro-opiomelanocortin</td>
</tr>
<tr>
<td>PTX</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td>RAMP</td>
<td>receptor activity modifying protein</td>
</tr>
<tr>
<td>RGS</td>
<td>regulator of G protein signaling</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethyl-ethylenediamine</td>
</tr>
<tr>
<td>UTP</td>
<td>uracyl triphosphate</td>
</tr>
<tr>
<td>VOCC</td>
<td>voltage operated calcium channel</td>
</tr>
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</table>
Chapter 1. General Introduction

1.1 Opium and morphine - a brief history

Opium (from the Greek word for juice - opion) comes from the seeds of the poppy Papaver somniferum. Evidence of early civilisations cultivating poppy seeds can be found from around 4000BC and by 2000BC knowledge of opium was widespread throughout Europe, the Middle East and North Africa, were it was considered a cure for all ailments. Opium was considered important in Greco-Roman pharmacy, and was used to alleviate pain as well as insomnia, coughs, bowel problems and a variety of other conditions. As well as opium's role as an analgesic, it was also used as a religious drug and as an aid to commit murder. In Europe the use of opium declined with the collapse of the Roman Empire, but re-appeared with the return of the crusaders in the 12th and 13th centuries. By the sixteenth century opium had an established role as a medicine in Europe, and became increasingly popular in the 18th century.

There existed two arguments in the 18th century as to the mode of action of opium. The first of these was the idea that opium caused the blood to become rarefied or thinned, an action due to absorption and circulation of the drug. The second argument was that it was acting on nerves to where it was applied and was distributed by 'consent' and this explained the fast mode of action of the drug.

In 1806 Friedrich Sertürner isolated a major active constituent of opium and named it morphine after Morpheus, the Greek god of dreams. By the early 1820's morphine was commercially available in Western Europe in standard measures of strength, and was widely used in the 19th century to combat pain, often given as an injection. The manufacture of heroin in 1898 lead to the synthesis of other opioid drugs, which came to be used clinically and in research as to the mode of action of opioids. Specific binding sites for opioids were first postulated in 1954 by Becket and Casy and elaborated by Portoghese (1965), and Goldstein et al., (1971). The identification of stereo-specific binding sites for opioids was made in 1973 (Pert and Synder, 1973; Simon et al., 1973; Terenius, 1973) and so began the field of opioid receptor pharmacology.
1.2 Endogenous opioids and their receptors

From the demonstration of stereo specific opioid binding sites (Pert and Synder, 1973; Simon et al., 1973; Terenius, 1973), it was postulated that receptors did not exist only for application of exogenous substances, but there were endogenous substances that produced 'morphine - like' effects (Hughes, 1975). Indeed a substance was isolated from mammalian brain that inhibited contractions of mouse vas deferens and guinea pig myenteric plexus (Hughes, 1975). This work led to the identification of two related opioid peptides, termed met- and leu-enkephalins (from the Greek ‘in the head’), which were isolated from porcine brain (Hughes et al., 1975). Soon afterwards it was discovered that the C fragment of lipotropin (a fat-mobilizing hormone) had opioid activity (Bradbury et al., 1976), which was later termed β-endorphin (from endogenous morphine). A third endogenous opioid peptide, termed dynorphin (from the Greek dynamis – power) was isolated from porcine pituitary in 1979 (Goldstein et al., 1979). Precursors for these endogenous opioid peptides have a distinct distribution in the periphery and CNS (see Mansour et al., 1988), and are shown in Table 1.1, Figure 1.1 shows the amino acid structure of the endogenous opioid peptides.

Due to the presence of multiple endogenous ligands it was suggested that there might be multiple opioid receptor subtypes. Evidence for this came from the work of Martin et al., who in 1976, suggested μ- (from morphine) and κ- (from ketocyclazocine) opioid receptors using cross-tolerance studies in dogs. A σ receptor (from SKF10-047) was also identified, but this was later found to be non-opioid (Manallack et al., 1986). The identification of the δ- (from vas deferens) opioid receptor was reported in 1977, following the identification of the enkephalins. (Lord et al., 1977). Twenty years later, two peptides were discovered that displayed high affinity and selectivity for the μ-opioid receptor (Zadina et al., 1997), termed endomorphin-1 and endomorphin-2 (again from endogenous morphine). Although both peptides have been isolated from mammalian brain, to date no precursor has been found.

In addition, other opioid peptides cleaved from endogenous precursors exist, these include morphiceptin and haemorphin-4 cleaved from casein and haemoglobin respectively (Yang et al., 1999) and others shown in table 1.1. Opioid peptides have also been isolated from amphibian skin -dermorphin and deltorphin - which are
selective for μ and δ-opioid receptors (Corbett et al., 1999). As well as the three main types of opioid receptor, other types of opioid receptor have been proposed, the ε receptor - specific for β-endorphin as well as τ, λ and ζ subtypes (Corbett et al., 1999). In addition, a receptor specific for the active metabolite of morphine, morphine-6-glucuronide (M6G) has been proposed (Paul et al., 1989; Brown et al., 1997).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Precursor</th>
<th>Receptor selectivity</th>
<th>Other active products (opioid)</th>
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<tr>
<td>Dynorphin</td>
<td>Pro-dynorphin</td>
<td>κ</td>
<td>Dynorphin B, Dynorphin A(1-8), α/β neoendorphin</td>
</tr>
<tr>
<td>Enkephalin</td>
<td>Pro-enkephalin</td>
<td>δ</td>
<td>-</td>
</tr>
<tr>
<td>β-endorphin</td>
<td>pro-opiomelanocortin</td>
<td>μ and δ</td>
<td>-</td>
</tr>
<tr>
<td>Endomorphin-1/2</td>
<td>?</td>
<td>μ</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1.1

Endogenous opioid peptides, their precursors and receptor selectivity
Dynorphin A
Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln

Met/Leu-Enkephalin
Tyr-Gly-Gly-Phe-Met/Leu

β-endorphin

Endomorphin-1/-2
Tyr-Pro-Trp-Phe/Tyr-Pro-Phe-Phe

Figure 1.1
Amino acid sequence of representative endogenous opioid peptides.

1.3 Alternative nomenclature
The International Union of Pharmacology subcommittee on opioid receptors has proposed an alternative classification of opioid receptors, these being OP₁ (δ), OP₂ (κ) and OP₃ (µ) (Dhawan et al., 1996), based on the order in which they were cloned and where OP = opioid peptide. However, this nomenclature has not been widely adopted by the scientific community, and throughout this thesis the traditional µ-, δ-, and κ-terminology is used.

1.4 Cloned opioid receptors
The first opioid receptor to be cloned and sequenced was the δ–subtype, cloned simultaneously by two independent groups (Keiffer et al., 1992; Evans et al., 1992). Soon after both the κ- (Yasuda et al., 1993) and µ- (Thompson et al., 1993) opioid receptors were formally identified. Following this it was shown definitively that opioid receptors belonged to the G protein receptor family, which contains many members, sharing common structural features (Birnbaumer et al., 1990). All consist of seven transmembrane-spanning domains, rich in hydrophobic amino acids, an extracellular N
terminus with multiple glycosylation sites and intracellular C-terminus in which there are potential phosphorylation sites (serine and threonine residues). Cysteine residues form a disulphide bridge between the first and second extracellular loop of the receptor (Law and Loh, 1999).

Each of μ-, δ-, and κ- opioid receptors has approximately 60% homology with each other, the greatest homology being in the transmembrane regions (73-76%) and the intracellular loops (86-100%). The greatest diversity is at the N and C termini and the extracellular loops (Law and Loh, 1999). Each sub-type of opioid receptor is able to differentially bind opioid ligands and all three subtypes are able to mediate analgesia, and share common second messenger systems.

1.5 Opioid receptor subtypes and ligands
Numerous subtype selective agonists and antagonists have been developed for each of the opioid receptor subtypes, some are in use clinically and others used for research purposes. Some ligands are based upon the simplification of morphine (figure 1.2), for example fentanyl, used clinically for the induction and maintenance of anaesthesia. Thebaine is an alkaloid, structurally related to morphine that is also found in opium. Although this compound is relatively inactive, commonly used non-selective antagonists such as naloxone, naltrexone and diprenorphine are derived from this compound. Other ligands are enkephalin analogues, protected so that they are not broken down. Non-peptide ligands, selective for either δ- or κ-opioid receptors have also been developed. Commonly used ligands are shown in table 1.2, which shows peptide analogues and synthetic ligands.

![Figure 1.2](image)

*Chemical structure of morphine*
<table>
<thead>
<tr>
<th>receptor</th>
<th>selective agonists</th>
<th>Selective antagonists</th>
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<tbody>
<tr>
<td>μ</td>
<td>DAMGO¹, Fentanyl</td>
<td>CTAP</td>
</tr>
<tr>
<td>δ</td>
<td>DPDPE¹, DSLET¹, DADLE¹</td>
<td>Naltrindole</td>
</tr>
<tr>
<td>κ</td>
<td>spiradoline, enadoline, U50488</td>
<td>NorBNI</td>
</tr>
</tbody>
</table>

**Table 1.2**

*Commonly used subtype selective agonists and antagonists. Enkephalin analogues*

From evidence based on differing affinities and actions of subtype selective ligands, it has been proposed that each opioid receptor subtype exists as multiple isoforms. These include μ₁, μ₂ (Pasternak and Wood, 1986) and μ₃ (Cruciani *et al*., 1994), δ₁ and δ₂ (Traynor and Elliot, 1993; Sofuoglu *et al*., 1991) and κ₁, κ₂ and κ₃ (Pasternak, 1993; Cheng *et al*., 1985). However, not all pharmacological studies agree with subtype division (e.g. Connor *et al*., 1997a). It is possible that μ₁ opioid receptors mediate supraspinal analgesia whilst μ₂ opioid receptors mediate spinal morphine analgesia, and it may be the μ₂ site that is responsible for respiratory depression (Pasternak and Wood, 1986).

To date, there is no molecular biological evidence to support the idea of subtype division, since only one example of each of the μ-, δ- and κ-opioid receptors has been cloned from any one species. However the possibility of post-translational modification or interaction with receptor activity modifying proteins (RAMPs, Foord and Marshall, 1999) remain a possibility. It is possible that opioid receptors may exist as complexes, composed of distinct but interacting μ-, δ- and possibly κ-opioid receptors. It has been proposed that there are two δ binding sites; δₙₑₓ which is not associated with any other opioid receptors, and is pharmacologically the δ₁ receptor and δₑₓ which is the δ receptor complexed with μ receptors (Rothman *et al*., 1992, Traynor and Elliott, 1993). Opioid receptors have also been shown to exist of heterodimers between κ- and δ-opioid receptors (Joarden and Devi, 1999) which display binding and functional properties distinct from either receptor. Indeed receptor dimerization of GPCRs as been shown to modulate ligand affinity and efficacy (Onaran and Gurdal, 1999).
Splice variants of the pharmacological μ1-opioid receptor have been isolated, these being MOR1 and MOR1B, where MOR1B is seven residues shorter than MOR1 (Zimprich et al., 1995). In addition, further alternative isoforms of MOR1 have been proposed, including MOR1C, MOR1D, MOR1E (Pan et al., 1999) and MOR1F (Pan et al., 2000). Again the differences occur in the C-terminal tail of the receptor and it is interesting to note that some of the variants displayed a differential regional distribution. Three mRNA variants of the κ-opioid receptor (Wei et al., 2000) have also been isolated. It remains to be seen what the full biological role of these splice variants are.

1.6 Location of opioid receptors

The distribution of opioid receptors within the body differs depending upon receptor type, although there is some overlap. It should be noted however, that the majority of localization studies have been performed on rat brain, and there are species differences in the anatomy of opioid receptors (Dhawan et al., 1996). The μ-opioid receptor has the widest distribution, being found in brain areas such as the caudate putamen, cortex, thalamus, nucleus accumbens, amygdala, globus pallidus, locus ceruleus and periaqueductal grey, pons and medulla (Mansour et al., 1987; Mansour et al., 1988; George et al., 1994). Receptors can also be found in the superficial layers of the dorsal horn of the spinal cord, as well as in peripheral sites such as the gut and vas deferens. δ-opioid receptors can be found in the olfactory cortex, caudate putamen, hippocampus and nucleus accumbens as well as in the dorsal horn. (Mansour et al., 1987; Mansour et al., 1988; George et al., 1994). Like the μ-opioid receptor it is also found in the periphery, such as the vas deferens (from which it was first identified). With regard to the κ-opioid receptor, there appear to be great species differences in distribution. However, this receptor is found in the amygdala, hypothalamus, thalamus, caudate putamen, nucleus accumbens, cortex, hippocampus, pons/medulla and brainstem (Mansour et al., 1987; Mansour et al., 1988; George et al., 1994).

1.7 Genetic pharmacological and behavioral studies

The cloning of opioid receptors has allowed genetic modifications to be made to receptors, thus determining regions of opioid receptors involved in structure and function. For example, using chimeric rat μ/δ receptors it was shown that the major
binding motif for DPDPE is within transmembrane regions V-VII and the intervening loops (Fukuda et al., 1995). This study also showed that the region spanning from the first intracellular loop to the amino-terminal half of transmembrane segment III is involved in binding of DAMGO, whereas the \( \mu \) selective alkaloids morphine and codeine bind to transmembrane segments V-VII (Fukuda et al., 1995). Specific amino acids have also been shown to be important in ligand binding. Lysine 108 of the \( \delta \)-opioid receptor prevents the \( \mu \) agonist DAMGO binding to the \( \delta \)-opioid receptor (Minami et al., 1996), Tryptophan 284 and Leucine 384 in the human \( \delta \)OR appear to be crucial for the binding of \( \delta \) selective ligands (Valiquette et al., 1996). Leucine 300, a hydrophobic region (amino acids 295-300) and arginine 291 of the human \( \delta \)-opioid receptor are important in ligand binding and Trp300 may block access of \( \delta \) selective ligands (DPDPE and SNC-121) to their docking site (Pepin et al., 1997). For further information regarding receptor domains mediating receptor function the reader is directed to the following review, Quock et al., (1999).

The C-terminal tail of opioid receptors has received much attention, since it is believed to have a role in the desensitization and down-regulation of opioid receptors. Again specific mutations have been made in this region of the receptor and have been correlated with receptor function. Several groups have reported that specific serine and threonine residues on the C-terminus of the \( \delta \)-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 \( \mu \)-opioid receptor but not the \( \delta \)-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).

As well as manipulating opioid systems at the level of the receptor, It is possible to genetically manipulate animals (usually mice) so that they do not express any of a certain type of opioid receptor (homozygotes) or approximately 50% reduction in receptor number (heterozygotes). These 'knockout mice' then can be used for pharmacological and behavioral studies of opioid receptor function. For example, mice lacking the \( \mu \)-opioid receptor gene do not display morphine induced analgesia and do not display symptoms of opioid withdrawal, but have no major differences in the
number and distribution of δ- and κ-opioid receptors (Matthes et al., 1996; Kitchen et al., 1997). κ-opioid receptor knock-out mice have shown that the κ-opioid receptor gene is involved in the perception of chemical visceral pain, and is necessary for other actions of U50-488H including hypo-locomotion and aversion (Simonin et al., 1998). Intriguingly, in mice lacking the δ-opioid receptor gene, there is retention of DPDPE induced supraspinal analgesia, although there is loss of morphine tolerance (Zhu et al., 1999). The reasons behind the analgesic effect of DPDPE remain to be clarified. Recently it has been demonstrated that mice lacking exon-1 of MOR1 retain M6G and heroin induced analgesia (Schuller et al., 2000), thus providing evidence that these opioids may act though a distinct receptor site not encoded for by exon-1 of MOR1.

1.8 G protein coupled receptor (GPCR) signaling

Opioid receptors are examples of GPCR family. G proteins allow cell surface receptors, which have no direct contact with effector molecules to mediate signal transduction by relaying information via a G protein to a second messenger which will activate/inactivate the final effector molecules.

Heterotrimeric G proteins comprise a larger α subunit and two smaller β and γ subunits that always remain in close contact with each other. At rest the α subunit has GDP bound and all subunits are in close association with each other, the G protein complex as a whole being associated with the cytoplasmic face of the membrane (Clapham, 1996). Upon agonist binding to the receptor, the intracellular domains undergo isomerization allowing the receptor to couple to the G protein. G protein activation causes GTP to replace GDP in a cleft in the α subunit, and in doing so causes a conformational change to occur in switch regions of the α subunit producing the dissociation of Gα and Gβγ (Lambright et al., 1996; Hamm, 1998). The α and βγ subunits are then able to interact with effector molecules, see figure 1.3.

After a few seconds of interaction with the effector the α subunit with its own intrinsic GTPase activity hydrolyses GTP to GDP. In doing so the α subunit inactivates itself, dissociates from the effector and re-associates with the βγ subunit. The intrinsic GTPase activity provides the basis of a timer and memory function in the signaling system (Bourne et al., 1990), so that an interaction between agonist and receptor lasting
milliseconds can be translated into an effect lasting many seconds. It is also possible that the effector molecule can act as a GTPase activating protein (GAP) accelerating the GTPase activity of the α subunit (Bourne and Stryer, 1992). G protein signaling may also be influenced by RGS (regulators of G protein signaling) proteins, a group of proteins may which act as GAPs to attenuate receptor signaling or as effector antagonists by causing inhibition of GDP release to prevent GTP binding (Hepler, 1999).

The complexity of G protein signaling is increased by the diversity of α and βγ subunits. At present there are at least 20α, 6β and 12γ subunits that have been identified (Hamm, 1998). Historically G proteins are classified using two types of bacterial toxin, pertussis (PTX) and cholera (CTX). Pertussis toxin ADP-ribosylates inhibitory G proteins (Gi) and prevents interaction with the receptor molecule. Cholera toxin prevents GTP hydrolysis to GDP and therefore irreversibly activates α-GTP of stimulatory G proteins (Gs). However, the use of G protein subunit specific antibodies has more accurately defined this classification.
Figure 1.3
Representation of the G protein cycle.
1) In the absence of agonist, the G protein α subunit has GDP bound and all the subunits are in close association with each other. 2) Agonist interaction with the receptor allows coupling of the receptor to the G protein. GTP then replaces GDP in a cleft in the α subunit causing the split of Ga and Gβγ. 3) The α and β subunits are then able to interact with effectors. The α subunit displays its own intrinsic GTPase activity, and is able to hydrolyses GTP to GDP following interaction with the effector. In doing so the α subunit inactivates itself, dissociates from the effector and re-associates with the βγ subunit (Ag = agonist).
Opioid receptor - G protein coupled effector systems

1.9 Adenylyl Cyclase

Adenylyl cyclase is the enzyme responsible for the generation of cAMP from ATP. To date there are nine cloned mammalian isoforms of adenylyl cyclase. All share a common structure; a cytoplasmic N-terminal region, a membrane anchoring hydrophobic domain consisting of 6 transmembrane helices, a large cytoplasmic domain, a second transmembrane domain and a second cytoplasmic domain at the C-terminus (Simmonds, 1999; Hurley, 1999). However, differences exist between the isoforms with respect to their location and regulation, with some isoforms (e.g. adenylyl cyclase type 2 and adenylyl cyclase type 8) being restricted to neuronal tissue, whilst other types (e.g. adenylyl cyclase type 7) are found in brain and non-neuronal tissue (e.g. kidney, spleen). All types of mammalian adenylyl cyclase are stimulated by Gs subunits of G proteins, and all but type nine are stimulated by forskolin, which is often used experimentally to elevate cAMP levels. G protein βγ subunits, PKA, PKC, Ca²⁺/CaM can also differentially regulate each of the isoforms (Simmonds, 1999), thus allowing integration from various signaling pathways.

Inhibition of adenylyl cyclase is a universal signaling pathway activated by opioids that was first demonstrated as early as 1975 (Sharma et al., 1975). More recently, each of µ, δ-, and κ-opioid receptors have been shown to couple negatively (via a PTX sensitive G protein) to adenylyl cyclase to cause a reduction in cAMP formation (Smart et al., 1997; Hirst et al., 1997; Hirst et al., 1998). Functional consequences of cAMP inhibition may be diverse; for example cAMP causes activation of the enzyme PKA, which may phosphorylate various cellular targets (Levitan, 1985). cAMP may modulate gene expression by modulation of the cAMP response element binding protein (CREB) (McMillan, 1995). Opioids may reduce neuronal excitability by modulating the hyperpolarization activated cation current, Iₜ (Ingram and Williams, 1994) (probably not via PKA, but a direct action, Larckman et al., 1995) and this may be one of the mechanisms by which opioids produce analgesia.

1.10 Voltage operated Ca²⁺ channels

Voltage operated Ca²⁺ channels may be divided into several categories, according to their activation threshold, inactivation rate and conductance, see table 1.3. Each type of
channel also differs in its sensitivity to various pharmacological agents, with L-type Ca\(^{2+}\) channels being sensitive to the dihydropyridines and other types varying in their sensitivity to toxins such as \(\omega\)-conotoxin (Hille, 1991).

<table>
<thead>
<tr>
<th>Property</th>
<th>L</th>
<th>N</th>
<th>P/Q</th>
<th>R</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conductance (pS)</td>
<td>11-25</td>
<td>10-20</td>
<td>10-20</td>
<td>15</td>
<td>7-10</td>
</tr>
<tr>
<td>Activation threshold</td>
<td>high</td>
<td>low</td>
<td>low</td>
<td>low</td>
<td>low</td>
</tr>
<tr>
<td>Inactivation rate</td>
<td>rapid</td>
<td>intermediate</td>
<td>slow</td>
<td>rapid</td>
<td>rapid</td>
</tr>
<tr>
<td>Location</td>
<td>cardiac, smooth, neuronal, neuronal, neuronal widespread skeletal muscle, neuroendocrine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 1.3*

*Characterization of voltage sensitive Ca\(^{2+}\) channels (adapted from Triggle, 1999).*

Opioids have been shown to have an inhibitory effect on N-, P/Q-, R- and T-types of Ca\(^{2+}\) channels (Seward *et al*., 1990, Soldo and Moises, 1998, Piros *et al*., 1996, Corbett *et al*., 1999). Since it is accepted that neurotransmitter release is a Ca\(^{2+}\) dependent process, inhibition of Ca\(^{2+}\) channels at nerve terminals may lead to a reduction in neurotransmitter release. Opioids have been reported to have both an inhibitory effect (Morikawa *et al*., 1995) or promote opening (Smart *et al*., 1995) of the predominately non-neuronal L-type Ca\(^{2+}\) channel.

**1.11 Inwardly rectifying K\(^+\) channels (K\(_{ir}\))**

Inwardly rectifying K\(^+\) channels favour the entry of K\(^+\) when the cell is hyperpolarized, but do not allow it to exit under depolarization. In addition to this they also carry outward current and by doing so maintain the resting potential of the cell near the equilibrium potential for K\(^+\) and therefore stabilize the membrane potential (Hille, 1991). All three types of opioid receptor have been shown to activate inwardly rectifying K\(^+\) channels (Christie *et al*., 1987; Henry *et al*., 1995, Han *et al*., 1999). The results of this activation will be movement of the membrane potential to a more
negative value and thus reduce the likelihood of an action potential producing depolarization.

1.12 Opioids and neurotransmitter release
The combined effect of these actions of opioids (closure of VOCC's, activation of $K_{ir}$ and inhibition of cAMP formation) is to bring about a reduction in neurotransmitter release, as summarized in figure 1.4. Indeed opioids have been shown to inhibit neurotransmitter release from a variety of neuronal preparations e.g. glutamate (Nicol et al., 1996a), acetylcholine, substance P and noradrenaline (Leslie et al., 1987). Opioid receptors are also located post-synaptically, and may 'dampen down' neurotransmission by having similar effects on postsynaptic neurons.

---

**Figure 1.4**

Pre-synaptic opioid receptors are thought to decrease neurotransmission via a reduction in cAMP levels, closure of voltage-operated calcium channels and activation of inwardly rectifying potassium currents. These coordinated changes lead to a reduction in the release of excitatory neurotransmitters and analgesia.
1.13 Stimulatory effects of opioids

Opioids have also been reported to produce stimulatory effects on neurotransmission and neurotransmitter release, which was first explained as a suppression of opioid inhibition or ‘disinhibition’. For example the stimulatory effect of opioids on acetylcholine release from caudate nucleus was suggested to result from presynaptic suppression of an inhibitory dopaminergic system (Vizi et al., 1977). More recently, direct stimulatory actions of opioids have been shown in a variety of tissues. Opioids increase adenosine secretion from spinal cord synaptosomes (Cahill et al., 1993), increase release from dopaminergic neurons (Devine et al., 1993), cause the release of a variety of neurotransmitters from DRG neurons (Bourgoin et al., 1994; Cahill et al., 1995) and stimulate noradrenaline release from SK-N-SH cells (Same et al., 1994). Indeed, opioids have been shown to increase action potential duration (when the Ca\(^{2+}\) component of the action potential is prolonged) and decrease K\(^+\) current in DRG neurons (Crain and Shen, 1990; Shen and Crain, 1994). Opioids induced increases in neuronal firing rate have also been demonstrated, due to post-synaptic action (Lin and Carpenter, 1994).

