Generation of an inducible expression system to perform molecular pharmacology studies with mu opioid receptors

Author: Arlet María Acanda de la Rocha

Scientific Supervisor: Dr. Juan Francisco López-Giménez

Institute of Biomedicine and Biotechnology of Cantabria (IBBTEC)
CSIC-UC-SODERCAN, Santander, Spain

Molecular Biology and Biomedicine Master Program
University of Cantabria
Introduction

G-protein-coupled receptors

G protein-coupled receptors (GPCRs) are proteins very highly conserved through evolution and thereby expressed in nearly all organisms ranging from yeast to human beings. GPCRs are encoded by almost 1% of the human genome and targets more than 40% of the drugs nowadays used in therapeutics (Ma and Zemmel, 2002). The chemical diversity found among the endogenous ligands is exceptional. Thus they include biogenic amines, peptides, glycoproteins, lipids, nucleotides, ions, and proteases. Moreover, the sensation of exogenous stimuli, such as light, odors, and taste, is mainly mediated via this class of receptors (Kolakowski, 1994; Hoon et al., 1999).

According to a commonly used classification scheme (Horn et al., 2003), most GPCRs are grouped in classes A–E. Class A is the most widespread and contains rhodopsin-like GPCRs, class B contains the secretin-like GPCRs, while class C the metabotropic glutamate/pheromone receptors. These are the main classes of receptors in animals. The rhodopsin family is by far the largest and most diverse of these families, and members are characterized by conserved sequence motifs that imply shared structural features and activation mechanisms. The link between an activated GPCR and the cell physiological responses is a heterotrimeric G-protein (αβγ) within the interior of the cell that interacts with the activated receptor. Four families of G-proteins are defined in the literature, i.e. $\text{G}_{\alpha i/o}$, $\text{G}_{\alpha q/11}$, $\text{G}_{\alpha s}$ and $\text{G}_{\alpha 12/13}$, (Elefsinioti et al., 2004), based on sequence similarity among different α subunits that are the main determinants of G-protein coupling specificity.

Despite their molecular and functional diversity, all GPCRs share a similar structure consisting of seven transmembrane domains linked by alternating intracellular and extracellular loops, presenting the amino terminus located on the extracellular side and the carboxy terminus in the intracellular side (Rosenbaum et al., 2009). Extracellular domains, which vary among the different classes of GPCRs, contribute to ligand recognition and binding, whereas coupling to G proteins is determined mainly by interactions with intracellular domains (Kristiansen, 2004). When a GPCR agonist binds to the extracellular domain it induces a change in the conformation of the receptor. This, in turn, leads to coupling to and activation of one or more G proteins inside the cell.

GPCRs act at the heterotrimeric G proteins as guanine-nucleotide exchange factors; thus, the activated receptor induces a conformational change in the associated G protein α-subunit leading to release of GDP followed by binding of GTP (Bourne et al., 1991). Subsequently, the GTP-bound form of the α-subunit dissociates from the receptor as well as from the stable βγ-dimer. Both the GTP-bound α-subunit and the released βγ-dimer might modulate several cellular signaling pathways. These include, among others,
stimulation or inhibition of adenylate cyclases and activation of phospholipases, as well as regulation of potassium and calcium channel activity (Hamm et al., 1998).

To date, four opioid receptors have been cloned: μ-, δ-, κ-, and nociceptin/orphanin FQ receptors (Waldhoer et al., 2004). The opioid family assignment is based on sequence and functional homology, and conservation of exon structure and gene regulation (Law et al., 2004). The μ-, δ-, and κ-opioid receptors are about 60% identical to each other, with the greatest identity found in the transmembrane domains (73–76%) and intracellular loops (86–100%). The greatest divergent areas were found in the N-terminus (9–10%), extracellular loops (14–72%) and the C-terminus (14–20%) (Chen et al., 1993).