1.14 Opioid stimulation of Adenylyl Cyclase

It is widely accepted that activation of opioid receptors leads to a reduction in cAMP formation (see section 1.9), but there are reports of a stimulatory effect of opioids on adenylyl cyclase activity in a variety of tissues, e.g. DRG neurons (Mackman et al., 1988), Xenopus oocytes transfected with the κ receptor (Kaneko et al., 1994) and human kidney cells transfected with the δ receptor (Tsu et al., 1995).

Some studies have found that the stimulation of adenylyl cyclase was G\(_{\text{s}}\) linked (Shen and Crain, 1994 Ammer and Shulz, 1997), whereas in other studies it was found to occur in a PTX sensitive (G\(_{\text{i/0}}\)) manner involving βγ subunit stimulation of type II adenylyl cyclase (Olinas and Onali, 1994; Kaneko et al., 1994; Tsu et al., 1995). In some situations, agonist dependent up-regulation of adenylyl cyclase activity was noted following prolonged agonist treatment (Avidor-Reiss et al., 1996; Ammer and Shulz, 1997). In some studies a stimulatory effect is only seen at nM concentrations of opioid agonist, whereas at higher concentrations an inhibitory effect is seen (Wang and Gintzler 1995; Fields and Sarne 1997). In addition in rat olfactory bulb μ- and δ-opioids
stimulate basal cAMP formation but inhibit stimulated cAMP formation (Olinas and Onali, 1994).

It is possible that the stimulation of cAMP formation may be involved in tolerance and dependence to opioids, Wang and Gintzler (1997) found that in opioid naive tissue, inhibition of cAMP was G₁ linked, but in tolerant tissue, previously inhibitory doses (micromolar) of opioid lead to facilitation of cAMP formation in a CTX sensitive manner. It is at present unclear whether this and other examples of cAMP stimulation may contribute to produce tolerance or are an effect of tolerance.

1.15 Stimulation of phospholipases / Ins(1, 4,5)P₃ formation

In addition to being cellular building materials, phospholipids serve as a reservoir from which cells are able to generate extra and intracellular messengers. Phospholipases are the enzymes responsible for the breakdown of phospholipids, and can be divided into three groups, PLA₂, PLC and PLD. Each form of phospholipase differs in their cleavage site on phospholipids, and therefore the products of this breakdown. PLA₂ breaks down phospholipids to yield fatty acids and lysophospholipids, PLC yields DAG and inositol phosphates whilst PLD produces phosphatidic acid (Prescott, 1997).

PLC breakdown of the membrane phospholipid phosphatidylinositol(4,5)bisphosphate (PIP₂) produces the second messengers Ins(1,4,5)P₃, and DAG. Ins(1,4,5)P₃ is then able to release Ca²⁺ from intracellular stores, whilst DAG activates PKC, or is cleaved to produce arachidonic acid. In addition, a reduction in the amount of PIP₂ may also play a signaling role, since PIP₂ is a cofactor for PLD and a substrate for PI3-kinase (Rhee and Bae, 1997). There are at present 10 isoforms of PLC - β(4), -γ(2) and -δ(2). PLCβ is the most well characterized isoform and is able to be regulated by all subunits of the G₉ class and also by Gβγ subunits (Rhee and Bae, 1997).

Activation of both recombinant and endogenous opioid receptors have been shown to stimulate phosphoinositide turnover, leading to the production of Ins(1,4,5)P₃ in a variety of cell lines. These include SH-SY5Y cells (Smart et al., 1995), via Ca²⁺ channel opening, and in NG108-15 cells (Jin et al., 1994, Smart and Lambert, 1996), Both recombinant μ and δ opioid receptors have also been shown to stimulate PLC
when expressed in CHO cells (Smart et al., 1997, Hirst et al., 1998). Phosphoinositide hydrolysis occurs in spinal cord-DRG neurons (Barg et al., 1993), where opioid stimulation of phosphoinositide hydrolysis has been proposed as a possible mechanism for the opioid induced release of adenosine in dorsal root ganglion neurons (Cahill et al., 1995).

PLA₂ exists as cytosolic, secretory and calcium-independent isoforms (Leslie et al., 1997). One of the products of cytosolic PLA₂ hydrolysis of phospholipids is arachidonic acid, which may be involved in modulation of PKC or PLC. In addition arachidonic acid can be converted via lipoxygenase or cyclooxygenase pathways to form eicosanoids, mediators of the inflammatory pathway (Leslie et al., 1997).

In PAG neurons, it has been shown that activation of PLA₂ can lead to K⁺ channel activation, which brings about a reduction in GABA release, thereby potentiating neurotransmission to the nucleus raphe magnus, which has an inhibitory output on dorsal horn neurons. In this way, activation of PLA₂ may be involved in descending inhibitory control, see section 1.20 (Vaughan et al., 1997). Breakdown of phospholipids in the PAG area produces arachidonic acid, which via a 12-lipoxygenase pathway is converted to 12HPETE (hydroperoxyeicosatetraenoic acid). This may be reduced to 12HETE (hydroxyeicosatetraenoic acid), which activates a voltage and dendrotoxin sensitive K⁺ channel or may be reduced to leukotrienes and hepoxilins (Vaughan et al., 1997). It is possible that 12HPETE and hepoxilins may also modulate K⁺ channel activity. However, leukotrienes are inflammatory mediators, and it is not known what effect these would have on PAG neuron activity, but it is also possible that the precise products and routes of arachidonic acid metabolism differ depending on the cellular environment, and it may be that in the brain a pathway is favoured that has reduced levels of inflammatory mediators as metabolites.

1.16 Release of intracellular Ca²⁺

Ins(1,4,5)P₃ produced from the breakdown of PIP₂ by PLC is able to release Ca²⁺ from intracellular stores on the endoplasmic reticulum. Ins(1,4,5)P₃ receptors exist as three principle types, which differ in their distribution (Patel 1999); Ins(1,4,5)P₃ type 1 is the most ubiquitous and most well characterized. The Ins(1,4,5)P₃ receptor has several structural motifs, a ligand recognition site at the N-terminus, a membrane anchoring
region, a group of regulatory sites and an intrinsic ligand gated ion channel (Furuichi and Mikoshiba, 1995; Patel, 1999). Functional Ins(1,4,5)P3 receptor channels are formed by the association of four Ins(1,4,5)P3 receptor subunits (Wilcox et al., 1998). Due to the presence of regulatory sites, Ca\textsuperscript{2+} release may be modulated by a variety of factors such as calmodulin (Levitan, 1999), Ins(1,4,5)P3 receptor phosphorylation (e.g. Burgrim, 1999) and Ca\textsuperscript{2+} itself (Berridge, 1997a). For example phosphorylation of the Ins(1,4,5)P3 receptor by PKC enhances Ca\textsuperscript{2+} mobilization (Furuichi and Mikoshiba, 1995). Ryanodine receptors, which are structurally similar to Ins(1,4,5)P3 receptors serve as intracellular release channels for Ca\textsuperscript{2+} stores within the sarcoplasmic reticulum. Cyclic adenosine diphosphate (cADP) ribose, synthesized from ATP may be the endogenous trigger for calcium release (Berridge, 1993). In addition a novel Ca\textsuperscript{2+} release mechanism activated by the pyridine nucleotide, nicotinic acid adenine diphosphate (NAADP) has been identified (Genazzani and Galione 1997; Peterson and Cancela, 1999). However, the location of the NAADP sensitive pool is unknown and Ca\textsuperscript{2+} release does not involve Ins(1,4,5)P3 receptors or ryanodine receptors.

Ca\textsuperscript{2+} mobilization from Ins(1,4,5)P3 stores is a highly regulated process, with release being spatially, frequency and amplitude encoded (Berridge, 1997b). The release of Ca\textsuperscript{2+} from intracellular stores is quantal in nature, with Ins(1,4,5)P3 receptors differing in their sensitivity to Ins(1,4,5)P3, and giving rise to elementary events such as bumps, puffs and sparks - names reflecting their spatial temporal properties (see Berridge, 1997a). The pattern of Ca\textsuperscript{2+} release can differ, being seen as a peaks and plateaus, waves, oscillations and spikes depending on the nature of the release.

There is much debate at present as to the nature of refilling of the stores once Ca\textsuperscript{2+} has been released. The capacitive model has been proposed (Putney 1986; Berridge 1995). In this model the emptying of intracellular stores causes entry of extracellular Ca\textsuperscript{2+}, and thus Ca\textsuperscript{2+} entry is regulated by the filling state of the stores. The channel through which extracellular Ca\textsuperscript{2+} enters the cell was identified as I\textsubscript{crac} (calcium release activated calcium) by Hoth and Penner, (1992). However, it is now known that this is not the only store operated Ca\textsuperscript{2+} channel in existence (see Clementi and Meldolesi, 1996). Numerous mechanisms have been proposed as to how the stores ‘signal’ to the Ca\textsuperscript{2+} channel that the store is emptying, such as a calcium influx factor (Randriamampita and
Tsien, 1992), cGMP, small G proteins or direct coupling (Clementi and Meldolesi, 1996) or by a decrease in Ca$^{2+}$ concentration in the subplasmalemmal space (Barritt, 1998). However the capacitive model has been questioned, especially at low agonist concentrations where an alternative pathway involving PLA$_2$/arachidonic acid has been proposed (Shuttleworth, 1999).

Opioid receptor activation has been shown to produce an increase in Ca$^{2+}$ in cells expressing endogenous receptors, such as NG108-15 cells, (Jin et al., 1994; Smart and Lambert, 1996) and SH-SY5Y cells (Connor and Henderson, 1996). In the latter cell line the authors have found that synergism with other agents was necessary to produce an opioid mediated response, whilst others have not noted an increase in Ca$^{2+}$ (Smart et al., 1995). Recombinant opioid receptors have also been shown to cause an increase in [Ca$^{2+}$]$_i$, (Kaneko et al., 1994; Smart et al., 1997). In addition to opioid mobilization of intracellular calcium from Ins(1,4,5)P$_3$ sensitive stores, there has been a report to suggest that the δ-opioid receptor SK-N-BE cells (Allouche et al., 1996) and the κ-opioid receptor in rat myocytes (Tai et al., 1992) can regulate the activity of ryanodine receptors.

1.17 MAP kinase

Mitogen-activated protein kinases are highly conserved serine / threonine protein kinases (Kranenburg et al., 1999). Within this group there are extracellular signal regulated kinases (ERKs, also known as p42/p44MAPK), P38 MAPkinases and C-jun N-terminal kinases / stress activated protein kinases (Gutkind et al., 1998; Zhang et al., 1999a). Activation of the ERK cascade leads to the activation of protein kinases, transcription factors and membrane proteins leading to regulation of cell growth and differentiation (Davis 1993; Zhang et al., 1999). Activation of opioid receptors has been shown to activate MAPK in a variety of cell lines including C6 glioma (Gutstein, et al., 1997; Bohn et al., 2000a), HEK293 (Schmidt et al., 2000), CHO (Fukuda et al., 1996; Li et al., 1996; Li et al., 1999) and COS-7 cells (Ignatova et al., 1999). Activation of MAP kinases by a variety of G protein coupled receptors is dependent upon receptor internalization in a number of cases (Lefkowitz, 1998; Kranenburg, 1999). In the case of opioid receptors, it has been shown that internalization may (Ignatova, 1999) or may not be (Li et al., 1999, Schmidt et al., 2000) required for MAPK activation. Conversely,
MAPK activation has been implicated in, and may be required for receptor internalization (Polakiewicz et al., 1999; Schmidt et al., 2000).

1.18 Divergence of signaling pathways
Why then, if all three subtypes of opioid receptors mediate common signal transduction mechanisms, do there exist subtypes of opioid receptor? Although there is overlap, each type of opioid receptor differs in location and sensitivity to endogenous ligands. On a more molecular level, it is possible that activation of a specific opioid receptor subtype may lead to the activation of different G protein subunits, as summarized in table 1.4.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>G protein coupling (native tissue)</th>
<th>possible (transfected systems)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu )</td>
<td>( G_{11}, G_{12}, G_{13}, G_z )</td>
<td>( G_{11}, G_{15}, G_{16} )</td>
</tr>
<tr>
<td>( \delta )</td>
<td>( G_{11}, G_{12}, G_{13}, G_{12} )</td>
<td>( G_{13}, G_{2}, G_{16} )</td>
</tr>
<tr>
<td>( \kappa )</td>
<td>( G_0, G_{12}, G_{13} )</td>
<td>( G_{2}, G_{16} )</td>
</tr>
</tbody>
</table>

Table 1.4
Opioid receptor coupling to G proteins, adapted from Connor and Christie, 1999; Standifer and Pasternak, 1997).

For example endomorphin-1 has been shown to activate \( G_{11}, G_{13} \) and \( G_z \), whilst endomorphin-2 activates \( G_{11}, G_{13}, G_z \) as well as \( G_{12} \) (Sanchez-Blanzquez et al., 1999). These two \( \mu \)-selective peptides may also differentially regulate adenylyl cyclase isoforms (see Nevo et al., 2000). \( \mu \)-opioid receptors may down-regulate differently, in neuro2A cells, \( \delta \)-opioid receptor down-regulation is independent of G protein coupling whereas the \( \mu \)-opioid receptor requires the formation of a high affinity G protein complex (Chakrabarti et al., 1997). It is possible that actions of one subtype of opioid receptor can oppose the actions of another type of receptor, particularly between \( \kappa \)- and \( \mu \)-opioid receptors. For example, the \( \kappa \)-opioid receptor has been shown to antagonize brain mediated actions of the \( \mu \)-receptor, including analgesia, tolerance and reward (Pan, 1998). In addition in C6 glioma cells, \( \mu \)-opioid agonists inhibit \( \kappa \)-opioid receptor stimulated ERK phosphorylation (Bohn et al., 2000b). There is also evidence for differential regulation of subtypes of opioid receptors, e.g. in mouse pituitary cells the \( \kappa \)-opioid receptor displays homologous desensitization whilst the \( \mu \)-receptor displays heterologous (agonist specific) desensitization. In SH-SY5Y cells, activation of the \( \mu \)-
opioid receptor activates a VOCC, allowing Ca\(^{2+}\) entry to stimulate PLC, but this is not the case with the \(\delta\)-opioid receptor expressed in this cell line (Smart et al., 1995). These examples may highlight subtle differences in signaling pathways between receptor subtypes / ligands or how the activity of one receptor may modulate the actions of another.

1.19 Nociceptin receptors

In 1994 several groups identified a receptor with considerable homology (~60%) to opioid receptors (Bunzow et al., 1994, Wick et al., 1994, Mollereau et al., 1994, Nishi et al., 1994, Fukuda et al., 1994, Wang et al., 1994). Although the homology between the nociceptin receptor and opioid receptors is almost equal to the homology between \(\mu\)-, \(\delta\)- and \(\kappa\)-opioid receptors, it does not bind traditional opioid ligands, and so became known as the orphan receptor. However, the endogenous ligand for this receptor was isolated a year after the discovery of the receptor (Meunier et al., 1995, Reinscheid et al., 1995) and named orphanin F/Q (due to its terminal amino acids) or nociceptin (due to its ability to produce a pro-nociceptive response). Nociceptin / OFQ is a heptadecapeptide (figure 1.5) cleaved from pro-nociceptin and shows similarity to dynorphin-A (Henderson and McKnight, 1997). The 'orphan' receptor has been given numerous names, ORL-1 (opioid receptor like), NCR (nociceptin receptor), OP\(_4\) or is still sometimes referred to as the orphan receptor. At present, the recommended IUPHAR nomenclature for this receptor is ORL-1.

<table>
<thead>
<tr>
<th>Nociceptin</th>
</tr>
</thead>
</table>

**Figure 1.5**

Amino acid structure of nociceptin. **Bold amino acids show identical sequences to classical opioid peptides**

ORL-1 is distributed widely throughout the CNS, in the cortex, hippocampus, hypothalamus, PAG, locus ceruleus and spinal cord (laminae I, II and X), indeed areas where classical opioid receptors are found. An exception to this is the caudate putamen, in which ORL-1 is absent (Darland and Grandy, 1998). On the whole, spinal actions of
nociceptin are to produce analgesia, whist supraspinally nociceptin produces hyperalgesia (reviewed in Calo' et al., 2000). At a cellular level, actions of nociceptin are similar to those of opioids, inhibition of adenylyl cyclase, activation of $K_r$ (Henderson and McKnight, 1997), closure of VOCCs and increases in intracellular $Ca^{2+}$ (Connor et al., 1996) and reduction in neurotransmitter release (e.g. Nicol et al., 1996b). Nociceptin can also produce anxiolytic-like actions, modulation of spontaneous locomotor activity, stimulation of food intake, hypotension and bradycardia and inhibition of gastrointestinal transit, but in contrast to classical opioids, it does not produce rewarding effects (Calo' et al., 2000). There is much debate at present as to whether this receptor is an opioid receptor, and much depends on what criteria are used to classify a receptor (e.g. genetics, pharmacology).

1.20 Pain Pathways

Many phrases have been formulated as to the definition of pain (see Wall and Melzack, 1994), but due to its complexity a strict definition is difficult. In many cases, it may be defined as a 'subjective perception of a nociceptive input'. In most situations pain serves as an important warning signal of actual or potential tissue damage, but in some cases chronic pain is a severely debilitating process. Treatment of pain with opioids, e.g. morphine, fentanyl or pethidine is common in clinical practice.

Nociceptive inputs from nerve endings are carried by Aδ and C fibres. C fibres are non-myelinated, have low conduction velocities (<2.5ms⁻¹) and are predominantly responsible for dull, aching pain. Aδ fibres are myelinated, have faster conduction velocities (2.5-20ms⁻¹) and are predominantly responsible for sharp, pricking pain (Greenstein and Greenstein, 1999; Rang et al., 1999). Afferent nociceptive fibres enter the spinal cord via the dorsal roots and end in the grey matter of the spinal cord, the majority in laminae I and II (substantia gelatinosa) (Rang et al., 1999). Excitatory transmitters such as glutamate, substance P, NKA and CGRP are released from these fibres, and there are many ascending nociceptive tracts projecting from the spinal laminae to the spinal cord, the most prominent being the spinothalamic tract, which originates in laminae I and V-VIII and terminates in the thalamus (Greenstein and Greenstein, 1999).
Many areas of the brain are known to be involved in the control of pain, such as the prefrontal cortex, the somatosensory cortex, hypothalamus, PAG and the nucleus raphe magnus. Indeed, electrical stimulation of the PAG area of the mid brain can cause enough analgesia so that in conscious rats, surgery can be performed without the need for anaesthetic, and this stimulation induced analgesia is reversed by naloxone, suggesting opioid activity in this phenomenon (reviewed in McNally, 1999). One particular pathway from the PAG runs to the nucleus raphe magnus then via the dorsolateral funiculus to dorsal horn interneurons (Rang et al., 1999). The PAG is an area rich in opioid receptors and opioid peptides (Rang et al., 1999). This descending pathway is an important site of action for opioid analgesics and a schematic representation of how opioids are thought to produce analgesia in this pathway is shown in figure 1.6. Enkephalinergic neurons are also found in the pathway from the nucleus raphe magnus which projects to dorsal horn interneurons. Activation of this pathway by opioids ultimately results in a reduction of activity of spinothalamic neurons (see figure 1.6).

Transmission of nociceptive impulses can be modulated at the level of the spinal cord, and this has given rise to the 'gate control theory' (Wall and Melzack, 1994). Simplistically, this proposes that the substantia gelatinosa, which contains mostly short inhibitory interneurons, responds to the activities of both large afferent non-nociceptive fibres and nociceptive fibres entering the cord. In this way it is able to allow transmission from one group of fibres regulate the transmission of others (Greenstein and Greenstein, 1999). An example would be the application of mechanical pressure (e.g. rubbing, Aα fibres) which would decrease the perception of a nociceptive impulse (e.g. pricking with sharp object, Aδ fibres).

As well as being involved in descending inhibitory control, opioids are also able to have a direct action on the dorsal horn of the spinal cord, either by inhibiting the release of excitatory peptides from primary afferent nociceptive neurons (pre-synaptic effect) or by having direct (post-synaptic) actions on dorsal horn post synaptic neurons (McNally, 1999).
Figure 1.6
Schematic representation of the action of opioids on descending inhibitory control. Activation of opioid receptors on PAG neurons leads to activation of PLA$_2$, which via an arachidonic / 12-lipoxygenase pathway activates dendrotoxin-sensitive $K^+$ channels, resulting in hyperpolarization and thus decreased GABA release (Vaughan et al., 1997). This increases neurotransmission to the NRM, which in turn causes increased release of the inhibitory neurotransmitters such as enkephalin and 5HT. This brings about (together with other modulatory inputs, e.g. NA from the locus ceruleus) inhibition of spinothalamic neurons, thus reducing nociception / pain. There are also feedback loops from dorsal horn interneurons to the NRM and the PAG (Greenstein and Greenstein, 1999; Rang et al., 1999). Therefore transmission through the dorsal horn is controlled by the amount of activity reaching the thalamus.
1.21 Non-analgesic and peripheral actions of opioids

In addition to producing analgesia, opioids are also able to produce modulatory effects on a variety of functions. For example opioid receptors are found on most immune cells, and it is known that B and T cells produce and secrete enkephalin (Sanders, 1995). T lymphocytes may also act as vectors, delivering β-endorphin to inflamed tissue and thus allowing highly specific opioid control of peripheral analgesia (Webster, 1998). Indeed this localised release of opioid peptides may mediate analgesia following conditions such as joint injury (Schaible and Grubb, 1993), and it has been demonstrated that inflammation causes an increase in the number of peripheral opioid receptors (Zhou et al., 1998).

With respect to cardiovascular modulation, opioid receptors are found both in brain area involved in cardiovascular regulation, especially the hypothalamic nuclei and also at peripheral sites such as the heart, blood vessels, kidney, sympathetic ganglia and adrenal medulla. Responses to opioid agonists differ according to subtype, dose and can produce both tachycardic and bradycardic responses as well as either hypo- or hypertension (Paakkari and Feuerstein, 1995). Indeed endomorphins produce in vivo cardiovascular effects consistent with opioids such as decreased cardiac output and total peripheral resistance, vasopressor and hypotensive responses (Zadina et al., 1997; Stone et al., 1997; Goldberg et al., 1998, Champion et al., 1997a,b,c; Czapla et al., 1998).

Another action of opioids is effects on gastro-intestinal function. All three types of opioid receptor are found in longitudinal muscle, myenteric plexus, intestinal smooth muscle and gastrointestinal nerves within the enteric nervous system also possess opioid receptors. Opioids may affect gastrointestinal motility, transit secretion and absorption (Burks, 1995). μ-opioids in particular slow gastric emptying (Crighton et al., 1998); this may either be seen as an unwanted side effect (constipation) or a beneficial effect (treatment of diarrhoea).

1.22 Receptor Desensitization (GPCR)

Receptor desensitization may be viewed as a mechanism to reduce or attenuate receptor signaling in response to a repeated or long lasting stimulus. Desensitization may be defined as loss of a functional response and also be accompanied by receptor
sequestration or down-regulation from the cell surface. The majority of preliminary studies on receptor desensitization and down-regulation were performed using the β-adrenoreceptor, but it is known that many members of the G protein coupled receptor family undergo desensitization (Lohse, 1993). Receptors may uncouple from G proteins following prolonged agonist exposure, this is mediated by receptor specific kinases such as GRK and β-arrestin and is often referred to as homologous or agonist specific desensitization. There are at least 6 members of the GRK and β-arrestin family; binding of GRK to an agonist bound receptor increases the affinity of the receptor for β-arrestin (Lefkowitz, 1998). β-arrestin then facilitates uncoupling of receptor from G protein (Lohse, 1993). β-arrestin may also serve as an adapter protein for clathrin (see later). Effector kinases such as PKC and PKA may also be involved in uncoupling of receptor from G protein, by phosphorylating Ser / Thr kinases on the C-terminal tail of the receptor. This type of desensitization may be homologous (agonist -non-specific) since activation PKC or PKA may phosphorylate sites on other receptors and thus desensitize their signaling pathways.

Receptors may also be sequestered / down-regulated from the cell surface, originally this was thought to be the 'slow step ' in the process of desensitization / down-regulation (Lohse, 1993), but it is known that in many systems this process can be rapid and occur within minutes of agonist treatment (Koenig and Edwardson, 1997). Multiple mechanisms may be involved in this process, receptors may be endocytosed in a dynamin (GTPase) dependent manner into clathrin coated pits (Murray et al., 1988), whereby they may be taken into acidic endosomes, then subjected to lysosomalous degradation or recycling back to the cell surface. Receptor phosphorylation and endocytosis may be related, in that phosphorylation of the β-adrenergic receptor causes interaction with β-arrestin, which promotes association of activated receptors with clathrin (Krupnick and Benoviv, 1998). Indeed, this may be the case with certain opioid receptor pathways in that removal of potential phosphorylation sites on the C-terminal tail of the δ-opioid receptor (expressed in CHO cells) prevents endocytosis (Trapaidze et al., 1996). However other studies have shown that phosphorylation of the δ-opioid receptor expressed in HEK293 cells is not required for endocytosis (Murray et al., 1998) and therefore cell type differences may exist.
Receptor endocytosis may not only be seen as a mechanism whereby there is attenuation of a signaling pathway, but also an activation of another pathway. In some cases it has been shown that receptor endocytosis is required for MAPK activation (Lefkowitz, 1998) but this may not always be the case (e.g. Li et al., 1999). The role of receptor sequestration may serve not only as a mechanism of desensitization, but also as a method of receptor recycling, and therefore re-sensitization, allowing the receptor to initiate another response (Koeing and Edwardson, 1999).

1.23 Opioid Tolerance
Tolerance is defined as reduction in the pharmacological effect of a drug following repeated exposure, or the need to increase the dose of the drug in order maintain the same effect. In the case of opioids, this effect may be analgesia, but tolerance also develops to other effects such as respiratory depression. There is much discussion as to the clinical implications of tolerance, and the reader is directed to the following review (Collett, 1998).

Many mechanisms have been implicated in the development of tolerance to opioids, and the following description is by no means exhaustive. Some studies have found that in tolerant tissue, opioid signaling is altered so that it occurs via Gs G proteins (Crain and Shen, 1996, Wang and Gintzler, 1997) and may be mediated by GM1 ganglioside (Crain and Shen, 1998). NMDA receptor antagonists given to opioid tolerant animals will reverse tolerance (e.g. Trujillo and Akil 1994; Pasternak et al., 1995) and nitric oxide may have a function in opioid tolerance (Babey, 1994). In ORL-1 knockout mice there is partial loss of tolerance to opioids (Ueda et al., 1997). It is also probable that receptor desensitization and down-regulation may have a role to play in the development of opioid tolerance (see section 1.22). Up-regulation of cAMP may also be involved in this phenomenon, since tissue from opioid tolerant animals often have increased levels of cAMP (Wang et al., 1996) but it is not known if this is cause or effect, since if opioid receptor coupling is lost, then this may lead to up-regulation of cAMP due to loss of an inhibitory tone. Stimulatory effects of opioids have also been implicated in tolerance (Smart and Lambert, 1996). Inhibition of Ca²⁺/CaM dependent protein kinase in the hippocampus of the rat has been shown to attenuate morphine tolerance and dependence (Fan et al., 1999). Ultimately, tolerance to opioids probably
results in interplay between numerous mechanisms leading to neuronal plasticity and adaptive changes.
1.24 Thesis aims

The primary aim of this thesis is to examine signal transduction pathways activated by recombinant opioid receptors expressed in cultured cell lines, in particular

1. Provide a basic characterization of recombinant opioid receptors expressed in cell lines.

2. To examine the stimulatory effects of opioids, in particular the opioid induced increase in $[\text{Ca}^{2+}]_i$ in CHO cells expressing the recombinant $\delta$-opioid receptor. Since the C-terminus of GPCRs has been implicated in receptor function, to determine the role of the C-terminus of the $\delta$-opioid receptor on this effect.

3. To further probe opioid induced increases in $[\text{Ca}^{2+}]_i$ in single adherent CHO cells expressing recombinant opioid receptors to assess if this correlates with what is seen in cell suspensions.

4. Endomorphin-1 and -2 have been proposed as endogenous ligands at the $\mu$-opioid receptor. To characterize the effect of two novel opioid peptides, endomorphin-1 and endomorphin-2 on recombinant $\mu$-opioid receptors expressed in CHO cells and SH-SY5Y cells in terms of receptor binding, inhibition of cAMP and increases in $[\text{Ca}^{2+}]_i$. If endorphins are endogenous ligands at the $\mu$-opioid receptor, then they should display actions typical of other opioid peptides.

5. To further characterize the cellular actions of endomorphin-1. Receptor desensitization and down-regulation plays a role in receptor responsiveness, and occurs with a range of opioid receptor agonists. To establish if endomorphin-1 causes desensitization and down-regulation of the recombinant receptor expressed in CHO cells.
Chapter 2 Materials and Methods

2.1 Sources of chemicals

Sigma Chemical company, Dorset, UK Probencid, amastatin, bestatin, EDTA, EGTA, Tris-HCl, Tris base, Triton-X100, GTPγS, acrylamide/bis acrylamide, PEI, DPDPE, ATP, carbachol, UPT, fura-2 AM, fentanyl, morphine, BSA, TEMED, brilliant blue, sigma markers, bromophenol blue, ammonium persulfate, lauryl sulfate, tween 20, antifoam A, IgG-FITC conjugate, IgG-alkaline phosphatase conjugate, cAMP, DAMGO, naloxone, DMSO, IBMX, forskolin, captopril, BCIP/NBT tablets, Folin's reagent

SEMAT, Hertfordshire, UK Endomorphin-1, endomorphin-2, spiradoline (U62066)

Fisher chemicals / Fisher scientific, Leicestershire, UK Sodium chloride, Potassium chloride, calcium chloride, magnesium sulfate, glucose, potassium di-hydrogen orthophosphate, di-sodium hydrogen orthophosphate, glycine, glycerol, methanol, propan-2-ol, 13mm coverslips, microscope slides, all plastic-ware, Folin and Ciocalteau's reagent, Optiphase hi safe 3, Optiphase safe

USB (Amersham), Ohio, USA HEPES

Life Technologies, Paisley, UK Hams-F12, minimum essential media, Dulbecco's modified minimum essential media, penicillin / streptomycin, fungizone, fetal calf serum, goat serum, trypsin, L-glutamine, geneticin (G418).

NEN, Hounslow, UK [3H]cAMP, [3H]DPN

Amersham Pharmacia Biotech, Buckinghamshire, UK [3H]-DPN

Calbiochem, Nottingham, UK μ-opioid receptor primary antibody

Peptide Institute, Osaka, Japan phosphoramidon
All other reagents were of the highest purity available.

2.2 Buffer composition

**Krebs-HEPES (mM)**

\[ \begin{align*}
Na^+ & (143.3), K^+ (4.7), Ca^{2+} (2.5), Mg^{2+} (1.2), Cl^- (125.6), H_2PO_4^- (1.2), SO_4^{2-} (1.2), \\
& Glucose (11.7), \\ 
& \text{HEPES (10) pH7.4 with 10M NaOH. BSA (0.5%)}\
\end{align*} \]

BSA (0.5%) was included for studies involving peptides.

**Krebs-HEPES (Ca^{2+} imaging) (mM):**

\[ \begin{align*}
Na^+ & (127.2), K^+ (6.0), Ca^{2+} (2.4), Mg^{2+} (1.2), Cl^- (134.4), H_2PO_4^- (1.2), Glucose (11.1), \\
& \text{HEPES (5.0) and BSA 0.05%, pH7.3 with 1M NaOH. This Krebs-HEPES buffer is slightly different to the composition} \\
& \text{described above, but is routinely used for Ca^{2+} imaging studies.}\
\end{align*} \]

**Tris buffer (radioligand binding studies)**

\[ \begin{align*}
50mM \text{ Tris buffer (BSA 0.5%), pH7.4 with 10M HCl. This buffer was Na^+ free due to the possibility of a Na^+ sensitive site on the} \\
& \text{intracellular region of μ-opioid receptors.}\
\end{align*} \]

**Phosphate buffered saline**

\[ \begin{align*}
Na^+ (143.4) \text{ Cl}^- (139.6) K^+ (4.2) H_2PO_4^- (6.5) HPO_4^{2-} (1.5) \\
\end{align*} \]

**Harvest buffer**

\[ \begin{align*}
50mM \text{ Tris-Cl, 4mM EDTA, pH7.4 with 10M NaOH} \\
\end{align*} \]

**cAMP assay buffer**

\[ \begin{align*}
10mM \text{ HEPES buffered saline (0.09%), 0.05% EDTA, pH7.4 with 10M NaOH} \\
\end{align*} \]

**Loading buffer (electrophoresis)**

\[ \begin{align*}
50mM \text{ Tris-Cl, pH6.8, 100mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol} \\
\end{align*} \]

**Running buffer (electrophoresis)**

\[ \begin{align*}
25mM \text{ Tris base, 250mM glycine, pH 8.3, 0.1% SDS} \\
\end{align*} \]

**Transfer buffer (immuno-blotting)**

\[ \begin{align*}
39mM \text{ glycine, 48mM Tris-base, 0.037% SDS, 20% methanol, pH 8.3} \\
\end{align*} \]

**Blocking solution (immuno-blotting)**

\[ \begin{align*}
5\% \text{ low fat milk in 20mM Tris-HCl, 0.5M NaCl, 0.05% tween 20, 0.01% antifoam A} \\
\end{align*} \]

2.3 Tissue culture

**CHOΔ** (L.A. Devi, New York University Medical School, USA), **CHOμ**, and **CHOκ** (D.K. Grandy, Vollum Institute, Oregon Health Sciences University, USA) opioid receptor transfects

CHO cells were cultured in Hams F12 medium supplemented with fetal calf serum 10%, penicillin 100U/ml, streptomycin 100μg/ml and fungizone 2.5μg ml⁻¹ at 37°C in 5% carbon dioxide humidified air. Stock cultures, which also contained
G418 250μg ml\(^{-1}\) were subcultured twice weekly using trypsin and used when confluent (3-5 days). Experimental cultures were G418 free for at least 3 days.

N18 δ-opioid receptor transfects (L.A. Devi, New York University Medical School, USA) were cultured in Dulbecco's minimal essential medium supplemented with fetal calf serum 10%, L-glutamine 2mM, penicillin 100Uml\(^{-1}\), streptomycin 100μg ml\(^{-1}\) and fungizone 2.5μg ml\(^{-1}\) at 37°C in 5% carbon dioxide humidified air. Stock cultures, which also contained G418 250μg ml\(^{-1}\) were subcultured twice weekly using trypsin and used when confluent (4-6 days). Experimental cultures were G418 free for at least 3 days.

HEK293 µ-opioid receptor transfects (R. Howells, University of New Jersey Medical and Dental School, USA) were cultured in Dulbecco's minimal essential medium supplemented with fetal calf serum 10%, L-glutamine 2mM, penicillin 100Uml\(^{-1}\), streptomycin 100μg ml\(^{-1}\) and fungizone 2.5μg ml\(^{-1}\) at 37°C in 5% carbon dioxide humidified air. Stock cultures, which also contained G418 250μg ml\(^{-1}\) were subcultured twice weekly using trypsin and used when confluent (4-6 days). Experimental cultures were G418 free for at least 3 days.

G418 is a protein synthesis inhibitor which selects for vectors containing genes with aminoglycoside-3'-phosphotransferase which inactivates G418 by phosphorylation. The resistance gene for G418 is present on the plasmid that encodes for the opioid receptor. Therefore any cells that do not have a transfected opioid receptor will lack the G418 resistance gene and will not survive; hence opioid receptor expression is maintained.

SH-SY5Y cells (J. Beadler, Memorial Sloane Kettering Center, NY, USA) were cultured in minimal essential medium supplemented with fetal calf serum 10%, L-glutamine 2mM, penicillin 100U ml\(^{-1}\), streptomycin 100μg ml\(^{-1}\) and fungizone 2.5μg ml\(^{-1}\) at 37°C in 5% carbon dioxide humidified air. Stock cultures were subcultured weekly and used when confluent (5-8 days).

CHO opioid receptor transfects, N18 δ-opioid receptor transfects, HEK293 µ-opioid receptor transfects and SH-SY5Y cells were routinely grown in 80cm\(^2\) or 175cm\(^2\) tissue
culture flasks, fed every 48h with appropriate media and used when confluent. For details of more specific cell culture, the reader is directed to the specific experimental section.

2.4 Cell Harvesting
On reaching confluence (3-8 days depending on cell line), media was removed from the tissue culture flask and cells incubated with HEPES (10mM) buffered saline (0.9%), 0.05% EDTA, pH7.4 for 5min. Following detachment, cells were re-suspended in 25ml of appropriate assay buffer, centrifuged at 440g for 2min at 4°C. This was repeated twice more.

2.5 Membrane preparation
Membranes were prepared by harvesting CHO, N18, HEK293 and SH-SY5Y cells as described above, and homogenizing with an Ultra Turrax for 30 seconds at 13500rpm at 4°C. The membranes were centrifuged at 20370g, 4°C for 10 minutes, washed with Tris buffer and homogenized and centrifuged twice more, as above.

2.6 Desensitization studies and washing procedure
CHOµ cells (grown in 12 well plates) were incubated with 10µM endomorphin-1 and 10µM of each of the peptidase inhibitors amastatin, bestatin, captopril and phosphoramidon (in order to prevent peptide breakdown) for various times in serum free media. Adherent cells were washed 5x at 4°C in Krebs HEPES buffer to remove bound peptide prior to experiments.
For displacement studies, confluent cells were washed 5x with 50mM Tris buffer containing BSA 0.5%, then harvested by scraping. Membranes were prepared as described in section 2.5

2.7 Measurement of cAMP in whole cell suspensions
Cells were harvested and washed as described in section 2.4. Whole cell suspensions (0.3ml) were incubated in the presence of isobutylmethylxanthine (1mM), forskolin (1µM), [with 10µM amastatin, bestatin, captopril and phosphoramidon for experiments using peptides] and opioid agonist (usually 10⁻¹¹-10⁻⁵M) and naloxone (10µM) if appropriate in various combinations for 15 minutes at 37°C. Reactions were terminated
by the addition of 20μl HCl (10M). The pH was equilibrated with the addition of 20μl NaOH (10M) and 200μl Tris-HCl buffer (pH7.4). Following centrifugation (12 000g) for 5min, the concentration of cAMP in the supernatant was measured using a specific protein-binding assay (Brown et al., 1971), with binding protein prepared from bovine adrenal cortex, see sections 2.9 and 2.10

2.8 Measurement of cAMP in adherent cells
Cells used in desensitization studies were grown in 12 well plates, fed every 48h with appropriate media and used when confluent. cAMP desensitization studies were performed on adherent CHO cells in 12 well plates for 5min at 37°C in 0.6ml volumes of Krebs-HEPES buffer containing forskolin (10μM), IBMX (1mM) BSA (0.5%), peptidase inhibitors (10μM) and varying concentrations of E1 (10⁻⁵-10⁻¹⁰M). Reactions were terminated by the addition of 40μl HCl (10M). The pH was equilibrated with the addition of 40μl NaOH (10M) and 300μl Tris-HCl buffer (1M, pH7.4). Following detachment of cells by scraping and centrifugation (12 000g) for 5min, cAMP was assayed as described in section 2.7.

2.9 cAMP assay and principle
The assay utilizes binding protein prepared from bovine adrenal glands with a limited number of binding sites for which both labelled and unlabelled ligands compete in a ratio proportional to their respective concentrations. cAMP dependent protein kinases are probably responsible for binding both labelled and unlabelled cAMP. There is an inverse relationship between the amount of unlabelled and labelled cAMP (radioactivity) present in the samples. As the mass of unlabeled cAMP present in the assay increases, there will be less labelled cAMP bound and therefore less radioactivity. Increasing amounts of unlabelled ligand of known mass form the standards (0, 0.25, 0.5, 1, 2, 4, 6, 8, 10 pmol 50μl⁻¹). This results in a concentration related displacement curve of labelled relative to the unlabelled cAMP. Displacement of radiolabel, produced by the unknown quantity of unlabelled cAMP in the sample being measured, is compared against the standard curve (as shown in figure 2.1) and the concentration of unlabeled cAMP is read from the curve (Riasmart software, Packard, Berkshire, UK).
The assay was carried out at 4°C on ice. 100μl of a low concentration (~0.5nM final) of [³H]cAMP and 150μl (diluted ~ 1/25) of binding protein were added to each standard or sample, then equilibration was allowed to occur for at least 3h at 4°C. Bound and free radioactivity were separated by addition of 250μl of charcoal mixture (250mg charcoal, 100mg BSA per 25ml 50mM Tris-HCl buffer, 4mM EDTA, pH7.4). Each tube was allowed to stand for 1min before centrifugation at 12 000g in a Sarstedt microfuge (Leicester, UK) at 22°C. 350μl of the supernatant was then mixed with 1ml of Optiphase hi-safe 3 liquid scintillation cocktail and radioactivity counted using liquid scintillation spectroscopy.

2.10 Purification of cAMP binding protein.

Bovine adrenal glands were dissected, to obtain adrenal cortex tissue. The adrenal cortex tissue was then homogenized using a Ultra Turrax at 13500rpm, 20x 20 second bursts in 2 volumes of Tris-HCl, 3mM EDTA buffer pH7.4 on ice. The adrenal cortex slurry was separated into 50ml high-speed centrifuge tubes and centrifuged at 20374g for 20 minutes at 4°C. The supernatant was removed and re-centrifuged as above until a clear red-brown solution was obtained. The supernatant contained cAMP binding protein at ~25 fold higher concentration than that required in the assay.
Figure 2.1

A representative (from n>20) cAMP standard curve. Curve fitting and extrapolation of unknown cAMP concentrations was by Riasmart (Packard, Berkshire, UK) software. $R^2 = 0.998$
2.11 Radioligand binding studies - Principle and theory

Two main methods of radioligand binding studies are employed in this thesis. The first of these are saturation studies. In this type of experiment, increasing amounts of radiolabeled ligand (usually high affinity antagonist) are used, up to a concentration at which all the available binding sites for that ligand are occupied. This is termed the $B_{\text{max}}$, or maximum number of binding sites. From this type of experiment the equilibrium dissociation constant, $K_d$ can also be determined.

For a ligand-receptor interaction,

$$
\frac{k_{\text{on}}}{k_{\text{off}}} \\
\text{Ligand + Receptor} \leftrightarrow \text{Ligand-receptor complex}
$$

Equilibrium is reached when new ligand-receptor complexes are formed at equal rates to the dissociation of ligand-receptor complexes;

$$
K_d \text{ equilibrium} = \frac{k_{\text{off}}}{k_{\text{on}}} = \frac{[L][R]}{[LR]}
$$

When the ligand occupies half the receptors, the concentration of unoccupied receptors equals the concentration of occupied receptors: $[R] = [LR]$. This can only be true when $K_d$ equals $[L]$, i.e., the $K_d$ is the concentration of ligand that will bind to half the receptors at equilibrium.

There are three possible ways of representing data from saturation experiments. Two of the most common ones are binding hyperbolae (left panel figure 2.2), and a Scatchard plot (right panel figure 2.2).
Figure 2.2

Diagram showing rectangular binding hyperbolae (left panel) analysis and Scatchard analysis (right panel) of hypothetical binding data.

Binding hyperbolae are useful for displaying the relationship between specific and non-specific binding, and for showing saturation has been achieved. A Scatchard plot transforms data into a linear form where it is easier to view, especially changes in $B_{\text{max}}$ or $K_d$. Throughout this thesis, it was first established that binding data has reached saturation then data was analysed using Scatchard plots (Scatchard, 1949).

The second type of radioligand binding study to be used is displacement experiments. These are useful for determining the affinity of a ligand for a receptor when it is not available as a radiolabel. Instead a fixed low concentration of radiolabel is applied to the tissue and then displaced by increasing amounts of the unlabeled ligand of interest (usually plotted on a log scale). An estimate of the $K_d$ value, the $K_i$ (equilibrium dissociation constant) corrected for the amount of radiolabel used can be obtained using the Cheng and Prusoff equation (Cheng and Prusoff, 1973);

$$K_i = \frac{IC_{50}}{1 + ([\text{ligand}] / K_d)}$$

where $IC_{50} = \text{half maximal inhibition of binding (nM)}$

$K_d = \text{equilibrium dissociation constant (nM)}$

If a higher concentration of radioligand is used, it will take a larger concentration of unlabeled drug to compete for the binding. It takes more unlabeled drug to compete for
a high affinity radioligand than for a low affinity radioligand. The Cheng and Prussoff equation alters the position of the curve to take these factors into account. In saturation and displacement experiments, the non-selective opioid antagonist diprenorphine (DPN) was used as a radiolabeled compound. An antagonist was used since agonists may discriminate between high and low affinity sites, thus complicating results. Indeed it has been shown that DPN binds with equal affinity to high and low affinity sites (Lee et al., 1999).

2.12 Saturation Experiments.

Cells were harvested and washed (as described in section 2.4) and membranes prepared from these cells (as described in section 2.5). Binding of \([^3H]DPN\) to membranes was measured in 1ml assay volumes of Tris buffer (pH 7.4, 10M HCl) containing 7-8 increasing concentrations of \([^3H]DPN\) (~0.02-2.5nM) at 22°C for 60min. Non-specific binding was defined using 10μM naloxone, membranes were added last to initiate the reaction. Bound and free radioactivity were separated by rapid vacuum filtration using a Brandel cell harvester onto Whatman GF/B filters and washed with 3x4ml aliquots of cold Tris buffer. Radioactivity was extracted for at least 6hrs in Optiphase safe scintillant and bound radioactivity measured by liquid scintillation spectroscopy.

2.13 Displacement experiments

Cells were harvested and washed (as described in section 2.4) and membranes prepared from these cells (as described in section 2.5). Displacement studies were performed in 1ml volumes of 50mM Tris, pH7.4 at 20°C for 60min with a fixed concentration of \([^3H]DPN\) (~0.4nM), membranes and increasing concentrations of opioid (usually 10⁻¹¹-10⁻⁵M). Non-specific binding was defined using 10μM naloxone. The peptidase inhibitors amastatin, bestatin, captopril and phosphoramidon (10μM of each) were included to prevent degradation of peptides when used. Bound and free radioactivity were separated by rapid vacuum filtration using a Brandel cell harvester onto Whatman GF/B filters and washed with 3x4ml aliquots of cold Tris buffer. Radioactivity was extracted for at least 6hrs in Optiphase safe scintillant and bound radioactivity measured by liquid scintillation spectroscopy.
2.14 Binding for desensitization studies
Radioligand binding studies were performed at 4°C (to prevent receptor recycling) for 3h on adherent cells in 12-well plates in 1ml volumes of Krebs-HEPES buffer containing a saturating concentration of [3H]-diprenorphine (~2.5nM). Non specific binding was defined using 10μM naloxone. Reactions were terminated by removal of the buffer (and 2x wash) and incubation with 0.4M PCA. Following detachment of cells by scraping, radioactivity was measured as described as in 2.12.

2.15 Measurement of intracellular Ca\(^{2+}\) in whole cell suspensions
Confluent cells were harvested and washed in Krebs HEPES buffer (3x10ml) as described in 2.4. Cell suspensions were incubated with 3μM fura 2 acetoxymethylester (AM) for 30 minutes at 37°C. Fura-2AM is uncharged and enters the cell by the way of the hydrophobic group interacting with membrane lipids. Once inside the cell, non-specific cellular esterases cleave the AM group. The remaining fura 2 is negatively charged and remains inside the cell. Cells were washed and then incubated at 20°C for 20 minutes to allow for de-esterification. Intracellular calcium concentrations were measured at 37°C (in the presence of 10μM amastatin, bestatin, captopril and phosphoramidon for peptide experiments) using a Perkin-Elmer LS50B fluorometer at 340/380nm excitation and 510nm emission, resolution = 1s. Two ml of cell suspension was placed into a quartz cuvette containing a magnetic stirrer and maintained at 37°C. [Ca\(^{2+}\)]\(_i\) was calculated from the 340/380 ratio according to Grynkiewicz et al., (1985), with R\(_{\text{max}}\) (maximum fluorescence) and R\(_{\text{min}}\) (minimum fluorescence) being determined using 0.1% Triton-X100, and 4.5mM EGTA (pH>8) respectively.

\[
[\text{Ca}^{2+}]_i = K_d \{ (R - R_{\text{min}}) / (R_{\text{max}} - R) \} \text{ Sbf}
\]

were K\(_d\) is the dissociation constant of fura-2 for Ca\(^{2+}\) and is 225nM at 37°C

Sbf = 380min / 380max (i.e. ratio of baseline fluorescence at 380nm under Ca\(^{2+}\) free and Ca\(^{2+}\) bound dye conditions)

Figure 2.3 shows the excitation spectra for fura-2 under Ca\(^{2+}\) saturating and Ca\(^{2+}\) free conditions.
2.16 Measurement of $[\text{Ca}^{2+}]_{i}$ in adherent cell populations

When $[\text{Ca}^{2+}]_{i}$ is measured in whole cell suspensions, it is not possible to remove ligands or other agents once they have been added to the cuvette. A perfusion system allows adherent cells to be perfused with ligands then allows their removal from the system. CHO cells were grown on 47mm size 1.5 glass coverslips, fed every 48h and used when confluent. Coverslips were positioned in a custom-made perfusion chamber (figure 2.4) inserted into a Perkin-Elmer LS-50B luminescence spectrophotometer and perfused at a rate of 1.5ml min$^{-1}$ with Krebs-HEPES buffer. Cells were perfused for 10min prior to experiments to allow for de-esterification. Intracellular calcium concentrations were measured at 37°C at 340/380nm excitation and 510nm emission. Cells were perfused with 1µM of opioid agonist for 1 minute.