**Mu-Opioid Receptors**

Mu-opioid receptors (MOP) are members of GPCRs superfamily, and they mainly modulate the function of effector molecules, such as adenylate cyclase and protein kinases (Standifer and Pasternak, 1997). They are coupled primarily to G proteins of the Gi/Go family. Signaling from MOP receptors is rapidly regulated by a well-characterized and highly conserved cascade of events involving receptor phosphorylation by G protein-coupled receptor kinases (GRKs) and subsequent β-arrestin recruitment (Ferguson, 2001). These processes contribute directly to rapid GRK- and β-arrestin-mediated desensitization by facilitating the uncoupling of the receptor from its G protein.

Following desensitization by GRKs and β-arrestin, opioid receptors are then rapidly endocytosed into intracellular compartments. This process occurs following even brief agonist exposure and independently of signal transduction (Arden et al., 1995). The speed and conservation of this process is ideal for modulating signaling from endogenous ligands, such as neurotransmitters, that are released in a pulsatile manner. Following their endocytosis, receptors can then be recycled back to the membrane, thereby restoring the functional complement of receptors, a process termed “resensitization.” In contrast, chronic exposure of MOP receptors to agonist, for example, during exogenous drug administration, can also lead to receptor desensitization/uncoupling.

Receptors that have been desensitized and rapidly endocytosed are uniquely poised to make an important decision with substantial impact on future signal transduction. As mentioned above, following endocytosis receptors can be recycled thereby restoring the functional complement of receptors. Alternatively, receptors that have been endocytosed can be targeted for degradation, thereby decreasing the functional complement of receptors ultimately resulting in receptor downregulation. Although endocytosis and subsequent degradation of receptors are not the only means of
producing receptor downregulation, they can produce receptor downregulation rapidly, even following brief exposure to agonist (Tsao and von Zastrow, 2000).

Endocytosis occurs by various mechanisms, which can be divided into those that are clathrin dependent and those that are clathrin independent (Conner and Schmid, 2003; Doherty and McMahon, 2009). In clathrin dependent endocytosis (CDE), the cytoplasmic domains of plasma membrane proteins are specifically recognized by adaptor proteins and packaged into clathrin-coated vesicles that are brought into the cell. CDE is facilitated by numerous accessory proteins, requires the GTPase dynamin for vesicle scission and has been widely studied (Conner and Schmid, 2003; Doherty and McMahon, 2009). Clathrin-independent endocytosis (CIE), in contrast, might occur in many forms and has been less well studied. There are also specialized actin-driven CIE pathways (Swanson, 2008), such as macropinocytosis and phagocytosis, and these might be stimulated forms of CIE (Donaldson et al., 2009). There seem to be several distinct mechanisms of CIE (Mayor and Pagano, 2007) that might reflect, to some extent, the different cell types and cargo molecules monitored. Caveolar endocytosis involves the caveolin coat, is dynamin dependent and is the mechanism that glycosphingolipids use for cell internalization (Mayor and Pagano, 2007).

The role of endocytosis in modulating signaling from the receptor comprises both endocytic events (desensitization) and post-endocytic sorting events (resensitization or downregulation).

Many GPCRs display a certain level of basal signaling activity and thus can activate G proteins in the absence of agonists, this process is known as constitutive activity. MOP receptors have also been shown to exhibit basal signaling activity in SH-SY5Y cells and in transfected HEK293 cells (Wang et al., 1994; Burford et al., 2000) and display more elevated constitutive activity following chronic exposure to morphine (Wang et al., 1994).

Morphine and other derivatives of this alkaloid compound are drugs widely known for its analgesic properties in clinical practice as well as by its consumption as recreational drugs. In the nervous system, MOP receptors drive the initial steps of both the positive effects of morphine, i.e. relief of intense pain, and its addictive effects.

Morphine and other opioids remain the analgesics of choice for the treatment of chronic pain. However the major limitation to their long-term use is the development of physiological “tolerance,” a profound decrease in analgesic effects observed in most patients during prolonged drug administration. In addition to tolerance, physiological “dependence,” which results in the necessity for continued administration of increasing doses of drug to prevent the development of symptoms of opioid withdrawal, can ensue in some patients. Not all opioid drugs generate the same degree of tolerance and dependence in experimental animals when administered chronically at equi-effectives
analgesic doses, therefore this fact leads to the consideration that binding of these agonists to MOP receptor should produce different effects at the cellular and molecular level (Lopez-Gimenez and Milligan, 2010). Morphine is distinguished from other agonist drugs that bind and activate mu opioid receptors by its marked ability to generate tolerance and dependence when is used for prolonged treatments. Interestingly and in contrast to other alkaloid drugs, morphine is inefficient in promoting MOP receptor endocytosis. This peculiarity has generated a plethora of scientific debates and controversy (von Zastrow et al., 2003; Bailey and Connor, 2005; Koch and Hollt, 2008).