Figure 2.3

*Excitation spectra for fura-2 free acid under Ca$^{2+}$ saturating and Ca$^{2+}$ free conditions (re-drawn from Hirst, 1997).*
2.17 Measurement of \([\text{Ca}^{2+}]_i\) in single cells (performed at Parke-Davis Neuroscience Research Centre, Cambridge).

Cells for \(\text{Ca}^{2+}\) imaging studies were grown on glass coverslips (22mm diameter) in 6 well plates seeded at a density of 200,000 cells well\(^{-1}\) in media as described in section 2.3 and used when 24-48h old.

Cells on coverslips were washed in Krebs-HEPES buffer (single cell) then incubated with 2\(\mu\)M fura-2 AM for 2hrs at 20\(^\circ\)C. Coverslips were then placed into a perfusion chamber that was positioned onto the stage of a Nikon inverted microscope with a x40 objective and a field of between 10-60 cells chosen for experiments. Coverslips were perfused with Krebs-HEPES buffer for 5min prior to experiments to remove any free fura-2 AM and to allow hydrolysis of intracellular fura-2 AM. Cells were subjected to excitation at 340 and 380nm using a spectral Wizard monochromator (Life Science Resources, Cambridge, UK) with a 700ms exposure time, with emission measured at 510nm. Cells were perfused (at a rate of 1ml per min) at room temperature with 1\(\mu\)M of opioid agonist for 1 minute and imaged using a cooled charge-coupled device camera and Merlin software (Life Science Resources, Cambridge, UK). Background fluorescence, determined at the start of each experiment was subtracted from each wavelength.
2.18 Fluorescence microscopy / confocal microscopy

The antibody used for fluorescence and confocal microscopy, and immuno-blotting was purchased from a commercial supplier (Oncogene Research Products / Calbiochem, Nottingham, UK). It is a rabbit polyclonal antibody raised against amino acids 384-398 of the rat μ-opioid receptor. Antibody specificity was determined by the manufacturer in rat caudate putamen and spinal cord.

For fluorescence / confocal microscope studies, CHO cells were grown to 10-50% confluence on 13mm diameter glass coverslips in 12 well plates. Coverslips were placed onto inverted microtube lids (to create surface tension) on filter paper soaked in phosphate buffered saline (PBS, to create humidity). Cells were washed twice before fixation in 3% formaldehyde. Cells were then washed 3x 10min in PBS and then incubated with primary antibody directed against the rat μ-opioid receptor (diluted 1:100 – 1:300 in 10% goat serum) for 20 h at 4°C. Cells were then washed 3x10min in PBS before incubation with fluorescin isothiocyanate (FITC) conjugated anti-rabbit IgG for 20 h at 4°C. Cells were then washed 3x10min in PBS, excess buffer was removed and coverslips were mounted invert on glass slides using Fluoromount.

For confocal images, cells were viewed under x100 objective on a Leica TLF4D microscope (Milton Keynes, UK) and recorded using Scanware software (Leica, Milton Keynes, UK). For fluorescence images, representative fields of cells were viewed under X40 objective on a Nikon fluorescence microscope and recorded using NIH Image software (Bethesda, USA).

2.19 Protein determination

Protein was determined according to the method of Lowry, et al (1957), which relies on two basic assay principles; 1. Reaction of protein with copper in alkali, 2. Reduction of Folin’s reagent by copper-treated protein.

Protein samples were diluted 1/25 in 0.5ml volumes 0.1M NaOH. A series of BSA stands were made up in 0.1M NaOH at concentrations of 0, 50, 100, 150, 200, 250μg ml⁻¹. Reagents A, B and C (where A = NaHCO₃ in 0.1M NaOH, B = 1% CuSO₄ and C = 2% Na⁺ K⁺ tartrate) in a 100:1:1 ratio were added in 2.5ml volumes to each sample.
and standards of known concentration. Following incubation at 22°C for 10min, 250μl of Folin's reagent (1 in 4 dilution) was added. Tubes were vortexed and incubated at 22°C for 30 min. Absorbance was read at 750nm on a Corning spectrophotometer. A standard curve was generated using linear regression on Graphpad Prizm and unknown concentrations of protein extrapolated from it (figure 2.4)

![Graph](image_url)

**Figure 2.4**

Representative protein assay standard curve (from n>20). Absorbance is read at 750nm and is directly related to protein concentration

### 2.20 Electrophoresis and immunoblotting

SDS poly-acrylamide gel electrophoresis allows separation of proteins according to their molecular mass. SDS is a strong ionic detergent, which binds denatured proteins and causes them to become negatively charged. The amount of SDS bound is usually proportional to the molecular mass of the polypeptide, therefore SDS-polypeptide complexes migrate through polyacrylamide in accordance with their size when an electric current is applied. The system used is composed of two different gel types, a stacking gel and a running gel. The stacking gel allows the formation of a moving boundary between Cl− ions and glycine molecules, which allows the samples to be
deposited on the surface of the running gel. The higher pH of the running gel causes ionization of glycine and frees the samples to move through the gel according to their size. The immobilized proteins are then blotted onto a nitrocellulose filter before detection with an antibody. By running a series of pre-determined molecular mass markers it is possible to determine the size of the protein of interest.

**SDS poly-acrylamide gel electrophoresis**

8% running gel 4.6ml H₂O, 2.7 ml 30% acrylamide / bis acrylamide in 29:1 ratio, 2.5 ml 1.5mM tris base, pH8.8, 100μl 10% SDS, 100μl 10% ammonium persulfate, 6μl TEMED

5% stacking gel 1.4ml H₂O, 333 μl 30% acrylamide / bis acrylamide in 29:1 ratio, 250μl 1mM tris base, pH6.8, 20μl 10% SDS, 20μl 10% ammonium persulfate, 2μl TEMED

CHOμ membranes were prepared as described in section 2.5. Approximately 60μg protein per 10μl (as determined by Lowry protein assay, section 2.19), and (prestained) molecular markers were denatured (therefore able to bind SDS) by heating to 100°C for 3min with 5μl of loading buffer. Samples were resolved on SDS-PAGE gels, composed of an 8% running gel with a 5% stacking gel at 22°C, 175-200V for 45min in running buffer.

**Transfer to nitrocellulose**

Gels were then placed onto nitrocellulose paper between 6 sheets of filter paper. Separated proteins were transferred to nitrocellulose using a semi-dry blotter, 60mA for 2.5h at 22°C in transfer buffer. Transferred proteins on nitrocellulose paper were blocked overnight in blocking solution at 4°C. Protein markers were either visualized by staining with Brilliant Blue (0.25g in 45% H₂O, 45% methanol, 10% glacial acetic acid) at 4°C overnight followed by destaining in 45% H₂O, 45% methanol, 10% glacial acetic acid for ~3h at 4°C, or prestained markers were used.

**Detection**

Nitrocellulose filters were incubated with primary antibody directed against the rat μ-opioid receptor diluted 1 in 2000 in blocking solution for 2h at 22°C. Filters were
washed 3x10 min in 20mM Tris-HCl, 0.5M NaCl before incubation with goat anti-rabbit IgG alkaline phosphatase conjugate, diluted 1 in 10 000 in blocking for 2h 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. Optical densities of immuno-blotted bands were assessed using Image Master system (Amersham Pharmacia Biotech, Bucks. UK).

2.21 Data analysis.
Details of data representation (e.g. mean±SEM or representative data) and n values are to be found in the figure legend to each graph. Details of statistical analysis are also to be found in specific figure legends.

All statistical analysis, curve fitting and linear regression was performed on GraphPad Prism, version 2.0 (GraphPad Software Inc., San Diego, USA)
Saturation hyperbolae were analysed using nonlinear regression (fit), one site binding (hyperbolae). Scatchard transformation of specific binding data was made using linear regression. pK_i values were obtained from displacement curves values were determined using non-linear regression, by modeling the points to a one site fit and corrected using the Cheng-Prussoff equation as described in section 2.12. pEC_50, E_max and slope factors pEC_50 / pIC_50 values were obtained by fitting a sigmoidal curve with a variable slope

pK_i is the negative log concentration of competitor that competes for half the specific binding corrected for the amount of radiolabeled ligand used. E_max is the maximal response produced (expressed as a percentage), pEC_50 is the negative log concentration of agonist producing half-maximal response, and pIC_50 is the negative log concentration of antagonist producing half-maximal response.
Chapter 1. Basic Characterization of cells transfected with recombinant opioid receptors

3.1 Introduction

Prior to the cloning of the opioid receptor family it was known that this class of receptors coupled to adenylyl cyclase, voltage-operated Ca\(^{2+}\) channels, K\(^+\) channels and phosphoinositide turnover via G\(_{i/o}\) G proteins (Piros et al., 1996). Since the cloning of the opioid receptor family in the early 1990's (Kieffer et al., 1992; Evans et al., 1992; Thompson et al., 1993; Yasuda et al., 1993), major advances have been made in the field of opioid receptor pharmacology. The ability to express opioid receptor DNA in cell lines devoid of endogenous opioid receptor allows an homogenous receptor population to be studied. Differing levels of receptor expression can be studied, and mutated and chimeric receptors can be transfected into cell lines. Recombinant \(\mu\), \(\delta\), and \(\kappa\) receptors have been shown to have characteristics consistent with endogenous opioid receptors when expressed in COS-7, CHO and PC-12 cells respectively (Raynor et al., 1993) and this has since been confirmed by many investigators.

Numerous cell lines have been used to express recombinant opioid receptors, including both neuronal and non-neuronal systems. In this thesis, Chinese hamster ovary (CHO), N18 and human embryonic kidney (HEK) cells are used, Table 1.1 In addition SH-SY5Y cells, which express endogenous opioid receptors (Table 1.1) are also studied.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Morphology</th>
<th>receptor expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>non-neuronal - epithelioid</td>
<td>transfected (\mu), (\delta), (\kappa)</td>
</tr>
<tr>
<td>HEK293</td>
<td>non-neuronal - epithelioid</td>
<td>transfected (\mu)</td>
</tr>
<tr>
<td>N18</td>
<td>neuronal - rat x mouse hybrid glioma</td>
<td>transfected (\delta)</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>neuronal - human neuroblastoma</td>
<td>endogenous (\mu), (\delta^*)</td>
</tr>
</tbody>
</table>

Table 1.1

Summary of characteristics of cell lines used in this thesis

*SH-SY5Y cells express predominately \(\mu\)- (~80%), and \(\delta\)-opioid receptors (Hirst, 1997), possibly also \(\kappa\)-opioid receptors (Cheng et al., 1995)
Opioid (and indeed other) receptor pharmacology may be studied using many different parameters, from studying end-organ responses following application of a drug to an organ bath though to molecular biology techniques utilizing phosphorylation-state specific antibodies. When using cell lines expressing opioid receptors, information is needed about the size, location, number and binding affinity of cell surface receptors, as well data regarding functional consequences of receptor activation. One of the easiest ways to ascertain information about receptor number and ligand binding affinity is to perform radioligand binding saturation experiments, which yields information about maximum number of binding sites ($B_{\text{max}}$) and equilibrium dissociation constant ($K_d$). Measurement of the second messenger, cAMP, provides an indication of opioid receptor activation. SDS-polyacrylamide gel electrophoresis separates protein according to their molecular weight. Following blotting onto nitrocellulose and labeling with specific antibody, this can be compared to markers of known molecular mass and the mass of the protein of interest determined. Cellular receptors may be viewed using fluorescence or confocal microscopy following incubation with a primary antibody directed against a specific receptor and subsequent incubation with a fluorescent secondary antibody.

### 3.2 Aims

The aims of this chapter are to provide a basic characterization of CHOδ, CHOμ, N18δ and HEK293μ cell lines. $B_{\text{max}}$ and $K_d$ values will be determined and compared to those already determined for other cell lines used in this thesis. Coupling of opioid receptors in N18 and HEK293 cells to cAMP formation will be examined and again compared to what is already known for other cell lines which are to be used in later chapters. Since the μ-opioid receptor expressed in CHO cells is used extensively in later chapters, immunoblotting and confocal / fluorescence microscopy studies on this cell line will be performed. This work will provide important preliminary information about the cell lines that are to be used in this thesis and necessary information (such as receptor number) which may aid the interpretation of subsequent work which is to be carried out.
3.3 Materials and methods

For details of chemicals, tissue culture, membrane preparation, radioligand binding protocols, fluorescence and confocal microscopy and immunoblotting the reader is directed to chapter 2. Details of data analysis are also found in this chapter.

3.4 Results [\textsuperscript{3}H]DPN binding

[\textsuperscript{3}H]DPN produced concentration-dependent and saturable binding in membranes prepared from CHO\textdelta WT, CHO\textdelta 37, CHO\mu, HEK293\mu and N18\delta cells (figures 3.1 and 3.2). The relationship between total, NSB and specific binding can be seen in these graphs and the NSB varies from ~15% of total for HEK293\mu to ~75% for N18\delta cells. B\textsubscript{max} and K\textsubscript{d} values from these are shown in table 3.1. For completion B\textsubscript{max} and K\textsubscript{d} values obtained from CHO\kappa and SH-SY5Y membranes are also shown in this table (Hirst, 1997)

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Receptor species</th>
<th>B\textsubscript{max} (fmol mg protein\textsuperscript{-1})</th>
<th>K\textsubscript{d} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO\textdelta WT</td>
<td>mouse</td>
<td>225.6±9.2</td>
<td>0.39±0.03</td>
</tr>
<tr>
<td>CHO\textdelta 37</td>
<td>mouse</td>
<td>85.8±12.0</td>
<td>0.23±0.03</td>
</tr>
<tr>
<td>CHO\kappa</td>
<td>rat</td>
<td>231.0±24.0</td>
<td>0.15±0.01</td>
</tr>
<tr>
<td>CHO\mu</td>
<td>rat</td>
<td>754.7±61.3</td>
<td>0.17±0.01</td>
</tr>
<tr>
<td>N18\delta</td>
<td>mouse</td>
<td>52.5±3.5</td>
<td>0.42±0.06</td>
</tr>
<tr>
<td>HEK293\mu</td>
<td>human</td>
<td>613.0±155.7</td>
<td>0.32±0.03</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>human</td>
<td>98.4±6.5</td>
<td>0.16±0.02</td>
</tr>
</tbody>
</table>

Table 3.1

B\textsubscript{max} and K\textsubscript{d} values for different cell lines expressing opioid receptors. Data are mean±SEM for n=6-12. Experiments were performed at room temperature in 1ml volumes of Tris buffer for 60 min with 7-8 increasing concentrations (~0.02-0.5nM) of [\textsuperscript{3}H]DPN. Non-specific binding was defined in the presence of 10\mu M naloxone. Values in bold type were taken from Hirst, (1997).
Continued overleaf
Figure 3.1
Hyperbola representation of total, NSB and specific binding data for CHOSWT, CHOS37, CHOP, N188 and HEK293μ cells. Data are representative of n=6-12. Experiments were performed at room temperature in 1ml volumes of Tris buffer for 60 min with 7-8 increasing concentrations (~0.02-0.5nM) of [3H]DPN. Non-specific binding was defined in the presence of 10μM naloxone.
3.5 Opioid receptor mediated inhibition of cAMP formation

It has previously been reported that CHOδWT, CHOδ37, CHOδ, CHOμ and SH-SY5Y cells couple negatively to adenylyl cyclase to cause an inhibition of cAMP formation (table 3.2; Hirst, 1997). In HEK293μ cells fentanyl produced a concentration dependent inhibition of cAMP formation (figure 3.6, table 3.2) with pEC_{50} and E_{max} values of 8.22±0.15 (6.03nM) and 77.64±2.98% respectively. In N18δ cells DPDPE also produced a concentration dependent inhibition of cAMP formation (figure 3.7, table 3.2) with pEC_{50} and E_{max} values of 7.74±0.42 (18.20nM) and 31.5±3.3% respectively.
Figure 3.3
Fentanyl produces a concentration dependent inhibition of forskolin stimulated cAMP formation in HEK293 cells. Data are mean±SEM for n=5. Cyclic AMP was measured by a specific protein binding assay.

Figure 3.4
DPDPE produces a concentration dependent inhibition of forskolin stimulated cAMP formation in N18δ cells. Data are mean±SEM for n=5. Cyclic AMP was measured by a specific protein binding assay.
3.6 Immunoblot analysis of the \(\mu\)-opioid receptor expressed in CHO cells

Immunoblot analysis of the \(\mu\)-opioid receptor expressed in CHO cells revealed a band of between 66 and 84KDa (figure 3.8). Figure 3.9 shows relative distance travelled by proteins within the gel relative to their molecular mass.

Figure 3.8

Immunoblot analysis of the \(\mu\)-opioid receptor, figure shows two separate lanes (1 and 2) and is representative of 4 individual experiments performed in triplicate. Proteins were resolved on SDS-polyacrylamide gel, transferred to nitrocellulose and incubated with primary antibody directed against the \(\mu\)-opioid receptor. Bands were revealed by alkaline phosphatase conjugated secondary antibody.

Figure 3.9

Representative graph of molecular mass of protein markers against relative distance travelled from the gel front. Data is representative from \(n=4\).
3.7 Fluorescence / confocal microscope studies of the \( \mu \)-opioid receptor expressed in CHO cells

Fluorescence and confocal microscopy revealed that when CHO\( \mu \) cells were incubated with primary antibody against the \( \mu \)-opioid receptor and a secondary FITC conjugated antibody, fluorescence was observed (figures 3.10a and 3.11a,b,c). All cells labelled with primary antibody fluoresced and the fluorescence was homogeneous. Fluorescence was not present in cells that had not been incubated with primary antibody (figures 3.10b and 3.11d)

![Figure 3.10](image)

**Figure 3.10**

Representative (from \( n = 7 \)) images of CHO\( \mu \) cells incubated with primary antibody against the \( \mu \)-opioid receptor and secondary FITC conjugated antibody (a) and CHO\( \mu \) cell incubated with secondary antibody only (b). Cells were viewed under x40 objective of a Nikon microscope.
Figure 3.11

Representative (from n=5) confocal images of CHO\(\mu\) cells incubated with primary antibody (a,b,c) against the \(\mu\)-opioid receptor and a secondary FITC conjugated antibody or secondary antibody only (d). Cells were viewed under x100 objective on a Leica microscope with Scanware software.

Scale: \[\text{---} = 10\mu\text{M}\]
3.10 Discussion

The data presented in this chapter show that $[^3\text{H}]$DPN produces a concentration dependent and saturable binding to opioid receptors expressed in a range of cell lines. There is considerable variation between the levels of receptor expression in these cell lines, for example CHOr cells express approximately 15 fold more receptors than N18δ cells. Due to these differences in receptor number, care should be taken when comparing functional responses (e.g. cAMP inhibition) in different cell lines since differences in receptor number may affect this response. If a cell line expresses a large number of receptors then it is possible that a receptor reserve exists that may not be present in endogenous tissues. This is most clearly demonstrated when in endogenous systems, a ligand may act as a partial agonist but in a recombinant system may display full agonist properties due to high levels of receptor expression (Berger et al., 2000). Knowledge of $B_{\text{max}}$ values will allow this information to be taken into account. However, it must also be appreciated that receptor-G protein stoichiometry may affect signaling pathways (Kenakin et al., 1994) and this may be altered in transfected cell lines.

The non-selective opioid antagonist $[^3\text{H}]$DPN binds with high affinity to cloned and endogenous opioid receptors. In the CHOδ cell line, removal of the final 37 C-terminal amino acids increases the affinity of $[^3\text{H}]$DPN. The reasons behind this are at present unclear, but are in agreement with Hirst et al., (1998). It may be that removal of this proportion of the receptor somehow alters the ligand-binding domain of the receptor. This is in contrast to two other studies examining the effect of C-terminal deletion of the $\mu$-opioid receptor (Capeyrou et al., 1997) and the $\delta$-opioid receptor (Zhu et al., 1997) found that truncation of the receptor did not affect $[^3\text{H}]$DPN binding.

It should be the case that the equilibrium dissociation constant for $[^3\text{H}]$DPN should not alter between different cell types. This is indeed true for the mouse $\delta$- receptor when expressed in CHO ($K_d = 0.39\text{nM}$) and N18 ($K_d = 0.42$) cells. However, there is a difference between $K_d$ values for the $\mu$-receptor in CHO and HEK293 cells, but this could be explained between species differences; rat and human $\mu$-opioid receptors display 95% homology with each other but have been shown to have differing affinities for ligands (Raynor et al., 1995). Since SH-SY5Y cells express a combination of human
opioid receptor subtypes it is difficult to compare $K_d$ values from this cell line to others displaying only one type of receptor. The $K_d$ values for $[^3H]DPN$ binding to recombinant opioid receptors presented in this chapter are in agreement with other studies (e.g. mouse $\delta = 0.3nM$; Cvejic et al., 1996, rat $\kappa = 0.26nM$, rat $\mu = 0.35nM$; Xue et al., 1995).

It is well established that opioid receptors couple negatively to adenylyl cyclase to cause an inhibition of cAMP formation (Childers, 1991). In HEK293$\mu$ cells, fentanyl inhibited cAMP formation with an $EC_{50}$ of 6.03nM and an $E_{max}$ value of 77%. In N18$\delta$ cells DPDPE inhibition of cAMP formation had an $EC_{50}$ value of 18.6nM and $E_{max}$ value of 31%. These differences in $E_{max}$ values may reflect differing levels of receptor expression, since it has been shown that coupling of the $\kappa$-opioid receptor to cAMP is dependent on receptor expression (Hirst et al., 1997). However, in contrast to this the coupling of the $\mu$-opioid receptor expressed in CHO cells is independent of receptor expression (Smart et al., 1997). The data presented in these two cell lines are consistent with previous data in recombinant cell lines that activation of opioid receptors in transfected cell lines inhibit cAMP formation with $EC_{50}$ values in the nM range (see table 3.2).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CHO$\delta$WT</th>
<th>CHO$\delta$37</th>
<th>CHO$\kappa$</th>
<th>CHO$\mu$</th>
<th>SH-SY5Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>p$EC_{50}$</td>
<td>8.72</td>
<td>8.53</td>
<td>8.51</td>
<td>7.94</td>
<td>7.75</td>
</tr>
</tbody>
</table>

*Table 3.2*

$pEC_{50}$ values for opioid receptor mediated cAMP inhibition in other cell lines used in this thesis, taken from Hirst, (1997).

Immunoblot analysis of the $\mu$-opioid receptor expressed in CHO cells revealed a band of between 84 and 66Kda. This is in agreement with the $\mu$-opioid receptor in rat brain having a size of 60-70Kda (Eppler et al., 1993).

Fluorescence and confocal microscopy revealed that when CHO$\mu$ cells were incubated with primary antibody against the $\mu$-opioid receptor and a secondary FITC conjugated antibody, fluorescence was observed. Since this was not present in cells that had not
been incubated with primary antibody, fluorescence could be attributed to μ-opioid receptor sites (antibody specificity was determined by the manufacturer in rat caudate putamen and dorsal horn of the spinal cord). From figure 3.10 it can be seen that cells appear to have dense perinuclear staining. This could be due to cells having an ovoid shape, with the nucleus being in the middle portion with the largest diameter. When they are viewed between a coverslip and a slide they become squashed so that the area around the nucleus appears more fluorescent due to a greater proportion of cellular material in this area. Perinuclear staining is also visible in confocal images (figure 3.11, especially b), however membrane staining is also visible in this figure (especially 3.11c). For both fluorescent and confocal images it is important to note that all cells incubated with both antibodies showed fluorescence, indicative that all cells expressed receptors.

In this chapter, data has been gained about the number of cell surface opioid receptors \( B_{\text{max}} \) on various cell lines, and it has been shown that they bind the opioid antagonist \( [\text{H}]\text{DPN} \) with high affinity. Two previously un-characterized cell lines, HEK293μ and N18δ both couple negatively to adenylyl cyclase. In addition, rat μ-opioid receptors are visible by fluorescent techniques, and the size of this receptor is between 66 and 84Kda.
Chapter 4. The Effect of C-Terminal Truncation of the Recombinant δ-Opioid Receptor on Ca\(^{2+}\) Signaling.

4.1 Introduction

The cloning of G protein coupled receptors has allowed the assimilation of a wide range of structural and functional information. As described in chapter 1, section 1.4, G protein coupled receptors consist of seven membrane-spanning domains, an extracellular N terminus and an intracellular C-terminus. Present on the C-terminus of opioid and other G protein coupled receptors are repeated serine and threonine residues which may act as potential phosphorylation sites for cellular protein kinases (Trapaidze et al., 1996). It is hypothesized that the C-terminal tail of G protein coupled receptors acts as a 'docking' site for the interaction with G protein subunits (Dohlman et al., 1991). Therefore it is possible that phosphorylation of these residues may affect receptor – G protein coupling. Theoretically, any second messenger that couples via a G protein to a receptor may be affected by phosphorylation / removal of these sites. In the case of opioid receptors this could be cAMP, Ins(1,4,5)P\(_3\), Ca\(^{2+}\) channels, or potassium channels (Joarden and Devi, 1998). One possible mechanism of receptor desensitization is thought to be uncoupling of receptors from G proteins, and indeed it has been shown that these sites may be important in receptor regulation and desensitization (Trapaidze et al., 1996; Cvejic et al., 1996; Capeyrou et al., 1997; Pak et al., 1997).

Following the cloning of the opioid receptor family, it has become possible to generate mutations within a particular opioid receptor before transfection into a cell line. Studies utilizing mutated receptors provide information on opioid receptor structure – function relationships, and since it is hypothesized that the C-terminal tail of the opioid receptor plays an important role in receptor – G protein coupling, a receptor with a C-terminal tail truncation would be ideal to answer this question. A previous study has reported that truncation of the C-terminus of the δ-opioid receptor expressed in 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 Ins(1,4,5)P\(_3\) formation increased with C-terminal truncation and the response rapidly desensitized. Since Ins(1,4,5)P\(_3\) is the second messenger responsible for the release of stored intracellular Ca\(^{2+}\), differences may also
exist between cells expressing wild type and truncated receptors in the subsequent release of intracellular calcium.

4.2 Aims
The aims of this chapter are to examine the effect of truncation of the final 37 amino acids of the C-terminal tail of the recombinant δ-opioid receptor (CHOδ37) on the coupling of this receptor to [Ca\(^{2+}\)]\(_i\) compared to the full length receptor (CHOδWT). In addition to this, a detailed examination of the nature of the Ca\(^{2+}\) signal in CHOδWT will be made.

4.3 Methods
For details of chemicals, tissue culture, membrane preparation, measurement of cAMP and intracellular calcium, the reader is directed to chapter 2. Details of data analysis is also found in this chapter.

4.4 Results - Radioligand binding
The binding of \([^{3}H]DPN\) to membranes prepared from CHOδWT and CHOδ37 was concentration-dependent and saturable, \(K_d\) and \(B_{max}\) values are shown in Table 3.1 of chapter 3.

4.5 Increases in intracellular calcium
\([Ca^{2+}]_i\) was measured in a large number (n>50) of cultures of CHOδWT. It was found that there was considerable differences between batches (i.e. day to day between flasks) in the absolute change in \([Ca^{2+}]_i\) in response to DPDPE (e.g. 43 to 550 nM, 1 \(\mu\)M DPDPE, CHOδWT). However, within one batch (i.e. one subculture) the response was less variable (e.g. 52 to 78 nM, 1 \(\mu\)M DPDPE, CHOδWT). There was no loss of response with increasing passage number.

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±1.3s after DPDPE addition in CHOδWT and 23.9±1.2s in CHOδ37 (figure 4.1). The κ-opioid receptor selective agonist, spiradoline (1 \(\mu\)M CHOδWT, 30 \(\mu\)M CHOδ37) and the μ-
opioid receptor selective agonist [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin (DAMGO), (1 μM CHOδWT, 30 μM CHOδ37) had no effect on [Ca²⁺]i (data not shown).

![Graph showing time-dependent increase in [Ca²⁺]i](image)

**Figure 4.1**

DPDPE produced a time dependent increase in [Ca²⁺]i in CHOδWT and CHOδ37. The data shown is representative of n≥10. [Ca²⁺]i was measured in fura-2 loaded whole cell suspensions.

DPDPE produced a concentration-dependent increase in [Ca²⁺]i in CHOδWT and CHOδ37 cells (Fig. 4.2) with pEC₅₀ values of 8.43±0.13 (3.68 nM) and 6.08±0.25 (0.831 μM) respectively.
Figure 4.2

DPDPE produced a concentration-dependent increase in $[\text{Ca}^{2+}]_i$ in CHOδWT and CHOδ37 cells. Data are mean±SEM for $n=6$. Whole curves are significantly different from each other ($P<0.05$) by 2-way ANOVA. $[\text{Ca}^{2+}]_i$ was measured in fura-2 loaded whole cell suspensions.

4.6 Effect of probenecid on fura-2 leakage and response to agonists

CHO cells have been shown to express low levels of the multidrug resistance efflux pump, P-glycoprotein (Brezden et al., 1994). During the course of the work presented in this chapter it was noted that the CHOδ cells used appeared to leak fura-2. This is an effect that has previously been reported for CHO cells loaded with fura-2 (Edelman et al., 1994) and has no reflection on the health of the cell. It is possible that the P-glycoprotein pump is responsible for this efflux. Probenecid is an organic anion transport inhibitor, originally developed to prevent excretion of penicillin from the kidney. Probenecid, at a concentration of 2.5mM was used in Krebs-HEPES buffer in a series of experiments. Figure 4.3 shows the results of these experiments, ATP or
DPDPE was used as an agonist, the former was used since it gave a larger increase in 
$[\text{Ca}^{2+}]_i$ than DPDPE.

Figure 4.3 Representative time course (from n=3) showing effects of 2.5mM probenecid in CHOδ cells. Probenecid reduced fura-2 leakage in un-stimulated cells (d) when compared to un-stimulated control (c) and reduced the peak and plateau phases of 100μM ATP stimulated (b) and stimulated control (a). $[\text{Ca}^{2+}]_i$ was measured in fura-2 loaded whole cell suspensions.

When CHOδ cells were challenged with 10μM DPDPE, there was an increase in $[\text{Ca}^{2+}]_i$ in the absence of probenecid of 242nM, whereas in the presence of probenecid it was reduced to 41nM (n=1). Due to the inhibitory effect of probenecid on both ATP and DPDPE response in CHOδWT cells it was decided not to use probenecid for further experiments in this chapter or throughout this thesis. In addition it was decided not to conduct experiments at 22°C since other studies (cAMP and Ins(1,4,5)P₃)
measurements) using CHOδWT and CHOδ37 were conducted at 37°C and this would make comparisons between these and results gained in this chapter difficult.

4.7 Effect of naloxone, thapsigargin, pertussis toxin and extracellular Ca\(^{2+}\) on the δ-opioid receptor mediated increases in [Ca\(^{2+}\)]\(_j\)

Thapsigargin is an agent that causes release of Ca\(^{2+}\) from intracellular stores directly without the generation of any known second messengers. In both cell types the response to DPDPE was both naloxone reversible and could be blocked by naloxone (Fig. 4.4a and 4.5b) and thapsigargin sensitive (Fig. 4.4b). The mean increase in [Ca\(^{2+}\)]\(_j\) elicited by thapsigargin was 324±122 nM and 233±24 nM in CHOδWT and CHOδ37 respectively (n=5). 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\(^{2+}\)]\(_j\) in both CHOδWT and CHOδ37 cells (Fig. 4.4c Table 4.1).

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. 4.5a). 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\(^{2+}\)]\(_j\) 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 4.5c).
Figure 1A

a

[Ca^{2+}]_i (nM)

Time (s)

naloxone

control

naloxone

DPDPE

b

[Ca^{2+}]_i (nM)

Time (s)

Thapsigargin

control

thapsigargin

DPDPE

c

[Ca^{2+}]_i (nM)

Time (s)

Control

PTX

DPDPE

Legend overleaf
Figure 4.4

All specimen traces are representative of CHOΔWT, similar data was obtained for CHOΔ37. a). Specimen trace (from n=5) for CHOΔWT depicting naloxone reversibility of the DPDPE induced increase in [Ca^{2+}]_i. 1μM DPDPE was added at 60s. Naloxone (10μM) was added at peak response. b). Specimen trace (from n=5) depicting thapsigargin (100nM) sensitivity of the DPDPE (1μM) induced increase in [Ca^{2+}]_i. c). Specimen trace (from n=3) for CHOΔWT depicting pertussis toxin (PTX 100ng ml^{-1}, 24 h) sensitivity of the DPDPE (1μM) induced increase in [Ca^{2+}]_i. [Ca^{2+}]_i was measured in fura-2 loaded whole cell suspensions for each series of experiments.
a

\[ [\text{Ca}^{2+}]_i \text{ (nM)} \]

\[ \begin{align*}
\text{DPDPE} & \\
0 & 50 & 100 & 150 & 200 & 250 & \text{Time (s)}
\end{align*} \]

b

\[ [\text{Ca}^{2+}]_i \text{ (nM)} \]

\[ \begin{align*}
\text{control} & \quad \text{naloxone pre-treated for 5min} \\
0 & 50 & 100 & 150 & 200 & \text{Time (s)}
\end{align*} \]

c

\[ [\text{Ca}^{2+}]_i \text{ (nM)} \]

\[ \begin{align*}
\text{DPDPE stimulation} & \quad \text{no DPDPE stimulation} \\
0 & 100 & 200 & 300 & 400 & \text{Time (s)}
\end{align*} \]

Legend overleaf
Figure 4.5

a). Specimen trace (from n≥5 others) showing the effect of extracellular Ca²⁺ on the DPDPE response for CHOδWT, 1μM DPDPE added at 60s, similar data was obtained for CHOδ37. b). Specimen trace (from n≥5 others) illustrating naloxone (10μM) sensitivity of the DPDPE induced increase in [Ca²⁺]ᵢ in CHOδWT. Experiments were conducted in the absence of extracellular Ca²⁺. c). Specimen trace from n≥5 others showing that Ca²⁺ re-addition produces a Mn²⁺ quenchable increase in [Ca²⁺]ᵢ. 2.5mM Ca²⁺ was added at 250 sec and 1mM Mn²⁺ was added at 325s Trace shows Ca²⁺ increase with and without DPDPE stimulation. [Ca²⁺]ᵢ was measured in fura-2 loaded whole cell suspensions for each series of experiments.

<table>
<thead>
<tr>
<th></th>
<th>CHOδWT</th>
<th>CHOδ37</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PTX</td>
</tr>
<tr>
<td>Basal [Ca²⁺]ᵢ (nM)</td>
<td>51±8</td>
<td>54±5</td>
</tr>
<tr>
<td>Peak [Ca²⁺]ᵢ (nM)</td>
<td>425±57</td>
<td>67±11*</td>
</tr>
</tbody>
</table>

Table 4.1

Effect of pertussis toxin (100ng ml⁻¹, 24hrs) on basal and peak [Ca²⁺]ᵢ in CHOδWT and CHOδ37 cells. Data are mean±SEM (n=3). *Pertussis toxin significantly decreased peak [Ca²⁺]ᵢ in both CHOδWT and CHOδ37 compared to control (P<0.05 by unpaired Student's t-test. Experiments were performed in whole cell suspensions.
Further evidence that the release of Ca\textsuperscript{2+} from CHO cells may be monophasic came from experiments conducted in a perfusion chamber, where DPDPE was perfused onto the cells for 1 min. There was then a 10 min perfusion with Krebs-HEPES buffer before further challenges with DPDPE (fig 4.7). It can be seen that the increase in [Ca\textsuperscript{2+}]\textsubscript{i} appears to be monophasic, however this could also result from pulsatile agonist addition.

![Graph showing Ca\textsuperscript{2+} release from CHO cells](image)

**Figure 4.6**

DPDPE increases [Ca\textsuperscript{2+}]\textsubscript{i} in populations of adherent CHO\textsubscript{δWT} cells. Cells were perfused with 1 μM DPDPE for 1 min (indicated by the solid lines). Data is representative of 3 individual experiments, each with at least n=2 stimulations. Studies were performed on adherent fura-2 loaded CHO\textsubscript{δWT} cells. In this figure there appears to be a hint of desensitization with successive agonist application.
4.8 Discussion

In chapter 3 it was demonstrated that the affinity of [3H]DPN is increased by removal of the C-terminus of the δ-opioid receptor, consistent with a previous study (Hirst et al., 1998), however the reasons behind this remain unclear since 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 [3H]DPN binding. Although the Bmax values differ slightly in this study from those in the study by Hirst et al., (1998), there is consistency in that CHOδWT had a slightly higher Bmax than CHOδ37.

Various other studies have shown that activation of (full length) recombinant opioid receptors causes a mobilization of [Ca2+]i (Smart et al., 1997; Zimprich et al., 1995; Hirst et al., 1998; Spencer et al., 1998) and the results of this study are in agreement with these. It may be possible that the increase in [Ca2+]i observed was due solely to release from intracellular stores, and that activation of the δ-opioid receptor did not induce Ca2+ entry from extracellular sources. In some results it appears that a 'plateau' is present (e.g. fig. 2c and 3a). It is believed that this does not represent Ca2+ entry into the cell, but is instead leakage of fura-2 from the cell. The data presented in figure 4.6 adds further weight of this argument since in a perfusion system, where extracellular fura-2 is washed away, the response appears to be monophasic. However the possibility of Ca2+ entry cannot be completely ruled out as it is possible that it is masked by fura-2 leakage from the cell. Indeed, other studies have demonstrated that when transfected into host cells, recombinant opioid receptors cause an increase in [Ca2+]i that is independent of extracellular Ca2+, i.e. release is from stores (Zimprich et al., 1995; Spencer et al., 1998).

The use 2.5mM probenecid showed that this agent reduced leakage from CHOδWT cells, but also reduced the maximal responses to ATP (presumably working via endogenous purinergic receptors) and DPDPE. It is thought that probenecid reduces leakage by blocking the multidrug resistance pump P-glycoprotein which would otherwise cause fura-2 export from the cell. However, the mechanism by which it reduces agonist responses is unclear. It may be that it interferes with ligand - receptor interactions, or if probenecid is transported into the cell it may interfere with
downstream events, e.g. Ca\textsuperscript{2+} release. Because of the side-effects of this drug it was decided to discontinue its use since the response to DPDPE would have become very small.

The data presented in this chapter show that deletion of the C-terminus of the \(\delta\)-opioid receptor produces a marked rightward shift in the concentration-response for [Ca\textsuperscript{2+}]\textsubscript{i} release (EC\textsubscript{50} CHO\(\delta\)WT = 3.68 nM, CHO\(\delta\)37 = 0.831 \(\mu\)M, 225 fold difference). The study by Hirst \textit{et al.}, (1998), examined the effect of the removal of the C-terminus of the \(\delta\)-opioid receptor on phospholipase C coupling. Whilst there was little effect on EC\textsubscript{50} values for phospholipase C coupling (CHO\(\delta\)WT = 55 nM, CHO\(\delta\)37 = 14 nM), there were differences in the time-course for Ins(1,4,5)P\textsubscript{3} accumulation between CHO\(\delta\)WT and CHO\(\delta\)37. In CHO\(\delta\)WT, the response peaked at 15s and remained elevated until sampling ended, whilst in CHO\(\delta\)37 the response was monophasic, reaching a peak at 120s. In CHO\(\delta\)WT cells there is a close temporal and concentration-response relationship between Ins(1,4,5)P\textsubscript{3} formation and increases in [Ca\textsuperscript{2+}]\textsubscript{i}, suggesting that the induced increase in Ins(1,4,5)P\textsubscript{3} formation leads to a mobilization of intracellular Ca\textsuperscript{2+}. It is worth noting that in this clone the peak Ca\textsuperscript{2+} declines (possibly masked by fura-2 leakage) despite elevated levels of Ins(1,4,5)P\textsubscript{3}, a phenomenon also described in CHO cells transfected with M3 muscarinic receptors (Tobin \textit{et al.}, 1993). However, in the CHO\(\delta\)37 clone the peak Ca\textsuperscript{2+} response occurs at the same time as in the CHO\(\delta\)WT despite the Ins(1,4,5)P\textsubscript{3} response only reaching a peak at 120s. There is a small increase in Ins(1,4,5)P\textsubscript{3} formation at 15s in CHO\(\delta\)37, and it may be possible that this is sufficient to cause mobilization of intracellular Ca\textsuperscript{2+}, even though the EC\textsubscript{50} values for Ins(1,4,5)P\textsubscript{3} formation and Ca\textsuperscript{2+} release do not seem to suggest this. However it should be noted that the EC\textsubscript{50} value for Ins(1,4,5)P\textsubscript{3} formation was taken at the maximal response time (120s) whereas EC\textsubscript{50} values for Ca\textsuperscript{2+} release were obtained at peak [Ca\textsuperscript{2+}]\textsubscript{i} following agonist stimulation. In cells expressing both the full length receptor and the truncated receptor the increase in [Ca\textsuperscript{2+}]\textsubscript{i} was thapsigagan sensitive, indicating release from intracellular stores, and pertussis sensitive, suggesting involvement of G proteins of the G\textsubscript{i/o} family. Therefore at present it remains unclear as to why truncation of the C-terminus of the \(\delta\)-opioid receptor produces such a marked difference in the Ca\textsuperscript{2+} concentration-response curves.
It could be argued that the data presented here resulted from differing levels of receptor expression. Indeed, it is possible that a receptor reserve could exist for $G_\alpha$ mediated responses but not for $G_\beta\gamma$ mediated responses. Therefore the increase in $[Ca^{2+}]_i$ (presumably $G_\beta\gamma$ response) could be dependent upon absolute levels of receptor expression, whereas $G_\alpha$ responses (e.g. inhibition of cAMP) would not be affected by differing levels of receptor expression due to the presence of spare receptors. Whilst expression differences cannot be completely excluded as an explanation for the data presented here, there are many reasons why this is unlikely; firstly the range of clones have similar levels of receptor expression (Cvejic et al., 1996). Indeed in the cells used in this chapter there is only a 2.6 fold difference in receptor number. Moreover in the previous study by Hirst et al., (1998) there was no modulation of either pEC$_{50}$ for cAMP ($G_\alpha$) or pEC$_{50}$ for Ins(1,4,5)P$_3$ (presumably $G_\beta\gamma$) 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 $\kappa$-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 $\kappa$-opioid receptor were $\sim$10 fold lower than $\mu$ and $\delta$ expression, maximal increases in $[Ca^{2+}]_i$ were reduced.

One possible explanation for the differences in $Ca^{2+}$ release between wild type and truncated receptors could be explained by phosphorylation states of the Ins(1,4,5)P$_3$ receptor. 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 CHO837 cell line $Ca^{2+}$ release occurred when there was less Ins(1,4,5)P$_3$ mass then it may be possible that there would also be less di-acylglycerol, resulting in a reduced level of protein kinase C activation. This would result in a reduced level phosphorylation of the Ins(1,4,5)P$_3$ receptor in the CHO837 cell line and therefore possibly shifting the CHO837 concentration-response curve to the right.

In a study utilizing two splice variants of the rat $\mu$-opioid receptor, MOR1 and MOR1B, where MOR1B is seven residues shorter than MOR1, Zimprich et al., (1995) demonstrated that when expressed in CHO cells both receptors mobilized $Ca^{2+}$ with
similar EC$_{50}$ values (EC$_{50}$ MOR1=1.5µM, EC$_{50}$ MOR1B=1.7µM). However, Koch et al., (1998) found that MOR1B desensitized more slowly and re-sensitized faster compared to MOR1 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 (Trapaidze et al., 1996; Cvejic 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 conclusion it has been demonstrated that activation of the wild type and C-terminal truncated δ-opioid receptor expressed in CHO cells produces a concentration-dependent, thapsigargin and PTX sensitive increase in [Ca$^{2+}$]$_i$. Truncation of the δ-opioid receptor produces a rightward shift in the concentration response curve for DPDPE mediated Ca$^{2+}$ release. In the next chapter, opioid mediated increases in Ca$^{2+}$ will be further studied, this time at the single cell level.
Chapter 5. Coupling of μ-, δ-, and κ-opioid receptors to [Ca\textsuperscript{2+}]\textsubscript{i} - single cell studies.

5.1 Introduction
Since Ca\textsuperscript{2+} plays an important role in a wide range of cellular functions, such as neurotransmitter release, cell proliferation and synaptic plasticity, it is likely that a diverse range of Ca\textsuperscript{2+} signals to regulate these differing functions exist (Berridge, 1996). In chapter 4 it was demonstrated that the δ-opioid receptor increases [Ca\textsuperscript{2+}], a study that was carried out in whole cell suspensions. This technique allows a range of information to be gained such as concentration dependence, source of Ca\textsuperscript{2+} used and sensitivity to various agents. However, the results gained are from the mean of a population of cells, so it is not possible determine what is happening at a single cell level.

Cellular and sub-cellular studies on Ca\textsuperscript{2+} signaling have demonstrated that Ca\textsuperscript{2+} release is spatially, frequency and amplitude encoded (Berridge, 1997b) and that release may be seen as oscillations and waves and there exists elementary events such as 'puffs', 'bumps' and 'sparks' (Berridge, 1997a). Release may be from Ins(1,4,5)P\textsubscript{3} sensitive stores within the cell, and may be modulated by a variety of factors such as calmodulin (Levitan, 1999), Ins(1,4,5)P\textsubscript{3} receptor phosphorylation (e.g. Burgrim, 1999) and Ca\textsuperscript{2+} itself (Berridge, 1996). Release of Ca\textsuperscript{2+} may also occur from ryanodine sensitive intracellular stores (Berridge, 1993). Mechanisms for store refilling have also been postulated, including the capacitive entry model (Putney 1986, Berridge, 1995) although this theory has been questioned (Shuttleworth, 1999).

Opioids have been shown to increase [Ca\textsuperscript{2+}], in whole cell suspensions and adherent monolayers (for example, Smart et al., 1997, Connor and Henderson, 1996 and see chapter 4). At a single cell level, [Ca\textsuperscript{2+}]; increases have been studied in NG108-15 cells expressing endogenous δ-opioid receptors (Jin et al., 1994) and in transfected neuro\textsubscript{2A} cells expressing μ- δ- and κ-opioid receptors (Spencer et al., 1998). Although it is expected that opioid agonists will increase [Ca\textsuperscript{2+}], in single adherent CHO cells, this has not been studied and would provide further information on stimulatory opioid signaling pathways.
5.2 Aims
The aims of this chapter are to explore the coupling of recombinant opioid receptors expressed in single adherent CHO cells to [Ca\(^{2+}\)]. SH-SY5Y cells will also be studied to allow comparison of endogenous μ-opioid receptors. In this particular cell line, activation of PLC may be Ca\(^{2+}\) entry driven (Smart et al., 1995).

5.3 Methods
For details of tissue culture, Ca\(^{2+}\) imaging studies and measurement of [Ca\(^{2+}\)]\(_i\) in whole cell suspensions the reader is directed to chapter 2

5.4 Results
Activation of recombinant opioid receptors by appropriate agonist produced an increase in Ca\(^{2+}\) in a proportion of cells, table 5.1

<table>
<thead>
<tr>
<th>Cell Line (Agonist)</th>
<th>No. of Cells Tested</th>
<th>% Responding</th>
<th>Δ340/380 (in responding cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHOm (fentanyl)</td>
<td>129</td>
<td>12.4</td>
<td>0.45±0.07</td>
</tr>
<tr>
<td>CHOd (DPDPE)</td>
<td>904</td>
<td>9.6</td>
<td>0.37±0.04</td>
</tr>
<tr>
<td>CHOk (spiradoline)</td>
<td>523</td>
<td>7.6</td>
<td>1.05±0.07</td>
</tr>
<tr>
<td>SH-SY5Y (fentanyl)</td>
<td>160</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5.1
Number of responding cells to appropriate agonist, together with Δ340/380 in responding cells. Data are mean±SEM, number of cells tested is indicated in the table. Experiments were performed in single adherent fura-2 loaded cells.