Heterologous Expression Systems

The development of recombinant cDNA methodology, combined with a wide range of molecular and cell biology techniques, has made feasible the expression of particular genes of interest into a vast variety of host cells. Heterologous expression systems provide a cellular context in which properties of a given receptor may be studied in the absence of the native intricate background, alleviating the complexity due to the presence of multiple receptor sub-types and/or to coupling to more than one class of G-proteins. In most cases, heterologous expression allows the analysis of single receptor subtypes in a defined system, and in addition provides easy access to human receptors outside their native tissues.

Particularly in our field of interest, such heterologous expression systems have been extremely useful to investigate pharmacological and molecular properties of each opioid receptor subtype when expressed individually. Furthermore, these experimental models allow the expression of genetically modified receptors in a way that facilitates their study, for example containing a short amino acid sequence as an epitope that is recognized specifically by an antibody. The use of such epitope-tagged receptors has provided means to employ biochemical techniques to facilitate the cellular and molecular studies of these GPCRs.

In the present study we used the Flp-In™ T-REx™ as heterologous expression system in order to expressed our gene of interest, MOP receptor, in an inducible manner. Briefly, MOP receptor was sub-cloned into pcDNA5 vector and subsequently transfected into the Flp-In mammalian host cell line.
Parental Flp-In Hek293 cells contain the Flp Recombination Target site and expressed the Tet repressor (Fig. 1). After transfection, the pcDNA5 integrates at the Flp Recombination Target site and the Tet repressor protein binds to the Tet-Operon region of the integrated pcDNA5, which controls the gene expression by repressing it. Doxycycline binds the Tet repressor after addition to the cells, releasing therefore the Tet operator and enabling MOP receptor expression.

**Fluorescent Proteins**

The discovery and development of fluorescent proteins from a wide variety of organisms has initiated a revolution in the study of cell biology by providing convenient markers for gene expression and protein targeting in living cells and organisms. The resulting fusion product can be used to localize previously uncharacterized proteins or to visualize and track known proteins to further understand cellular events. The use of fluorescent proteins as a minimally invasive tool for studying protein dynamics and function has been stimulated by the engineering of genetic variants with improved brightness, photostability and expression properties. Cells that express gene products tagged with fluorescent proteins can be imaged with low light intensities over many hours to provide useful information about changes in the steady-state distribution of a protein over time (Tsien, 1998).
Red Fluorescent Proteins

The first coral-derived fluorescent protein to be extensively utilized was derived from Discosoma striata and is commonly referred to as DsRed. Maturation of DsRed fluorescence occurs slowly and proceeds through a time period when fluorescence emission is in the green region. Furthermore, DsRed is an obligate tetramer and can form large protein aggregates in living cells. Although these features are inconsequential for the use of DsRed as a reporter of gene expression, the usefulness of DsRed as an epitope tag is severely limited. A few of the problems with DsRed fluorescent proteins have been overcome through mutagenesis. The second-generation DsRed, known as DsRed2, contains several mutations at the peptide amino terminus that prevent formation of protein aggregates and reduce toxicity. In addition, the fluorophore maturation time is reduced with these modifications (Bevis and Glick, 2002)

mCherry is a red monomeric fluorescent protein derived from the tetrameric Discosoma protein DsRed. The mCherry coding sequence has been human codon-optimized to allow optimal expression in mammalian cells. mCherry matures very rapidly (t1/2=15 min.), making it possible to see results very soon after the gene expression (Shaner et al., 2004). Is highly photostable and resistant to photobleaching, and as a monomeric fluorescent protein is often ideal for fusions as they tend to be the least disruptive to the function of the protein to which it is fused. Properties of mCherry fluorescent protein are shown in Table 1.