As a positive control, all CHO cells were challenged with 3μM UTP. This agonist increased [Ca\(^{2+}\)]\(_i\) in 100% of cells, presumably acting via endogenous purinergic receptors. All SH-SY5Y cells were challenged with 1mM carbachol (activating muscarinic receptors), again to which all responded. Pseudo-colour images of CHOm, δ and κ cells are shown in figures 5.1, 5.2 and 5.3 respectively, and should be read top left to bottom right. These figures show time courses of responding cells to appropriate agonist. Images are pseudo-coloured on a 'rainbow' scale from blue (representative of
low 340/380 ratios) through to red (representative of higher 340/380 ratios). Cells were not calibrated.
Figure 5.1 Pseudo-colour time course for fentanyl (1μM) mediated increases in [$Ca^{2+}$]i, in CHOμ cells. Each panel represents 1 frame and is equal to 5.3s. Panel 1 is representative of basal pseudo-coloured 340/380 ratios, panels 2 through to 15 show increases in [$Ca^{2+}$]i with the addition of fentanyl, which reached the cells ~65sec before panel 2. [$Ca^{2+}$]i was measured in single adherent cells and imaged using Merlin software.
Figure 5.2 Pseudo-colour time course for DPDPE (1 μM) mediated increases in $[Ca^{2+}]_i$ in CHOδ cells. Each panel represents 1 frame and is equal to 5.3s. Panel 1 is representative of basal pseudo-coloured 340/380 ratios, panels 2 through to 15 show increases in $[Ca^{2+}]_i$ with the addition of DPDPE, which reached the cells ~60sec before panel 2. $[Ca^{2+}]_i$ was measured in single adherent cells and imaged using Merlin software.
Figure 5.3 Pseudo-colour time course for spiradoline [1\mu M] mediated increases in [Ca^{2+}], in CHO\kappa cells. Each panel represents 1 frame and is equal to 5.3s. Panel 1 is representative of basal pseudo-coloured 340/380 ratios, panels 2 through to 15 show increases in [Ca^{2+}], with the addition of spiradoline, which reached the cells \sim 35sec before panel 2. [Ca^{2+}], was measured in single adherent cells and imaged using Merlin software.
Time courses for 3 representative responding cells for each transfected cell line are shown in figure 5.4. From these time courses it can clearly be seen that responding cells do not increase \([\text{Ca}^{2+}]\), in the same temporal manner.
Figure 5.4
Time courses for 3 representative (from \(n \geq 10\)) responding cells for each transfected cell line (fentanyl = CHOµ, DPDPE = CHOδ, spiradoline = CHOx). The solid bar indicates addition of agonist to the perfusion system, and takes 60s to reach the cells in the perfusion chamber. Studies were performed in fura-2 loaded single adherent cells.
Due to the low response rate to opioid agonist it was not possible to conduct further studies (e.g. naloxone sensitivity) on these cells. Preliminary experiments revealed that the low response rate was not due to experiments being conducted at room temperature, since the response rate was not increased by raising the temperature to 37°C (data not shown). Furthermore, experiments conducted in whole cell suspensions using cells of the same passage number, split on the same day, using identical temperatures, drugs and buffers systems revealed that there was an increase in $[\text{Ca}^{2+}]_i$ in suspension (figure 5.5) but a low response rate in single adherent cells.

![Figure 5.5](image.png)

*Figure 5.5*

Representative traces (from $n=3$) showing increase in $[\text{Ca}^{2+}]_i$ in $\text{CHO}_{\delta}$, $\text{CHO}_\mu$ and $\text{CHO}_\kappa$ cells. Solid bar indicates addition of appropriate agonist to the cuvette. Studies were performed in fura-2 loaded whole cell suspensions.

In addition to figure 5.5, further confirmation of opioid induced increases in $[\text{Ca}^{2+}]_i$ in whole cell suspensions comes from the work presented in the previous chapter with the $\delta$-opioid receptor (see figure 4.2). With regard to $\kappa$- and $\mu$-opioid receptor, $1\mu\text{M}$ spiradoline increases $[\text{Ca}^{2+}]_i$ by $123.8\pm40.2\text{nM}$ ($n=4$) in $\text{CHO}_\kappa$ cells, and $1\mu\text{M}$ fentanyl increases $[\text{Ca}^{2+}]_i$ by $80.0\pm16.4\text{nM}$ in $\text{CHO}_\mu$ cells (Smart *et al.*, 1997).
5.5 Discussion

The results presented in this chapter show that activation of recombinant opioid receptors in CHO cells increases \([Ca^{2+}]_i\), but the response rate is relatively low. This may be the underlying reason why, when \([Ca^{2+}]_i\) is measured in the same cell lines in whole cell suspensions, the increase in \([Ca^{2+}]_i\) is relatively small (1μM agonist increase ~100-200nM, or ~0.1 ratio unit from figure 5.5). In this chapter, between ~130-900 cells were sampled for each cell line. In a cell suspension in a cuvette there are probably ~1x10^5- 1x10^6 cells. Therefore if 10% of these cells responded then it is plausible that the increase in \([Ca^{2+}]_i\) that would be 'seen' in a mean population would low, but would occur due to large numbers of cells.

The underlying reason as to this low response rate is unclear. Transfected CHO cells are routinely grown in G418 to select for receptor expression and from fluorescence / confocal microscopy (Chapter 3, section 3.9) all cells appear to express receptors as shown for CHOp. Since it was shown in chapter 4 that the δ-opioid receptor mediated increase in \([Ca^{2+}]_i\) in suspension was PTX sensitive, it is possible that the opioid mediated increase in \([Ca^{2+}]_i\) is through G protein βγ subunits. It may be that although both α and βγ subunits are liberated upon opioid receptor activation, βγ subunits are 'inefficient' at coupling to effectors, indeed it is known that βγ subunits are less effective at stimulating PLC than G_q α subunits (Rhee and Bae, 1997). 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 response rate and conversely, cells in suspension still respond to agonist when at room temperature

A low response rate to opioids (~20%) in adherent cells has also been demonstrated in another study using cells expressing endogenous opioid receptors (Same and Gafni, 1996), in which synchronization was found to increase the probability of detecting a response (up to 70%). Spencer et al., (1998) noted a variable response rate to opioid agonists in neuro-2A cells expressing recombinant opioid receptors. In this particular study the response rate was between 17-85% depending on the cell line tested. This may correlate with receptor number \(B_{max} = 0.22\ \text{pmol mg protein}^{-1}\), response = 17%; \(B_{max} = 2.06\text{pmol mg protein}^{-1}\), response = 85%; although when \(B_{max} = 4.7\ \text{pmol mg} \text{ protein}^{-1}\),
protein$^{-1}$, response = 43%). It may be that the limiting factor in this pathway is the receptor (Spencer et al., 1998), and increasing receptor number allows more $\beta\gamma$ subunits to activate PLC. Indeed in this chapter, CHO$\mu$ cells had a higher $B_{\text{max}}$ than CHO$\delta$ or CHO$\kappa$ and displayed a slightly higher response rate, although there may be a finite number of $\beta\gamma$ subunits and although CHO$\kappa$ cells displayed a lower response rate, the increase in $[\text{Ca}^{2+}]_i$ was larger.

Interestingly, there was no response to fentanyl in SH-SY5Y cells. It has previously shown that in SH-SY5Y cells that 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 $[\text{Ca}^{2+}]_i$, although $\mu$ opioid receptor activation in the same cells causes a small and unpredictable increase in $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$. (Connor and 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).

In summary it has been shown that recombinant opioid receptors expressed in CHO cells cause an increase in $[\text{Ca}^{2+}]_i$. However, this response rate is low.
Chapter 6. Characterization of the effects of endomorphin-1 and endomorphin-2 in CHO cells expressing recombinant μ-opioid receptors and SH-SY5Y cells.

6.1 Introduction

Endogenous opioid peptides play an important role in the modulation of nociceptive neurotransmission. This is achieved by interaction with opioid receptors and subsequent activation of specific brain and spinal cord pathways (see section 1.20, general introduction). The existence of an endogenous 'morphine – like' opioid that may be involved in this pathway was first postulated in 1975 (Hughes, 1975). This work led to the identification of two related opioid peptides, termed met- and leu-enkephalins (from the Greek ‘in the head’), which were isolated from porcine brain (Hughes et al., 1975). Soon afterwards it was discovered that the C-fragment of lipotropin had opioid activity (Bradbury et al., 1976), which was later termed β-endorphin (from endogenous morphine). A third endogenous opioid peptide, termed dynorphin (from the Greek dynamis – power) was isolated from porcine pituitary in 1979 (Goldstein et al., 1979). Precursors for these endogenous opioid peptides have a distinct distribution in the periphery and CNS and are termed pro-opiomelanocortin (POMC, from which β-endorphin is cleaved), pro-enkephalin and pro-dynorphin respectively. In addition to these endogenous ligands, the endogenous ligand for the ORL-1 receptor, nociceptin, was isolated in 1995 (Meunier et al., 1995, Reinscheid et al., 1995).

With the exception of nociceptin, none of the traditional endogenous opioid peptides show great selectivity for individual types of opioid receptors. Dynorphins bind preferentially to κ-opioid receptors, enkephalins have higher affinity for δ-opioid receptors, whilst endorphins bind with approximately equal affinity to μ and δ sites, (although is slightly preferential for δ-opioid receptors).

However, some 22 years after the isolation of the first endogenous opioid ligand, two peptides were discovered that displayed high affinity and selectivity for the μ-opioid receptor (Zadina et al., 1997). This group of opioid peptides was synthesised by amino acid substitution of the peptide Tyr-Pro-Trp-Gly-NH₂, a peptide with opioid related activity, to
yield two peptides termed endomorphin-1 and endomorphin-2 (again from endogenous morphine), shown in figure 6.1

<table>
<thead>
<tr>
<th>Endomorphin-1</th>
<th>Tyr-Pro-Trp-Phe-NH₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endomorphin-2</td>
<td>Tyr-Pro-Phe-Phe-NH₂</td>
</tr>
</tbody>
</table>

Figure 6.1

Amino acid structure of endomorphin-1 and -2

In the original paper describing endomorphins (Zadina et al., 1997), endomorphin-1 displayed high affinity ($K_i = 0.36\text{nM}$) and selectivity for $\mu$-opioid receptor (4 000 and 15 000 fold preference over $\delta$ and $\kappa$ receptors respectively). This was mirrored by endomorphin-2, with a $K_i = 0.69\text{nM}$ and a 13 000 fold and 15 000 selectivity over $\delta$ and $\kappa$ receptors respectively. Both peptides were more potent than DAMGO in vitro and produced analgesia in mice. Endomorphin-1 and -2 were originally isolated from bovine brain, and have subsequently been found to occur in human brain, at higher levels than that found in bovine brain (Hackler et al., 1997). Furthermore, immunoreactivity / binding for endomorphin has been shown to occur in many brain areas associated with opioid receptor activity. These include medulla, thalamus, striatum, frontal cortex as well as in the PAG and the spinal cord (Martin-Schild et al., 1997; Zadina et al., 1997; Pierce et al., 1998; Schreff et al., 1998; Goldberg et al., 1998). Based on this evidence, it was proposed that the endomorphins could in fact be endogenous ligands at the $\mu$-opioid receptor. However, to date no precursor for either of these two peptides has been isolated. It is interesting to note that the amino acid structure of the endomorphins differ from traditional opioid peptides in that they do not contain the amino acid sequence Tyr-Gly-Gly-Phe at the C-terminus of the peptide. It remains to be seen whether both endomorphin-1 and -2 are cleaved from the same precursor molecule, what indeed this molecule is and if it has any biological activity of its own.
Advances have been made into the possible mechanisms of endomorphin-2 translocation. There is evidence to suggest that endomorphin-2 may be synthesized in primary sensory neurons in ganglia, transported to the superficial dorsal horn and released near neurons expressing \( \mu \)-opioid receptors (Martin-Schild et al., 1997).

Although it has been demonstrated that endomorphins produce \textit{in vivo} effects consistent with opioids, such as analgesia and hypotension / bradycardia (Zadina et al., 1997; Stone et al., 1997; Goldberg et al., 1998; Champion et al., 1997a,b,c; Czapla et al., 1998), a basic understanding of the cellular effects of endomorphin is critical to the further understanding of the physiological role of these peptides.

### 6.2 Aims
The aims of this chapter are to investigate the cellular effects produced by endomorphin-1 and -2 in CHO cells expressing recombinant \( \mu \)-opioid receptors and in SH-SY5Y cells that express endogenous \( \mu \)-opioid receptors. In particular it was aimed to 1) characterize the binding of both peptides to membranes prepared from the above cells, 2) to determine the effect of both peptides on forskolin stimulated cAMP formation and 3) to determine the effect of both peptides on \([\text{Ca}^{2+}]\), in whole cell suspensions and at a single cell level. Although classical opioid signaling pathways are anticipated, the results of this chapter will provide a basic cellular characterization of endomorphin-1 and 2, and provide data necessary before any further work on endomorphins can be interpreted confidently.

### 6.3 Methods
For details of chemicals, tissue culture, membrane preparation, measurement of cAMP, intracellular calcium and displacement protocols the reader is directed to chapter 2. Details of data analysis is also found in this chapter.

### 6.4 Results - \[^{3}\text{H}]\text{DPN displacement}
Endomorphin-1 and endomorphin-2 concentration-dependently displaced \[^{3}\text{H}]\text{DPN} binding in CHO\(\mu\) and SH-SY5Y cells (figure 6.1a and b), which when corrected for the amount of radioligand used, yielded pK\textsubscript{i} values shown in table 6.1. In all cases the slope factor was
less than unity (table 6.1). There was a small but significant difference between pK<sub>i</sub> values for the 2 cell types when analysed by students t-test (9.5 compared to 2.9 nM). Analysis of endomorphin-2 displacement of [<sup>3</sup>H]DPN between CHO<sub>µ</sub> and SH-SY5Y cells yielded a small but significant difference in pK<sub>i</sub> values by t-test (15.1 compared to 3.7 nM). Endomorphin –1 and –2 bound weakly to membranes prepared from CHO<sub>δ</sub> and CHO<sub>κ</sub> cells, as shown in table 6.1
Figure 6.1
Endomorphin-1 (a) and endomorphin-2 (b) concentration-dependently displaced [³H]DPN from CHOµ and SH-SY5Y membranes. Data are mean±SEM for n=4-9. Studies were performed at room temperature in 1ml volumes for 60 min with a fixed concentration of [³H]DPN. Non-specific binding was defined in the presence of 10µM naloxone.
Table 6.1

<table>
<thead>
<tr>
<th></th>
<th>pKᵢ E₁ [nM]</th>
<th>slope factor</th>
<th>pKᵢ E₂ [nM]</th>
<th>slope factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHΩμ</td>
<td>8.02±0.09[9.5]</td>
<td>0.72±0.04</td>
<td>7.82±0.11 [15.1]</td>
<td>0.59±0.05</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>8.54±0.13[2.9]*</td>
<td>0.61±0.10</td>
<td>8.43±0.13 [3.7]*</td>
<td>0.69±0.11</td>
</tr>
<tr>
<td>CHOδ</td>
<td>5.77±0.04 [1700]</td>
<td>nd</td>
<td>5.76±0.03 [1700]</td>
<td>nd</td>
</tr>
<tr>
<td>CHOκ</td>
<td>&lt;5.72 [1900]</td>
<td>nd</td>
<td>4.74±1.09 [18000]</td>
<td>nd</td>
</tr>
</tbody>
</table>

Selectivity

μ over δ; 178

μ over κ; >200

μ over δ; 112

μ over κ; 1192

*pKᵢ values for endomorphin-1 and -2 displacement of [³H]DPN binding from CHΩμ, CHOδ, CHOκ and SH-SY5Y cell membranes, nM values are shown in parentheses. Graphical representation of data is shown in figure 6.1. Data are mean±SEM for n=3-9. *Indicates significant difference (p<0.05, unpaired Student's t-test) between pKᵢ values for SH-SY5Y and CHΩμ cells. Slope factors describe the steepness of the displacement curve. nd = not determined, curve incomplete

6.5 Inhibition of cAMP formation

In CHΩμ and SH-SY5Y cells endomorphin-1 and -2 produced a concentration-dependent inhibition of forskolin-stimulated cAMP formation (figure 6.2), with pEC₅₀ values and maximal inhibition shown in table 6.2. This inhibition of forskolin-stimulated cAMP formation was naloxone sensitive (see table 6.3). Analysis of pEC₅₀ values by Students t-test showed that there was no significant difference between CHΩμ and SH-SY5Y cells for either endomorphin-1 or -2. There was also no significant difference between Eₘₐₓ values between CHΩμ and SH-SY5Y cells for endomorphin-1, however there was a small but significant difference in Eₘₐₓ values between cell lines for endomorphin-2.
Figure 6.2
Endomorphin-1 (a) and endomorphin-2 (b) concentration-dependently inhibited forskolin stimulated cAMP formation in CHOµ and SH-SY5Y cells. Data are mean±SEM for n=4-5. Cyclic AMP was measured by radioreceptor assay.
Endomorphin-1 and -2 inhibit forskolin stimulated cAMP formation in CHOμ and SH-SY5Y cells. Graphical representation of data is shown in figure 6.2. Data are mean±SEM for n=4-5. *Indicates significant difference (p<0.05, Student's unpaired t-test) between E_max values for SH-SY5Y and CHOμ cells. Cyclic AMP was measured by radioreceptor assay.

<table>
<thead>
<tr>
<th></th>
<th>Endomorphin-1</th>
<th></th>
<th>Endomorphin-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CHOμ</td>
<td>SH-SY5Y</td>
<td>CHOμ</td>
</tr>
<tr>
<td>pEC50 (nM)</td>
<td>8.03±0.16(9.3)</td>
<td>7.72±0.13(19.1)</td>
<td>8.15±0.24(7.1)</td>
</tr>
<tr>
<td>% E_max</td>
<td>53.0±9.3</td>
<td>46.9±5.6</td>
<td>56.3±3.8</td>
</tr>
</tbody>
</table>

Table 6.2

Endomorphin-1 and -2 inhibition of cAMP formation is naloxone sensitive. *Indicates naloxone produced a significant reduction (p<0.05, Paired t-test) in cAMP inhibition compared to control. Data are mean±SEM for n=3-7. Cyclic AMP was measured by radioreceptor assay.

<table>
<thead>
<tr>
<th>% inhibition of cAMP formation</th>
<th>control</th>
<th>naloxone 10μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1 (10μM) CHOμ</td>
<td>53.4±6.4</td>
<td>21.9±7.9*</td>
</tr>
<tr>
<td>E2 (10μM) CHOμ</td>
<td>46.6±2.2</td>
<td>9.9±5.8*</td>
</tr>
<tr>
<td>E1 (10μM) SHSY-5Y</td>
<td>33.4±7.9</td>
<td>12.9±4.7*</td>
</tr>
<tr>
<td>E2 (10μM) SHSY-5Y</td>
<td>22.3±2.1</td>
<td>2.2±0.3*</td>
</tr>
</tbody>
</table>

Table 6.3

Endomorphin-1 and -2 increased intracellular calcium in CHOμ cell suspensions, this data is summarized in table 6.4 and can be seen in figure 6.3 and 6.4. In single adherent CHOμ cells endomorphin-1 and -2 increased intracellular calcium in a proportion of cells, this data is summarized in table 6.5 and is shown in graph 6.3 and 6.4.
Figure 3
Panels a and b; Typical traces (from n≥4) depicting a rise in [Ca^{2+}], with the addition of endomorphin-1 (a) or endomorphin-2 (b) in CHOμ cells. [Ca^{2+}], was measured in fura-2 loaded whole cell suspensions. Solid bar indicates addition of agonist to the cuvette.

Panels c and d; Three typical traces (from n≥4) of an individual single adherent CHOμ cell depicting a rise in [Ca^{2+}], with the addition of endomorphin-1 (c) or endomorphin-2 (d). [Ca^{2+}], was measured in single adherent fura-2 loaded cells. Solid bar indicates addition of agonist to the perfusion system, and takes 60sec to reach cells in the perfusion system.
Table 6.4
Summary of the effects of endomorphin-1 and -2 in various cell lines in whole cell suspensions, together with the effects of naloxone and thapsigargin. Data are mean±SEM for n=4-8

<table>
<thead>
<tr>
<th>cell line / treatment</th>
<th>increase in ([\text{Ca}^{2+}]_i)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>endomorphin-1 (1\mu M)</td>
</tr>
<tr>
<td>CHO(\mu)</td>
<td>106±28nM</td>
</tr>
<tr>
<td>thapsigargin (100nM)</td>
<td>0nM (450±87nM)(^a)</td>
</tr>
<tr>
<td>naloxone (10\mu M)</td>
<td>reversed</td>
</tr>
<tr>
<td>CHO(\delta)</td>
<td>none (DPDPE [10\mu M] response)</td>
</tr>
<tr>
<td>CHO(\kappa)</td>
<td>none (spiradoline [1\mu M] response)</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>none (cch [1mM] response)</td>
</tr>
</tbody>
</table>

---

Table 6.5
Summary of data showing the effects of endomorphin-1 and -2 in single adherent CHO\(\mu\) and SH-SY5Y cells. Data are expressed as a percentage of cells responding (from 223 CHO\(\mu\) cells, E1 and 172 CHO\(\mu\) cells, E2) and mean±SEM for \(\Delta 340/380\) ratio

<table>
<thead>
<tr>
<th></th>
<th>Endomorphin-1 (1\mu M)</th>
<th>Endomorphin-2 (1\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO(\mu) % responding</td>
<td>7.2% (UTP [3\mu M] in 100%)(^b)</td>
<td>7.4% (UTP [3\mu M] in 100%)(^b)</td>
</tr>
<tr>
<td>(\Delta 340/380) ratio</td>
<td>0.81±0.09</td>
<td>0.40±0.08</td>
</tr>
<tr>
<td>SH-SY5Y % responding</td>
<td>0% (cch [1mM] response in 100%)</td>
<td>0% (cch [1mM] response in 100%)</td>
</tr>
</tbody>
</table>

\(^a\) Indicates the increase in \([\text{Ca}^{2+}]_i\), produced in response to thapsigargin. cch = carbachol, acting via muscarinic receptors.

\(^b\) UTP is presumably acting via endogenous purinergic receptors.
Figure 6.4
Pseudo-colour images of CHOκ cells under basal and endomorphin-1 or endomorphin-2 stimulated conditions. Images are representative of 223 CHOκ cells stimulated with E1 and 172 CHOκ cells stimulated with E2. Studies were performed in fura-2 loaded adherent cells and were perfused with 1μM endomorphin for 1 min at room temperature.
6.7 Discussion

The data shown here indicate that endomorphin-1 and -2 bind to the recombinant μ-opioid receptor expressed in CHO cells and the endogenous μ-opioid receptor in SH-SH5Y cells with high affinity and relatively high selectivity. In addition to this both peptides produce a concentration-dependent inhibition of forskolin stimulated cAMP formation in each cell line. Furthermore, endomorphin -1 and -2 increase intracellular calcium in CHOμ cells, but fail to do so in SHSY5Y cells.

Endomorphin-1 and -2 produced a concentration-dependent displacement [3H]DPN binding in membranes prepared from CHOμ and SH-SY5Y cells, which when corrected for the amount of radioligand used yielded $K_i$ values as shown in table 1. These values are ~10 fold lower than the original paper (Zadina et al., 1997) in which rat brain was used as a source of μ- and δ-opioid receptors and guinea-pig cerebellum for κ-opioid receptors, together with subtype selective ligands as the radiolabel. However, they are consistent with those proposed by Hosohata et al., (1998) ($K_i$ values of 7.7nM and 5.9nM 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 0.58nM were obtained for endomorphin-1 and -2 displacement of the respective iodinated endomorphin, whilst in mouse brain, displacement of [3H]DPN gave $K_i$ values of 0.03 (endomorphin-1) and 0.12nM (endomorphin-2) (Goldberg et al., 1998). It 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, it is probable that both endomorphins display essentially similar effects in both cell types. However, a report suggests that endomorphin-1 and endomorphin -2 may produce anti-nociception through differential actions at pharmacological μ1 and μ2 subtypes (endomorphin-1 being μ2 selective and endomorphin-2 being μ1 selective) (Sakurada et al., 1999). It has been speculated the μ-opioid receptor expressed in SH-SY5Y cells is like the μ2 subtype (Elliott et al., 1994), whilst the μ receptor transfected into CHO cells displays pharmacology akin to the μ1 subtype (D.K. Grandy, Oregon Health Sciences University, personal communication). Therefore the data presented in this chapter do not agree with μ-opioid
receptor selectivity of endomorphin-1 and -2 since both E1 and E2 displayed similar pK$_i$ values in both cell lines and appear not to be subtype selective.

In addition, endomorphin-1 and -2 bound only weakly to membranes prepared from CHOδ and CHOκ cells, confirming that the endomorphins are indeed selective for the μ-opioid receptor. However, the selectivity is not as great as that as shown by Zadina et al., (1997), since the data presented in this chapter shows that both endomorphins have low affinity at the δ-opioid receptor.