Table 1. Compilation of properties of mCherry fluorescent protein. Along with the common name, the peak absorption and emission wavelengths (given in nanometers), molar extinction coefficient, quantum yield, relative brightness, and in vivo structural associations are listed. The computed brightness values were derived from the product of the molar extinction coefficient and quantum yield, divided by the value for EGFP.

<table>
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<tr>
<th>Protein (Acronym)</th>
<th>Excitation Maximum (nm)</th>
<th>Emission Maximum (nm)</th>
<th>Molar Extinction Coefficient</th>
<th>Quantum Yield</th>
<th>in vivo Structure</th>
<th>Relative Brightness (% of EGFP)</th>
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<td>587</td>
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Objectives

Taking into account the central role of MOP receptor in the different cellular signaling pathways and physiological effects promoted by morphine, the main objective of our research is the generation of an inducible heterologous expression system harboring MOP receptor to conduct further investigations on its molecular pharmacology. To meet this objective we set the following tasks:

- Generation of a stable cell line expressing MOP-Cherry receptor in an inducible manner
- Fluorescence microscopy studies
- Pharmacological studies

Materials and Methods

Cell Culture

Modified human embryonic kidney (Flp-In HEK293) cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (CLINOR), 10ml/L penicillin G/streptomycin (CLINOR), 1mg/ml Blasticidin (IBIAN Technologies) and 100mg/ml hygromicine (IBIAN Technologies). Cells were maintained at 37°C in a humidified environment containing 5% CO₂. When indicated, cells were incubated with 500 µg/mL concanavalin A (Con A) (SIGMA), 100 ng/ml Pertusis toxin (PTX) (SIGMA), 10⁻⁵ M naloxone hydrochloride or naltrexone (SIGMA) or 80 µM dynasore monohydrate (SIGMA) for the different periods showed in results.

Receptor Fusions with Fluorescent Proteins

A human mu opioid (MOP) receptor C-terminally tagged with enhanced mCherry fluorescent protein (mCherry) was constructed by amplifying the sequence corresponding to the receptor by PCR primers containing a Hind III endonuclease site at the 5`end and a Not I endonuclease site at the 3` end removing in the process the stop codon. This PCR product was ligated to the fluorescent protein sequence amplified by PCR and containing the same endonuclease restriction site (NotI). The final product of this ligation corresponds to a single open reading frame encoding the receptor fluorescent protein fusion. MOP-Cherry was subcloned into the vector pcDNA5/FRT/TO (Invitrogen) for the subsequent generation of Flp-In T-REx HEK293 cell line.

Generation of Stable Flp-In T-REx HEK293 Cell Line

To generate Flp-In T-REx HEK293 cell line able to express MOP-Cherry receptor in an inducible manner, cells were transfected with a mixture containing MOP-Cherry
receptor cDNA into pcDNA5/FRT/TO vector and pOG44 vector (1:9). DNA solution [1.5 µg] was mixed with 1mg/ml of Polyethylenimine (PEI) reagent (Polysciences) and 300 µL of DMEM medium (serum-free) and incubated for 10 min at room temperature. When co-transfected with the pcDNA5/FRT plasmid into the Flp-In mammalian host cell line, the Flp recombinase expressed from pOG44 mediates the integration of the pcDNA5/FRT vector containing the gene of interest into the genome via Flp recombination target (FRT) sites. Clones resistant to hygromicine were collected and MOP-Cherry receptor expression was assessed by fluorescence microscopy.

To induce expression of MOP-Cherry receptor, cells were treated with varying concentrations of doxycycline (Sigma) for different periods. The optimal expression of MOP-Cherry receptor was achieved after 24h of treatment with 0.01 µg of doxycycline/ml growth medium.

**Living Cell Epifluorescence Microscopy**

Cells expressing MOP receptors tagged with mCherry were grown on poly-D-lysine-treated coverslips. Coverslips were placed into a microscope chamber containing physiological saline solution (130mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES and 10 mM D-glucose, pH 7.4). Fluorescent images of the cells were acquired using a LEICA AF6500 Fluorescence Microscope equipped with a 63X (numerical aperture 1.3) oil immersion lens. For internalization experiments in real time, drugs diluted in physiological saline solution were perfused into the microscope chamber, and pictures were taken at the different time points displayed in every picture.

**Cell lysates**

Flp-In MOP-Cherry cells were split into 6-well plates in DMEM medium supplemented with 5% fetal bovine serum, adding doxycycline either at different concentrations and at different time points. Cells were placed on ice, DMEM medium was removed and cells were washed and lysed with RIPA 1x buffer (100mM HEPES, 300mM NaCl, 2% TritonX-100, 1% sodium deoxycholate, supplemented with 0.2% SDS, 250mM NaF, 0.5M EDTA-Na₂, 0.5M NaH₂PO₄, 5% ethylene glycol, pH 7.3) supplemented with a protease inhibitor cocktail tablet (Roche). Cellular extracts were then centrifugated for 10 minutes at 14000 rpm, 4°C and the supernatant was recovered. Total protein concentrations were determined by the Bradford method using BSA as standard, and cell lysates were stored at -20°C until use.

**Western Blotting**

Samples were resolved by 4-12% Bis-Tris gel electrophoresis (Invitrogen) and transferred to nitrocellulose membranes. Blots were incubated in 5% non-fat milk-0.1% Tween 20/TBS-buffered solution at room temperature on a rotating shaker for 2h to block non-specific binding sites. The membrane was then incubated with an anti-µ antibody against the last 43 amino acid from C-terminus in blocking buffer at 4°C overnight. Blots were washed three times with TBS-0,1% Tween-20 buffer for 10 min each at room temperature and incubated with secondary antibody (anti-Goat peroxidase
conjugated IgG) (SIGMA) at 1:200000 in TBS-0.1% Tween-20 buffer for 1 h at room temperature. Blots were washed and visualized by ECL Prime Western Blotting Detection Reagent (Amersham).

Cell Membrane Preparation
Harvested pellets from Flp-In MOP Cherry cells kept at -80°C were thawed and resuspended in 10 mM Tris and 0.1 mM EDTA, pH 7.4 (Tris/EDTA buffer). Cells were homogenized by 25 passes of a glass-on-Teflon homogenizer. The resulting suspension was centrifuged at 1200g for 10 min to remove unbroken cells and nuclei. The supernatant was subsequently centrifuged at 20000 rpm for 1h in an Avanti centrifuge J-30I (Beckman Coulter, Fullerton, CA). Resulting pellets were resuspended in Tris/EDTA buffer and passed 10 times through a 25-gauge needle. Protein concentration was assessed as above, and membranes were stored at -80°C until use.

Cell membranes (14 µg of protein) were incubated with[^3]H]diprenorphine (0.02-2 nM in saturation assays) in a total volume of 1 ml of buffer (50 mM Tris-HCl, 1 mM EDTA, and 10 mM MgCl₂, pH 7.4). Nonspecific binding was determined by the inclusion of 10⁻⁴ M naloxone. Binding was initiated by the addition of membranes, and tubes were incubated at 25°C for 60 min. The assay was terminated by rapid filtration using a cell harvester (Brandel Inc.) with three 5 ml washes of ice-cold phosphate-buffered saline (80 g NaCl, 2 g KCl, 14.4 g Na₂HPO₄, 2.4 g KH₂PO₄, pH 7.4). Filters were soaked in 3 ml of scintillation liquid, and radioactivity was determined in a scintillation counter (Beckman Coulter LS 6000IC).