Both endomorphin-1 and -2 had similar effects on cAMP formation in CHOμ cells, and this was comparable to that seen previously with fentanyl (Smart et al., 1997). In SH-SY5Y cells, endomorphin-1 and -2 also cause a concentration-dependent inhibition of forskolin-stimulated cAMP formation, similar to that seen in CHO cells expressing the recombinant receptors. However, the maximum inhibition was slightly lower, possibly reflecting absolute levels of receptor expression (CHOμ $B_{max} \approx 800$ fmol mg protein$^{-1}$, SH-SY5Y cells $B_{max} = 98$ fmol mg protein$^{-1}$). It has previously been shown that fentanyl also produces an inhibition of forskolin-stimulated cAMP formation in these cells, with an EC$_{50}$ value of 27nM (Lambert et al., 1993). When the human μ-opioid receptor was expressed in CHO cells, Gong et al. (1999) showed that endomorphin-1 and -2 caused an inhibition of cAMP formation with EC$_{50}$ values of ~35nM and an $E_{max}$ of ~60%. These inhibitory actions of opioids on cAMP 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 and Williams, 1994).

It is interesting to note that the competitive antagonist naloxone did not completely reverse the endomorphin-induced inhibition of cAMP formation. However, there may be a simple explanation for this. It may be that since the concentration of endomorphin used in this study was very high (10μM), naloxone, also at a concentration of 10μM may not be able to competitively block all μ-opioid receptor-binding sites.
At present there is some debate as to whether endomorphin-1 and -2 act as partial (low intrinsic efficacy) or full (high intrinsic efficacy) agonists. Various studies have demonstrated partial agonist activity of endomorphins, as measured by $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding (Sim et al., 1998; Hosohata et al., 1998; Alt et al., 1998). However other studies found that the endomorphins were as efficacious as other full $\mu$ agonists (Zadina et al., 1997; Higashida et al., 1998; Mima et al., 1997; Kakizawa et al., 1998; Harrison et al., 1998, Gong et al 1999). 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 cAMP inhibition, endomorphin-1 and -2 displayed similar efficacy (maximal inhibition) in CHO$\mu$ cells expressing $\sim 800 \text{fmol mg}^{-1} \text{ protein}$ and SH-SY5Y cells expressing 98$\text{fmol mg}^{-1} \text{ protein}$. Indeed this was similar to that produced by the well described full $\mu$-opioid agonist fentanyl, indicating the endomorphins were probably acting as full agonists in these tissues. However it may have been useful to include a direct comparison with either DAMGO or fentanyl in the experiments carried out in this chapter.

The endomorphin-1 and-2 induced increase in $[\text{Ca}^{2+}]_i$ observed in CHO$\mu$ cells in suspension is consistent with a previous report showing that the $\mu$-opioid fentanyl causes mobilization of $[\text{Ca}^{2+}]_i$ in CHO$\mu$ cell suspensions (Smart et al., 1997), in single adherent cells, chapter 5, section 5.4 and in adherent neuro-2A cells (Spencer et al., 1998). It is noteworthy that single adherent CHO$\mu$ cells appear to have a low response rate to endomorphins, but this is in general agreement with results presented in chapter 5.

In the present study, endomorphin-1 produced a greater increase in $[\text{Ca}^{2+}]_i$ than endomorphin-2. The reasons behind this are unclear, since at the level of inhibition of cAMP formation, both peptides appear to be full agonists. Of the other studies examining the effects of endomorphin-1 and -2 on $[\text{Ca}^{2+}]_i$, it has been demonstrated that both peptides cause $\text{Ca}^{2+}$ channel inhibition in NG108-15 cells expressing recombinant $\mu$-opioid receptors (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. In this chapter, 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. Interestingly, both endomorphins failed to mobilize Ca\(^{2+}\) from SH-SY5Y cells. Again for a possible explanation to this the reader is directed to chapter 5, section 5.5, fourth paragraph.

A report published after this study found that naloxone blocked endomorphin-1 but not endomorphin-2 induced inhibition of tachykinergic contractions of guinea pig isolated bronchus (Fisher and Undem, 1999). This suggests that in this preparation at least, the effect of endomorphin-2 may be due to its effects at non-classical opioid receptor subtypes. The reasons behind why this appears to be the only study that has observed this phenomena remains unclear. It is noteworthy that naloxone was able to reverse both endomorphin-1 and endomorphin-2 inhibition of acetylcholine release from guinea pig and human trachea (Patel et al., 1999).

In conclusion, endomorphin-1 and -2 bind with high selectivity and affinity at the \(\mu\)-opioid receptor and activate the \(\mu\)-opioid receptor to drive signal transduction pathways characteristic of opioids. This provides further evidence that these two peptides produce actions typical \(\mu\)-opioid ligands, and further studies of endomorphin-1 are presented in the next chapter, where \(\mu\)-opioid receptor desensitization is studied.
Chapter 7 Endomorphin-1 Induced Desensitization and Down-regulation of the Recombinant μ-opioid Receptor Expressed in CHO Cells

7.1 Introduction

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 signaling in response to the continued agonist presence. This allows control of cellular signaling to take place at the level of the receptor (Lohse, 1993), and may be agonist specific (homologous), where there is loss of responsiveness to only the desensitizing agent or non-agonist specific (heterologous) where there is loss of responsiveness to multiple ligands (Kuprinic and Benovic, 1988). Desensitization may also be accompanied by activation of another pathway, for example PKC or MAP kinase (Lefkowitz, 1998).

Desensitization and down-regulation occurs in a wide range of G protein coupled receptors (Lohse, 1993), although perhaps the most extensively studied receptor is the β-adrenergic receptor, where there is a correlation between receptor - G protein coupling, phosphorylation and receptor internalization (Koenig and Edwardson, 1997). Attenuation of this receptor - signaling pathway is known to involve β-adrenoreceptor kinase, protein kinase A and β-arrestin (Lohse, 1993). 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, which may lead to a reduced clinical effectiveness of opioids following repeated or long term exposure.

However it has become apparent that with certain opioid receptor signaling pathways the story may be more complicated. For example in transfected cell lines morphine activates the μ-opioid receptor, this may cause receptor phosphorylation but is unable to cause receptor internalization (Chakrabarti et al., 1998; Keith et al., 1998). In addition in HEK-293 cells expressing the δ-opioid receptor, phosphorylation is not required for internalisation (Murray et al., 1998). Therefore there may exist multiple pathways for opioid receptor desensitization. It was demonstrated in the previous chapter that endomorphin-1 displayed actions typical of opioids; displacement of [3H]DPN,
inhibition of cAMP formation and an increase in $[\text{Ca}^{2+}]_i$ - but the pattern of endomorphin-1-induced desensitization / down-regulation is not known.

7.2 Aims
The aims of this chapter are to ascertain whether endomorphin-1 causes desensitization and down-regulation of the $\mu$-opioid receptor expressed in CHO cells. Since it has previously been demonstrated that endomorphin-1 causes an inhibition of cAMP formation (chapter 6), this pathway will be studied, in particular; is there loss of this functional response? Is their up-regulation of cAMP? What are the $EC_{50}$ values for cAMP inhibition between treated and untreated cells (if desensitization occurs), and does desensitization occur via uncoupling of receptors from G protein? In addition it is intended to see if a loss of cell surface receptors occurs (down-regulation) and if this correlates with loss of functional response.

7.3 Materials and Methods
For details of desensitization protocols, cAMP measurement, radioligand binding protocols and immunoblotting, the reader is directed to chapter 2. Details of data analysis may also be found in this chapter.

7.4 Results - Desensitization at level of cAMP
Pre-treatment of CHO$\mu$ cells with 10$\mu$M endomorphin-1 for 0.5, 1, 2, 3, 4, 5 or 8h failed to significantly ($p<0.05$, paired Student's t-test) reduce forskolin stimulated cAMP formation compared to control when there was a subsequent challenge with 10 $\mu$M endomorphin-1. However, there was a significant reduction ($p<0.05$, paired Student's t-test) after 11h and 18h endomorphin-1 pre-treatment when compared to control values (figure 7.1).
Pre-treatment for 18h with 10μM endomorphin-1 causes a reduction in the level of cAMP inhibition when cells were re-challenged with 10μM endomorphin-1. No reduction in cAMP inhibition was seen for pre-treatments of up to 8h. Data are mean±SEM for n=3(in triplicate)-9, expressed as a percentage of their own control. *Indicates significant reduction in the level of cAMP inhibition between control and endomorphin-1 pretreated cells, p<0.05 by paired Student's t-test. Experiments were performed on adherent CHOμ cells, cAMP was measured by specific protein binding assay.

This suggests that it takes between 8-11h for the receptor to desensitize. The reduction in level of inhibition of cAMP formation was reversed by the opioid antagonist naloxone (10μM, figure 7.2). If endomorphin-1 is acting at the same receptor site to fentanyl, then it would be expected that if cells were pre-treated with fentanyl then re-challenged with endomorphin-1, desensitization to endomorphin-1 would also occur. Indeed pre-treatment with 10μM fentanyl also caused desensitization to endomorphin-1 (10μM) when this was used in a subsequent challenge (Figure 7.2).
Endomorphin-1 (10μM, 18h) desensitization of inhibition of cAMP formation does not occur when cells are pretreated in the presence of 10μM naloxone. *Indicates significance in the level of cAMP inhibition between cells pre-treated with endomorphin-1 and cells pre-treated with endomorphin-1 and naloxone, $p<0.05$ by paired Student's t test. Pre-treatment with 10μM fentanyl also caused reduction in the level of cAMP inhibition when cells were re-challenged with 10μM fentanyl. Data are mean±SEM for $n=4$ Experiments were performed on adherent CHOμ cells. cAMP was measured by specific protein binding assay.

Table 7.1 shows the absolute level of cAMP production (expressed in pmol well$^{-1}$) under basal and forskolin stimulated conditions in control, 0.5h endomorphin-1 pre-treatment (i.e. when there was no loss of cAMP inhibition) and 18h endomorphin-1 pre-treatment (i.e. when there was loss of cAMP inhibition).
Table 7.1

<table>
<thead>
<tr>
<th>E-1 pre-treatment</th>
<th>[cAMP] pmol well⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>Control</td>
<td>UD</td>
</tr>
<tr>
<td>0.5h</td>
<td>UD</td>
</tr>
<tr>
<td>18h</td>
<td>UD</td>
</tr>
</tbody>
</table>

Absolute levels of cAMP produced under basal and forskolin stimulated conditions in control (un-treated) and 0.5h and 18h endomorphin-1 pre-treated CHOμ cells. Data are mean±SEM for n=4. UD = undetectable *Indicates significant difference in the level of cAMP produced following 18h endomorphin-1 (10μM) pre-treatment compared to control, p<0.05, paired Student's t-test. Experiments were performed on adherent CHOμ cells, cAMP was measured by specific protein binding assay.

This data may be suggestive of receptor – G protein uncoupling and / or constitutive activity of the μ-opioid receptor. To further probe this effect, concentration-response curves for the inhibition of cAMP formation between untreated and cells pre-treated with 10μM endomorphin-1 for 18h were examined (figure 7.3). Again, there was a reduction in the maximal levels of cAMP inhibition when cells were re-challenged with high concentrations of endomorphin-1. Moreover, there was a small rightward shift in the concentration-response curves between the two treatments (pEC₅₀ control = 7.8±0.03, pEC₅₀ endomorphin-1 = 7.3±0.2), although this failed to reach statistical significance. In addition, at low concentrations of endomorphin-1 there was an enhancement of cAMP formation to levels above those of forskolin controls (depicted as a negative inhibition), and in agreement with mass measurements above (Table 7.1).
Figure 7.3
Endomorphin-1 produced a concentration-dependent inhibition of forskolin stimulated cAMP formation in control and 18h endomorphin-1 treated cells. A negative inhibition is indicative of cAMP levels above that of forskolin controls. Data is mean±SEM for n=4-7. Whole curves are significantly different (p<0.05) by 2-way ANOVA. *Indicates significant (p<0.05, unpaired Student's t-test) difference between control and 18h endomorphin-1 pre-treated cells when re-challenged with 10mM endomorphin-1, consistent with figure 7.1. Experiments were performed on adherent CHO μ cells, cAMP was measured by specific protein binding assay.

7.5 Radioligand binding 4°C time course
Since radioligand binding for desensitization studies was carried out at 4°C, it was necessary to perform a time course for binding at this temperature. Figure 7.1 shows the results of this, and from this it was decided to incubate all radioligand binding experiments conducted at 4°C for 3h.
Time course for binding of $[^3H]DPN$ to whole adherent $CHO_{\mu}$ cells at $4^\circ$C. Data are mean±SEM for $n=6$. Experiments were performed using a saturating concentration of $[^3H]DPN$ (~2.5nM), non-specific binding was defined using 10$\mu$M naloxone.

7.6 GTP$_\gamma$S shifts

In order to determine if G protein - receptor uncoupling occurred, endomorphin-1 displacement studies in the presence and absence of GTP$_\gamma$S, a non-hydrolyzable form of GTP, were performed. In membranes prepared from un-treated and 0.5 h treated cells, addition of GTP$_\gamma$S produced a significant rightward shift in the concentration response curves. This was not present in membranes prepared from cells that had been treated with endomorphin-1 for 18h (figure 7.5 and table 7.2).
Figure 7.5

Endomorphin-1 produced a concentration-dependent displacement of $[^3H]$DPN binding to CHOµ membranes in the presence or absence of 50µM GTPγS. Data are mean±SEM for n=5. Whole curves and $p_K_i$ values were significantly different (p<0.05, ANOVA, paired Student's $t$-test respectively) in the presence and absence of GTPγS for membranes prepared from untreated (0h) and 0.5h endomorphin-1 pre-treated cells. No significant difference was observed in membranes prepared from 18h endomorphin-1 pretreated cells. Studies were performed at 4°C in 1ml volumes of Tris buffer for 3h with a fixed concentration (~0.2nM) of $[^3H]$DPN and endomorphin-1 as the displacer. Non-specific binding was defined in the presence of 10µM naloxone.

<table>
<thead>
<tr>
<th>length of E1 pretreatment (h)</th>
<th>p$K_i$ [nM] control</th>
<th>GTPγS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.86±0.11 [13.8]</td>
<td>7.37±0.15 [42.7]$^*$</td>
</tr>
<tr>
<td>0.5</td>
<td>7.92±0.12 [12.0]</td>
<td>7.36±0.08 [43.7]$^*$</td>
</tr>
<tr>
<td>18</td>
<td>7.69±0.11 [20.4]</td>
<td>7.75±0.08 [17.8]</td>
</tr>
</tbody>
</table>

Table 7.2

$p_K_i$ values for endomorphin-1 displacement of $[^3H]$DPN in the presence and absence of 50µM GTPγS. nM values are shown in parentheses. Graphical representation of data is shown in figure 7.5. Data are mean±SEM for n=5, *$p_K_i$ values were significantly different by paired Student's $t$-test in the presence and absence of GTPγS for membranes prepared from untreated (0h) and 0.5h endomorphin-1 pre-treated cells. Studies were performed at 4°C in 1ml volumes of Tris buffer for 3h with a fixed concentration (~0.2nM) of $[^3H]$DPN and endomorphin-1 as the displacer. Non-specific binding was defined in the presence of 10µM naloxone.

7.7 Loss of cell surface receptors

To examine if the loss of cAMP inhibition correlated with loss of cell surface receptors, the effect of varying lengths of endomorphin-1 pretreatment on number of cell surface receptors was examined (Figure 7.6). Cells were pre-treated with 10µM endomorphin-1 for various times, and then the $B_{max}$ was determined using a saturating concentration of $[^3H]$DPN (as protein was not determined absolute values for $B_{max}$ in fmol mg protein$^{-1}$...
cannot be calculated). Endomorphin-1 pretreatment produced a rapid, time dependent loss of cell surface receptors, with all time points after 1 min being significantly reduced (p<0.05, paired Student's t-test) compared to control.

Figure 7.6
Pre-treatment with 10μM endomorphin-1 caused a rapid loss of cell surface receptors. Data are mean±SEM for n=5-20. All time points after 1min are significantly lower (p<0.05, paired Student's t-test) compared to their own control. Inset shows time course for up to 1h endomorphin-1 pre-treatment. Radioligand binding studies were performed at 4°C (to prevent receptor recycling and lipophilic ligands crossing the cell membrane) for 3h on adherent CHOμ cells in 12-well plates in 1ml volumes of Krebs-HEPES buffer containing a saturating concentration of [3H]-DPN (~2.5nM). Non specific binding was defined using 10μM naloxone.
Using crude membranes prepared from untreated and 0.5h endomorphin-1 pretreated cells there was a 63.5±3.8% reduction in cell surface receptors (B\textsubscript{max} control = 767.1±77.8, 0.5h endomorphin-1 = 271.9±24.7 fmol mg protein\textsuperscript{-1}). This was greater than the reduction seen in whole cells (~30%), but was accompanied by a 63.5±3.8% reduction in optical density of μ-opioid receptor-specific stained bands determined by immuno-blotting (see figure 7.7).

**Figure 7.7**

Experiments performed in membranes show a reduction in μ-opioid receptor specific antibody staining, as measured by immunoblotting (left panel) when analysed by optical density of bands. Data are mean±SEM for n=3 in triplicate, insert is representative immuno-blot from n=3 showing 4 lanes - 2 control and 2 0.5h endomorphin-1. Samples were resolved on SDS-PAGE gels, transferred to nitrocellulose, incubated with antibody and visualized using alkaline phosphatase conjugated secondary antibody.

A reduction in B\textsubscript{max} also occurred between membranes prepared from control and 0.5h endomorphin-1 pretreated cells (right panel). Data are mean±SEM for n=5. Experiments were performed at 4°C in 1ml volumes of tris buffer, a saturating concentration of [\textsuperscript{3}H]DPN (~2.5nM), non-specific binding was defined using 10μM naloxone.

*Indicates significant (p<0.05, paired Student's t test) reduction in optical density / B\textsubscript{max} in membranes prepared from 0.5h endomorphin-1 pre-treated cells compared to control.
7.8 Discussion

The data presented here show that endomorphin-1 causes desensitization and down-regulation of the rat \( \mu \)-opioid receptor. However it takes between 8-11h pre-treatment with 10\( \mu \)M endomorphin-1 for the receptor to desensitize. This desensitization is characterized by a reduction in maximal cAMP inhibition and an up-regulation of forskolin stimulated cAMP formation, which is probably due to receptor - G protein uncoupling. Endomorphin-1 also produces down-regulation of the \( \mu \)-opioid receptor; this loss of cell surface receptors is rapid, there is a possible degradation of receptors 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 endomorphin-1 in HEK 293 cells resulted in \( \mu \)-opioid receptor internalization. McConalogue et al., 1999 also showed that a 30 min treatment of KNRK (rat kidney) cells with either endomorphin-1 or endomorphin-1 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 are 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 endomorphin-1 (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 and Medzihradsky, 1997, Kato et al., 1998, Li et al., 1999, Pak et al., 1999). In CHO\( \mu \) cells over-expressing ~3pmol of \( \mu \) receptors, Pak et al., (1999) showed that down-regulation of opioid receptors occurred by 2 distinct pathways when cells were treated with 1\( \mu \)M DAMGO for 1h. 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 CHO\( \mu \) cells \( (B_{\text{max}} = 8\text{pmol mg protein}^{-1}) \) loss of cell surface receptors was
also independent of G protein coupling but the reduction in the level of cAMP 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 ($B_{\text{max}} = 750\text{fmol mg protein}^{-1}$, see chapter 3) 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 8-11h) to cause loss of functional coupling. Indeed in C6 glial cells, 12h agonist pretreatment was required to induce uncoupling (Yabaluri and 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. It is envisaged that receptors are removed from the cells 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 receptor loss has been observed strongly suggests that receptors have been degraded, even after 0.5h of agonist pretreatment although this will require further detailed study.

Because there was such a rapid loss of cell surface receptors, one view may be that this was not a true loss of cell surface receptors, but a reduction in $B_{\text{max}}$ due to endomorphin-1 still being bound to the receptor. Without having labeled endomorphin-1, it is difficult to dispute this unequivocally. However, wash-off experiments have been conducted, where endomorphin-1 was applied to cells, immediately (~3sec later) washed off, and $B_{\text{max}}$ found to be no different than control. However, the kinetics (i.e. on rate) of endomorphin-1 binding to the receptor are not known. Secondly, it would be expected that there would probably be some loss of cell surface receptors before 18h. In this series of experiments there was no difference between time points 0.5h and 18h, i.e. no further reduction after 0.5h. In addition, experiments conducted with membranes, which were conducted with cells that had been washed 5x and then subjected to a further 3x 40ml wash also resulted in a loss of receptors.
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 (Murray et al., 1998). 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 and Simon, 1999a, Kramer and Simon 1999b). 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, Zhang et al., 1999b). In addition in neuro2A 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 and 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 cAMP formation. A small shift to the right did occur but this did not reach statistical significance. In a study by Kato et al., (1998), there was an enhancement of forskolin response following 4h DAMGO pretreatment, but in agreement with our study, no change in the EC50 values for cAMP inhibition between control and DAMGO treated cells. An enhancement of forskolin stimulated cAMP 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 cAMP may be due to constitutive activity (i.e. is not affected by the presence of agonist) of the opioid receptor under investigation.

In conclusion, the results of this chapter demonstrate that endomorphin-1 causes a rapid loss of cell surface μ-opioid receptors. In addition, prolonged treatment causes an uncoupling of receptor from the 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 degraded.
Chapter 8 - General Discussion

The opioid receptor family is the site of action of many clinically important analgesics such as morphine and fentanyl. Endogenous opioids also play an important role in antinociception as well as other central and peripheral functions. Activation of opioid receptors produces effects on cellular signaling pathways which may be both inhibitory, such as those which bring about a reduction in neurotransmitter release and/or stimulatory, although the function of the latter actions is unclear.

8.1 Basic Characterization of cells transfected with recombinant opioid receptors
In chapter 3, The $B_{\text{max}}$ was determined for opioid receptors in a number of different cell lines using the radiolabeled non-selective opioid antagonist diprenorphine. It was also shown that activation of recombinant $\delta$- and $\mu$-opioid receptors expressed in N18 and HEK293 cells respectively caused a concentration dependent inhibition of forskolin stimulated cAMP formation. The $\mu$-opioid receptor expressed in CHO cells was further characterized by fluorescent and confocal microscopy as well as immuno-blotting.

8.1a Evidence for receptor subtypes?
The existence for further division of opioid receptor sub-types remains controversial, although some pharmacological evidence suggests subtypes, there is no genetic evidence for there being more that one, since only three homologous genes have been cloned. Knockout mice have provided further evidence for only one subtype of each receptor, for example knockout of the $\mu$-opioid receptor gene abolishes DAMGO (pharmacological $\mu_1$ and $\mu_2$ non-selective) binding to demonstrating that $\mu_1$ and $\mu_2$ arise from the same gene (Keiffer, 1999). However there remains several possibilities which could account for the pharmacological differences observed including; alternative splicing mechanisms (as discussed in chapter 1, section 1.5), post-translational modifications, or existence of receptor complexes (Rothman et al., 1992; as discussed in chapter 1, section 1.5). One interesting possibility is the interaction with RAMPs. Expression of these proteins have been found to define the pharmacology of the calcitonin receptor-like receptor (CRLR), possibly by participating in ligand binding and determining the glycosylation state of the receptor. A striking example of the role of RAMPs is that when RAMP2 and RAMP3 are expressed with CRLR, this leads to
the expression of the adrenomedullin receptor (Foord and Marshall, 1999). Since RAMPS are expressed more widely than the CRLR (Foord and Marshall, 1999), it is a possibility that these proteins may interact with other receptors, including opioids and may define their pharmacology such that further subtype division is evident.

8.1b Recombinant vs endogenous systems
The majority of studies performed in this thesis are performed using transfected cells and there are numerous advantages of using such systems. For example it allows receptor modifications (such as truncation of the C-terminus, as studied in chapter 4) to be made, transfection of different receptor numbers, and the receptor to be studied in isolation of other receptor systems. However, it must be remembered that the receptor is taken out of its natural environment and therefore may couple to systems (e.g. different G proteins) not normally present in such an environment, or may not be able to couple to effectors normally present in the native environment and thus display altered signaling pathways. As discussed in section 3.1, it is important to remember that expression levels may alter the pharmacology of ligands, e.g. from partial to full agonist and may have a role to play in the controversy as to the efficacy and potency of endomorphin (section 6.5).

8.2 The Effect of C-Terminal Truncation of the Recombinant δ-Opioid Receptor on Ca^{2+} Signaling.
In chapter 4 it was demonstrated that activation of the full length and C-terminal truncated δ-opioid receptor expressed in CHO cells caused a concentration dependent, thapsigargin and PTX sensitive increase in [Ca^{2+}]. Removal of the C-terminus of the δ-opioid receptor caused a significant rightward shift in the concentration response curve for DPDPE mediated Ca^{2+} release. This evidence suggests a role for the C-terminus in mediating the coupling of the receptor to the subsequent release of Ca^{2+}, and may be explained by the differences in the Ins(1,4,5)P_{3} accumulation time course between the full length and the truncated receptor.