Cell membranes (14 µg of protein) were incubated in buffer (20 mM HEPES, 100 mM NaCl, and 4 mM MgCl₂, pH 7.4) containing 10 µM GDP and DAMGO, as the agonist ligand, at different concentrations in dose response curves. For single point experiment cell membranes were incubated with DAMGO [10⁻⁵ M] and Naloxone [10⁻⁵ M], used as an inverse agonist ligand. All experiments were performed in triplicate. The reaction was initiated by the addition of cell membranes, and then membranes were incubated at 30°C for 60 min in the presence of 0.1 nM[^35]S]GTPγS. The reaction was terminated by rapid filtration with a cell harvester (Brandel Inc.) and three 5 ml washes with ice-cold phosphate-buffered saline. Radioactivity was determined as described above.
Results

We first proceeded to the amplification and ligation of a cDNA encoding for human MOP receptor to the sequence corresponding to mCherry fluorescent protein, in order to obtain a final single open reading frame chimeric protein by the fusion of mCherry to the carboxyl terminus of MOP receptor. This construct of 1920 bp was subcloned into pcDNA5/FRT/TO vector to be subsequently transfected into the tetracycline/doxycycline-inducible Flp-In locus of Flp-In T-REx HEK293 cells. After selection of positive clones by antibiotic resistance, we obtain a stable cell line expressing MOP-Cherry in an inducible manner (Fig. 2).

![Figure 2](image)

**Figure 2.** Schematic representation of the stable cell line generation. cDNA encoding for human MOP receptor was amplified and ligated to the sequence corresponding to mCherry fluorescent protein using enzymatic digestion. This construct of 1920 bp was subcloned into pcDNA5/FRT/TO vector to be transfected into Flp-In T-REx HEK293 cells to get its insertion in the inducible locus by homologous recombination.

The expression and cellular distribution of MOP-Cherry was assessed by means fluorescence microscopy in living cells. We performed a curve of doxycycline (DOX) concentrations and chose the optimal concentration (0.01 µg/ml) for our experimental model (Fig. 3C). Time course experiments of DOX treatment revealed, firstly, a MOP-Cherry expression dependent of DOX induction (Fig. 3B), i.e., red fluorescence was undetectable in the absence of DOX beside a modest signal corresponding to cell autofluorescence (Fig. 3A). Conversely, DOX treatment resulted in the expression of MOP-Cherry receptors observed as specific red fluorescence signal distributed in punctuated and moving vesicles throughout cells (Suplementary material 1). The addition of DOX to the cell growth medium showed a time-dependent increase in MOP-Cherry expression (Fig. 3B) that was accompanied by the augmentation of intracellular vesicles (Fig. 3A).
**Figure 3.** Evaluation of MOP-Cherry receptor expression by DOX treatment in Flp-In HEK293 cells. **A**, Images were obtained after illuminating living cells with settings to detect mCherry (gray) and Hoechst 33342 (blue) fluorescence. Cells were treated with DOX (0.01 µg/ml) for different times displayed at the upper left corner of each picture. Addition of DOX resulted in a time-dependent increase of MOP-Cherry receptor expression that was paralleled by the augmentation of intracellular vesicles. **B**, Western Blot (WB) analysis was performed with cell lysates from cells non treated (c), or treated with DOX for different periods. **C**, WB analysis performed with cell lysates treated with varying concentrations of doxycycline for 24 h.
To further investigate on the nature of these trafficking vesicles, cells were treated for different periods with Concanavalin A, an endocytosis inhibitor (Fig. 4). Inhibition of vesicle formation was not apparent after 30 min of treatment (Supplementary material 2), observing a total inhibition after 24h of endocytosis inhibitor presence (Supplementary material 3).

In our experimental model MOP-Cherry receptor endocytosis is constitutive, i.e., it takes place in the absence of activating agonists. Moreover, it has been extensively described for many GPCRs constitutive activity in functional terms, which means that these receptors coupled to G proteins in the absence of any agonist.

![Figure 4. Effect of Concanavalin A on vesicle trafficking. Fluorescent microscopy images were obtained from living cells treated with DOX (0.01 µg/ml) for 24h. Concanavalin A (500 µg/ml) was added in parallel at the time point displayed in every picture](image)
In order to test whether MOP-Cherry constitutive internalization is a G-protein coupling process, we treated cells with pertussis toxin (PTX), a drug capable of inhibiting the interaction between receptors and $G_{i/o}$ subunits, for 24h (Fig. 5A). After PTX treatments we observe a significant decrease of both intracellular vesicle formation and receptor expression levels (Fig. 5A, and Supplementary material 4).