8.2a Role of the C-terminus in second messenger coupling
The role of the C-terminus in second messenger coupling has perhaps being most well addressed by measuring cAMP as an endpoint. Cvejic et al., (1996) and Hirst et al.,
(1997) reported that removal of the final 37 amino acids of the rat δ-opioid expressed in CHO cells did not affect coupling to cAMP. Capeyrou et al., 1997 found that whilst mutation of all the Ser and Thr residues to Ala on the C-terminal tail of human μ-opioid receptor expressed in CHO cells did not alter EC₅₀ values for inhibition of cAMP, these mutations caused a reduction in the Eₘₐₓ. It will be of future interest to ascertain what residues of which subtype and species of receptor are important for G protein coupling, since it is generally perceived that this region is involved in attaching to the G protein (Dolman et al., 1991). A study which addressed the question of which regions of the δ-opioid receptor are important in G protein interactions used synthetic peptides corresponding to specific regions of the receptor (Merkouris et al., 1996). Peptides homologous to the third intracellular loop inhibited GTPase activity and reduced [³⁵S]GTPγS binding, pointing to a role of this region in G protein coupling. However, peptides homologous with the C-terminal tail residues 322-333 failed to alter GTPase activity or [³⁵S]GTPγS. Although at first this may imply that the C-terminal tail of the δ-opioid receptor is not involved in signal transduction, residues 322-333 are located on the portion of the C-terminal tail predicted to be close to the plasma membrane and are not ones truncated in δ37 receptors used in this thesis. One area of further study that could prove useful would be to perform GTPγS shifts, as in chapter 7 with CH0δWT and CH0δ37. GTPγS is an agent that uncouples receptors from G proteins by maintaining the α-GTP state. If these experiments were performed, receptors that were able to couple to G proteins would cause a GTP shift, whereas if G protein coupling were impaired there would be a smaller shift, which would be seen if the C-terminal tail of the δ-opioid receptor were involved in coupling, as is predicted by the results of chapter 4.

The effect of C-terminal mutations has also been examined in other GPCR, for example the human somatostatin receptor expressed in CHO cells has been examined (Hucovic et al., 1998). In this study mutant receptors with progressive C-terminal deletions (final 16, 25, 35 or 45 C-terminal amino acids) were used and showed progressive reduction in coupling to adenylyl cyclase, with 45 showing a complete loss of coupling. Intriguingly, mutant receptors retained their ability to associate with G proteins, since there was no correlation between length of C-terminal tail and GTPγS binding. In contrast to this, Schrufstatter et al., (1998) found that the C-terminal tail of the CXCR2
chemokine receptor was important in G protein coupling, as measured by GTPγS binding. Hizaki et al., (1997) found that truncation of the C-terminal tail of the EP3 prostaglandin receptor led to constitutive activity of the receptor, and concluded that in this situation at least, the length of the C-terminal tail is an important determinant for constraint of the receptor in the active form. Numerous studies have examined the effect of C-terminal truncation of GPCR and its involvement in desensitization and down regulation as discussed in chapter 4. 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 (Trapaidze et al., 1996; Cvejec et al., 1996; Murray et al., 1998), and indeed this is the case with other GPCR, for example internalization of the human B2 receptor requires phosphorylation of serine and threonine residues on its carboxy tail (Pizzard et al., 1999). Thus although not all studies agree with a role in coupling and desensitization of the C-terminal tail, many studies point to its role in receptor signaling and regulation, but there may be cell type, receptor type and subtype differences.

8.2b The gonadotropin receptor

The GnRH receptor also belongs to the family of GPCR, but unlike many other members of this family it is unusual in the fact that its intracellular C-terminal tail is only two amino acids long. This receptor undergoes G protein coupling to second messengers (PLC and Ca²⁺) and desensitization and down regulation despite the length of its intracellular C-terminus (Stojilkovic et al., 1994). However, as well as the absence of the intracellular C-terminus, the GnRH receptor displays very little homology to other GPCR, in particular there are differences in amino acid sequences between it and other GPCR from transmembrane (TM) segment 3 to intracellular loop2 as well as between TM2 and TM7 (Stojilkovic et al., 1994). Hence whilst data from this receptor points to lack of involvement of the C-terminal in receptor signaling, it is probable that this receptor uses other regions to mediate receptor signaling.

8.3 Coupling of μ-, δ-, and κ-opioid receptors to [Ca²⁺]i - single cell studies.

In chapter 5 it was demonstrated that activation of recombinant μ-, δ- and κ-opioid receptors expressed in CHO cells caused an increase in [Ca²⁺]i in ~10% of cells and the size of response was variable between individual cells. It is predicted that this a true
response rate to appropriate opioid agonist, and when \([\text{Ca}^{2+}]_i\) is measured in a cuvette system a more 'robust' response is seen due to the greater number of cells.

### 8.3a 'Inhibitory' GPCRs and increases in \([\text{Ca}^{2+}]_i\)

Opioid receptors are not unique to the family of \(G_{i/o}\) coupled GPCRs in causing increased in \([\text{Ca}^{2+}]_i\), other \(G_{i/o}\) coupled receptors (Watling, 1998) have been shown to produce an increase in \([\text{Ca}^{2+}]_i\), as shown in table 8.4

<table>
<thead>
<tr>
<th>receptor</th>
<th>other signaling pathways*</th>
<th>Major receptor functions*</th>
<th>selected references</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2 adrenoreceptors</td>
<td>↘cAMP ((G_i))</td>
<td>inhibition of sympathetic neurotransmission</td>
<td>Dorn et al., 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enkvist et al., 1996</td>
</tr>
<tr>
<td>neuropeptide Y</td>
<td>↘cAMP ((G_i))</td>
<td>modulation of LHRH + CRF release, cardio-respiratory effects, vasoconstriction</td>
<td>Vanderheyden et al., 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Connor et al., 1997b</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>↘cAMP ((G_o))</td>
<td>endocrine functions</td>
<td>Akbor et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>inhibition of cell proliferation</td>
<td>Wilkinson et al., 1997</td>
</tr>
</tbody>
</table>

**Table 8.4**

Examples of other \(G_{i/o}\) coupled receptors that cause an increase in \([\text{Ca}^{2+}]_i\) from intracellular stores. LHRH = luteinizing hormone releasing hormone, CRF = corticotropin releasing factor *from (Watling, 1998)

### 8.3b Physiological role of opioid induced increases in \([\text{Ca}^{2+}]_i\)

It may be argued that because the response rate in CHO cells is low, and no increase in \([\text{Ca}^{2+}]_i\) is seen in SH-SY5Y cells that endogenously express \(\mu\)-opioid receptors, then is what is the physiologically relevance of opioid induced increases in \([\text{Ca}^{2+}]_i\)? Increases in \([\text{Ca}^{2+}]_i\) probably do not result from over-transfection of receptors, since CHOδ cells, which express a lower level of receptors than SH-SY5Y cells produce a DPDPE mediated increase in \([\text{Ca}^{2+}]_i\).
The physiological role of increases in \([\text{Ca}^{2+}]_i\), remain unclear. It may be that intracellular release does not play a primary role in neurotransmission, but may augment \(\text{Ca}^{2+}\) increases from other sources. A number of cellular enzymes and other factors are able to be regulated by \([\text{Ca}^{2+}]_i\), these include PDE1, which catalysis cAMP / cGMP hydrolysis, PLC8 -a phosphatidylcholine specific phospholipase, protein phosphatase 2B (calcineurin) – involved in modulation of phosphorylation cascades (Watling, 1998) and adenylyl cyclase types I, III and VIII (Simmonds 1999). In addition \(\text{Ca}^{2+}\) may bind to calmodulin, which may in turn regulate a variety of calmodulin specific kinases, or regulate NOS (Stryer, 1988). To speculate, it may be that opioids are able to indirectly regulate a variety of functions mentioned above by causing an increase in \([\text{Ca}^{2+}]_i\). For example, an opioid induced increase in \([\text{Ca}^{2+}]_i\) may modulate NOS activation (via calmodulin), which in turn is known to enhance opioid actions (Machelska et al., 1997). Calmodulin has also been shown to interact with the 3\(^{rd}\) intracellular loop of the \(\mu\)-opioid receptor in a \(\text{Ca}^{2+}\) sensitive manner and may compete with G proteins for a shared binding site (Wang et al., 1999).

**8.4 Characterization of the effects of endomorphin-1 and endomorphin-2 in CHO cells expressing recombinant \(\mu\)-opioid receptors and SH-SY5Y cells.**

In chapter 6 it was demonstrated that endomorphin-1 and -2 bound with high affinity and relatively high selectivity to \(\mu\)-opioid receptor sites. In addition to these effects both peptides concentration-dependently inhibited forskolin stimulated cAMP formation in CHO\(\mu\) and SH-SY5Y cells and caused a mobilization of \(\text{Ca}^{2+}_i\) in the former cell line.

The selectivity profile (chapter 6, table 6.1) of these two peptides (~100-1000fold) over other opioid receptors is not as great as that originally reported by Zadina et al., (1997) who reported selectivity of between 4000-15 000 fold. However, even the 100-1000 fold selectivity determined in this thesis is relatively high selectivity when compared to other endogenous ligands (see table 8.1). Indeed it has been proposed that the endogenous \(\kappa\)-opioid receptor ligand, dynorphin A, may be a potential endogenous ligand for not just \(\kappa\)-opioid receptors but also \(\mu\)-, \(\delta\)- and ORL1 opioid receptors (Zhang et al., 1998b).
Ligand | Selectivity
---|---
β-endorphin | $\mu=\delta$, 40x over $\kappa$
met-enkephalin | $\delta$, 10x over $\mu$, 4480x over $\kappa$
dynorphin | $\kappa$, 6x over $\mu$, 20x over $\delta$

*Table 8.1*

*Selectivity of endogenous opioid receptor ligands, selectivity based on $pK_i$ values taken from Corbett et al., (1993)*

However in this chapter it was demonstrated that both peptides had high potency for the inhibition of cAMP (nM range) and these are in agreement with *in vitro* bioassays performed by Zadina’s group (1997) and with other published values.

8.4a Characteristics of endomorphin-1 / endomorphin-2

The endomorphins are structurally different from other opioid peptides in that they do not contain the sequence Tyr-Gly-Gly-Phe, but do contain Tyr$^1$ and Phe$^4$. Opioid peptides have been shown to consist of 2 components, a biologically active N-terminal message sequence and a C-terminal address sequence. The N-terminal sequence appears to require Tyr at position 1, an appropriate spacer and an aromatic group. It is envisaged that that endomorphin-1 and -2 are potent because of their aromatic group (Trp or Phe) in both positions 3 and 4, giving increased receptor binding and increased bioassay activity (Yang *et al.*, 1999) and Pro$^2$ as a stereochemical spacer (Paterlini *et al.*, 2000). Further evidence from NMR spectroscopy and molecular modeling has revealed that the selectivity of endomorphin-1 may be dependent upon spatially distinct selectivity pockets within the ligand (Podlogar *et al.*, 1998). It is interesting to note that nociceptin, whilst bearing a similar structure to dynorphin A only Phe$^4$ is identical to other opioid peptides and it is this amino acid that is influential for ORL-1 recognition (Calo’ *et al.*, 2000).

8.4b A precursor for endomorphin-1 / endomorphin-2?

One of the major challenges for endomorphin pharmacology is the identification of a precursor. Both endomorphin-1 and endomorphin-2 were originally identified by amino acid substitution of a peptide (Tyr-MIF-1, Tyr-Pro-Trp-Gly-NH$_2$) with some opioid activity, (i.e. synthetically) but have since been isolated from bovine (Zadina *et al.*, 2000).
1997) and human (Hackler et al., 1997) brain. Indeed evidence for both peptides has been demonstrated in a variety of mammalian species both by the original authors and other groups, see Table 8.2

<table>
<thead>
<tr>
<th>Species</th>
<th>areas</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat/monkey E2</td>
<td>Superficial dorsal horn (substantia gelatinosa marginal zone, nucleus proprius</td>
<td>Pierce et al., 1998</td>
</tr>
<tr>
<td>Rat E2</td>
<td>Nucleus accumbens septum, thalamic nuclei hypothalamic + amygdala locus ceruleus, PAG spinal cord dorsal horn</td>
<td>Schreff et al., 1998</td>
</tr>
<tr>
<td>Rat E2</td>
<td>Dorsal horn superficial laminae in medulla and spinal cord, DRG</td>
<td>Martin Scild et al., 1997</td>
</tr>
<tr>
<td>Bovine E1</td>
<td>Thalamus hypothalamus striatum frontal cortex</td>
<td>Zadina et al., 1997</td>
</tr>
<tr>
<td>Rat E1 E2</td>
<td>E2- posterior hypothalamus, more in spinal cord NST, more in brain</td>
<td>Martin Schild et al., 1999</td>
</tr>
<tr>
<td>Human E1 E2</td>
<td>fronto-parietal cortex</td>
<td>Hackler et al., 1997</td>
</tr>
</tbody>
</table>

Table 8.2

Localization of endomorphin-1 and/or endomorphin-2 immuno-reactivity in different species. PAG = periaqueductal grey DRG = dorsal root ganglion NST = nucleus of solitary trac.

The majority of endomorphin-1 / endomorphin-2 immuno-reactivity is located near μ-opioid receptor sites, but in other cases it is not, for example the striatum is an area in which there is an abundance of μ-opioid receptors, but no immunoreactivity for endomorphin-1 (Zadina et al., 1999). It may be that the peptide is synthesized in primary sensory neurons in ganglia, then transported to the superficial dorsal horn and released near μ-opioid receptor expressing neurons (Martin-Schild et al., 1997). Endomorphin peptides have also been found to be co-localized with excitatory neuropeptides such as substance P (Martin-Schild et al., 1997, 1998) and CGRP (Pierce et al., 1998), and therefore may modulate the release of these neurotransmitters.
However, despite these effects (and screening of known peptide sequences – J. Zadina, personal communication, International Narcotics Research Conference, 1999) to date no precursor has been identified. For each of the other opioid peptides, definite precursors have been identified. For example β-endorphin is cleaved from POMC (propiomelanocortin). The products of this precursor are not just opioids, but other neuropeptides. POMC contains a signal peptide and three structural domains. POMC is first cleaved to produce adrenocorticotropic hormone and β-lipotropin, β- lipotropin is then further cleaved to produce γ-lipotropin and β-endorphin. Further cleavage yields smaller peptides including melanocyte-stimulating hormone, met-enkephalin and corticotropic-like intermediary peptide (CLIP) (Greenstein and Greenstein, 1999), see figure 8.1. Therefore it would be interesting to see if the precursor for endomorphin-1 / endomorphin-2 is derived from an already known sequence for biologically active peptides.
Figure 8.1
The propiomelanocortin system (adapted from Greenstein and Greenstein 1999; www.isu.indstate.edu/thcme/mwking/peptide-hormones.html). POMC is a precursor for a number of biological peptides as well opioids. ACTH = adrenocorticotropic hormone, LPH = lipotropin, MSH = melanocyte stimulating hormone, CLIP = corticotropin-like intermediary peptide.

8.4c Endomorphins and pain
In chapter 7 it was shown that endomorphin-1 / endomorphin-2 displayed actions typical of opioids, the combined actions of which may be to bring about a reduction in neurotransmission, indeed it has been demonstrated that these peptides bring about a reduction in excitatory neurotransmission / neuronal firing (Chapman et al., 1997, Patel et al., 1999, Doi and Russel, 1997). The next step downstream of a reduction in neurotransmission is analgesia and again endomorphin-1 and endomorphin-2 produce analgesia, although the duration may be dependent upon the route of administration and / or species. For example i.c.v. administration of endomorphin-1 produced long lasting analgesia (over 1 h, ~6x longer than met-enkephalin) (Zadina et al., 1997) whereas i.t. injection of endomorphin-1 / endomorphin-2 produced short acting analgesia (Stone et al., 1997). One interesting finding is that endomorphins appear particularly useful in
models of neuropathic pain and exhibit antinociceptive potency in rats tolerant to morphine (Przewlocka et al., 1999a,b). This raises interesting possibilities at to future clinical uses of the peptides in certain pain states for which morphine is ineffective.

8.4d Differential actions of endomorphin-1 / endomorphin-2

Although many investigators have found that endomorphin-1 and endomorphin-2 display similar actions, there are numerous reports of differential actions of endomorphin-1 and endomorphin-2 as summarized in table 8.3.

<table>
<thead>
<tr>
<th>Endomorphin-1</th>
<th>Endomorphin-2</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduction in dorsal horn Aβ evoked response</td>
<td>No reduction in dorsal horn Aβ response</td>
<td>Chapman, et al., 1997</td>
</tr>
<tr>
<td>Potent spinal analgesia</td>
<td>Less potent than E1 spinally</td>
<td>Zadina et al., 1997, Goldberg et al., 1998, Przewlocki et al, 1999a</td>
</tr>
<tr>
<td>Anti-nociception via pharmacological μ2</td>
<td>Anti-nociception via pharmacological μ1</td>
<td>Sakurada et al., 1999</td>
</tr>
<tr>
<td>Naloxone reversible inhibition of tachykinergic bronchial contractions</td>
<td>Naloxone insensitive inhibition of tachykinergic bronchial contractions</td>
<td>Fischer and Undem, 1999</td>
</tr>
<tr>
<td>Anti-nociception via G₁₁, G₁₃ and G₂</td>
<td>Anti-nociception via G₁₁, G₁₃, G₂</td>
<td>Sanchez-Blanzquez et al., 1999</td>
</tr>
<tr>
<td>Analgesia via μ-opioid receptor</td>
<td>Activation of different μ-opioid receptor, induces dynorphin release and analgesia via κ receptor</td>
<td>Tseng et al., 2000</td>
</tr>
</tbody>
</table>

Table 8.3

Summary of some differential actions of endomorphin-1 and endomorphin-2

It should be noted that not all investigators have found these differences, for example both endomorphin-1 and endomorphin-2 display naloxone reversible inhibition of
acetylcholine release from guinea-pig and human trachea (Patel et al., 1999). Clearly further work is needed to clarify these differences, for example; are there species, preparation or methodological differences? At present the reasons behind them remain unclear.

8.5 Receptor desensitization and down regulation
In the final results chapter of this thesis it was demonstrated that endomorphin-1 caused desensitization of the recombinant μ-opioid receptor expressed in CHO cells. This desensitization was characterized by a reduction in maximal cAMP inhibition, which occurred between 5-18h of pretreatment with 10μM endomorphin-1, and was accompanied by an up-regulation of forskolin stimulated cAMP formation. This desensitization is probably due to receptor - G protein uncoupling. Endomorphin-1 also produces down-regulation of the μ-opioid receptor; this loss of cell surface receptors is rapid and the time course did not mirror that for loss of a functional response. It is also possible that there is degradation of receptors between 0-0.5h 10μM endomorphin-1 pretreatment.

8.5a Mechanisms of desensitization and down regulation
As discussed in chapter 7, it has become apparent that desensitization and down-regulation is dependent upon a large number of factors including cell type, subtype of receptor, species of receptor and ligands used. Therefore it is difficult to propose a simple model for opioid receptor desensitization and down regulation. However, a model of endomorphin-1 down regulation / desensitization in CHO cells expressing recombinant μ-opioid receptors was proposed in the previous chapter, and is summarized below in figure 8.2.
Proposed model for endomorphin-1 induced desensitization and down regulation in CHO cells. Blue hexagon = endomorphin-1, green square = G protein. Down regulation may be independent of G protein coupling, and may involve receptor degradation. Desensitization involves loss of G protein coupling.

In this model it is envisaged that receptors are endocytosed into clathrin-coated pits, taken into vesicles then degraded. Although this cannot be proved since it was not determined experimentally in this thesis, evidence for endocytosis comes from the fact that receptors co-localize with transferrin receptor after internalization and in which this receptor internalization can be blocked by the dominant negative mutant of arrestin or dynamin (Law et al., 2000). At 20,000g vesicles will be sedimented with the crude membrane preparation so a reduction in receptor number in binding or immuno-blotting experiments has been attributed to degradation since degraded receptors will not be centrifuged into the membrane preparation due to their mass. Clearly further work, possibly involving sucrose gradients to determine membrane and cellular fractions will be needed. Cell type specific differences have also been noted for other GPCRs, for example the human β3 adrenergic receptor desensitizes (measured by cAMP accumulation) in SK-N-MC and HEK293 cells but not in CHO cells or rat adipocytes (Chaudry and Grannemon, 1994).
In a recent study, Tsao and von Zastrow (2000) examined down regulation of both the δ-opioid receptor and the β-adrenergic receptor co-expressed at similar levels in HEK293 cells and found distinct differences in the endocytic pathway between the two receptors. Three hour pre-treatment with appropriate agonist causes negligible down regulation of the β-adrenergic receptor, whereas ~50% of δ-opioid receptors down regulate. β-adrenergic receptors undergo rapid recycling to the plasma membrane after agonist removal, whereas δ-opioid receptors are retained in the endocytic pathway then subjected to degradation. These observations suggest that different GPCRs can differ in their endocytic trafficking even when co-expressed in the same cell.

8.5b Is phosphorylation important?
It is often perceived that phosphorylation is the first step in decreased receptor responsiveness (Lefkowitz, 1998), and evidence may come from 1) directly measuring phosphorylation of the receptor and determining which kinases are involved 2) removing / mutating potential phosphorylation sites on the C-terminus of the receptor. Several different groups have implicated the role of kinases in opioid receptor desensitization /down-regulation including GRKs (Hasbi et al., 1998; Zhang et al., 1998a; Li et al., 1999) PKC (Kramer and Simon, 1999a; Kramer and Simon 1999b) and MAP kinase (Schmidt et al., 2000). Indeed receptor recycling may be mediated by de-phosphorylation (Hasbi et al., 2000). However cell specific differences are apparent and a definite link between phosphorylation, desensitization and down regulation is difficult to confirm, especially since phosphorylation is a rapid event and numerous studies have shown that uncoupling is not (Law et al., 2000). However the M1 muscarinic receptor expressed in CHO cells undergoes rapid desensitization (detectable at 10s) measured by Ins(1,4,5)P₃ accumulation, and is accompanied by casein kinase phosphorylation, but does not involve internalization (Waugh et al., 1999). It would be interesting to conduct phosphorylation studies on CHOδ receptors especially since it is postulated that desensitization and down-regulation proceed by distinct mechanisms. The role of the C-terminus has been examined for its involvement in internalization since it contained putative phosphorylation sites on the C-terminus, and as was discussed in Chapter 4 many groups have found this to be important in opioid receptor activities. This correlates with the findings that the C-terminus of other GPCR is important in internalization such as the angiotensin II receptor, where maximal endocytosis requires
phosphorylation of the C-terminus (Thomas et al., 1998) and the bradykinin receptor which requires phosphorylation of its C-terminal tail to internalize (Pizard et al., 1999).

8.5c Morphine and receptor regulation
Morphine is an opioid that is in widespread clinical use, and to which tolerance develops (Collette, 1998). However, morphine is unusual in that in the majority of cases it does not cause internalization (reviewed in Law et al., 2000). It has been suggested that this is because in the laboratory setting, morphine is a partial agonist (Lambert et al., 1993), and may thus regulate receptor function differently (Yabaluri and Medzihradsky, 1997; Schultz et al., 1999). Some studies have found that there is a link between down-regulation and agonist efficacy (e.g. Remmers et al., 1998) and attempts have been made to correlate this (Clarke et al., 1999) but again there is no clear answer from the wide range of published work available. The obvious question is that if morphine causes clinical tolerance, yet morphine does not down regulate the μ-opioid receptor like other ligands how does morphine cause this effect and what is the contribution of down regulation to effects seen clinically? The answers probably lie in the fact that clinically, tolerance does not result from one single mechanism but an interaction of many.
Further work

The data presented in this thesis has made a valuable contribution to our understanding of opioid receptor signaling pathways, however there are some areas in which further work would enhance our understanding of topics the addressed in this thesis, including;

1. Why is the opioid induced increase in $[\text{Ca}^{2+}]_i$ in CHO cells expressing recombinant opioid receptors low? What is the functional role for opioid induced increases in $[\text{Ca}^{2+}]_i$? Are there ways of increasing this so that it may be used for practical applications, e.g. screening?

2. What is the precursor for endomorphin?

3. Could endomorphin (or endomorphin analogues) be useful in certain pain states, e.g. neuropathic pain for which at present traditional opioid therapy is ineffective?

4. Does endomorphin 1 and -2 (and indeed other opioid) desensitization / down regulation depend upon the cell type? What is the desensitization / down regulation profile in cells endogenously expressing $\mu$-opioid receptors (e.g. SH-SY5Y cells)? Does it occur at physiological concentrations and does it have a role under (patho)physiological conditions?
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Publications arising from this thesis

Papers

Reviews, editorials and book chapters

Abstracts