GPCRs constitutive activity might be inhibited as well by drugs known as inverse agonists. Thus, we treated cells with Naloxone (Nal) or Naltrexone (Nalt), to test their opioid inverse agonist properties (Cruz et al., 1996), at a saturating concentration [10^{-5} M] for different periods (Fig. 5B and 5C). Any evident effect was observed, suggesting that in our experimental cell model neither naloxone nor naltrexone acts as inverse agonists.

**Figure 5.** Effect of Pertussis toxin, Naloxone and Naltrexone on constitutive receptor endocytosis. Fluorescent microscopy images were obtained from living cells treated with DOX (0.01 μg/ml) for 24h to induce MOP Cherry receptor expression. **A**, cells treated with doxycycline for 24h (control) or with pertussis toxin (PTX 100ng/ml) for 24h. **B**, cells treated with Naloxone (Nal 10^{-5}M) for 30 min and 24h. **C**, cells treated with Naltrexone (Nalt 10^{-5}M) for 24h and 48h.
To get further insight in the cellular mechanisms involved in MOP-Cherry constitutive receptor internalization, we designed experiments to probe the role of dynamin in the endocytosis of MOP-Cherry receptors. On one hand, we treated cells with dynasore for 30min and 24h. Dynasore is a cell-permeable inhibitor of dynamin GTPase activity that facilitates the formation of coated pits in the process of endocytosis (Macia et al., 2006; Nankoe and Sever, 2006). Dynasore treatment did not inhibit MOP-Cherry receptor internalization, since intracellular vesicles are observed in dynasore treated cells (Fig. 6B).

Otherwise, cells were also transfected with a mixture containing a dominant-negative mutant N272 dynamin and GFP fluorescent protein (Fig. 6A) to monitor the effects of dynamin on membrane protein internalization. N272 dynamin is a dominant-negative dynamin mutant which lacks the complete GTP-binding domain (Werbonat et al., 2000). As shown in Fig. 6A, cells transfected with the mixture also contains intracellular vesicles, suggesting that N272 dynamin did not affect MOP-Cherry internalization process.

**Figure 6.** Effect of a dynamin dominant-negative mutant transfection and dynasore on constitutive receptor endocytosis. **A**, cells were transfected with a mixture containing dominant-negative N272 dynamin and GFP fluorescent protein (0.1 μg/ml). Image were obtained after illuminating living cells previously treated with DOX (0.01 μg/ml) for 24h, with settings to detect mCherry (gray) and GFP (green) fluorescence. **B**, cells were treated with dynasore (80 μM) for 30 min and 24h. To induce MOP-Cherry receptor expression cells were treated with DOX (0.01 μg/ml).
Next, we proceed to evaluate the pharmacological profile of MOP-Cherry receptors by \([^3\text{H}]\text{diprenorphine}\) and \([^{35}\text{S}]\text{GTPyS}\) binding assays. Saturation of specific \([^3\text{H}]\text{diprenorphine}\) binding sites in membranes from cells treated with DOX for different periods showed affinity values in agreement with the expected one for this antagonist: \(K_d = 0, 24 \pm 0, 04\text{ nM}\) after 24h of DOX treatment and \(K_d = 0, 27 \pm 0, 03\text{ nM}\) after 96h of DOX treatment. Regarding receptor expression levels, we observed a significant increase in receptor expression in samples from those cells treated with DOX for longer periods compared to membranes from cells untreated (Fig 7 and Table 2). Pertussis toxin treatment significantly decreases MOP-Cherry receptor expression (Fig 7 and table 2), result that was also corroborated by western blot assays (Fig. 8C). Pharmacological parameters are displayed in Table 2.

**Table 2.** Pharmacological parameters of \([^3\text{H}]\text{diprenorphine}\) saturation binding assays performed with membranes from cells treated with DOX for 24h, with DOX for 96h, or with DOX plus pertussis toxin for 24h. Parentheses indicate number of independent experiments performed. Values are presented as mean ± S.E.M. The values in parentheses represent \(n\).

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<th>(B_{\text{max}}) (fmol/mg)</th>
<th>(K_d)</th>
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<tr>
<td>24h DOX</td>
<td>2229,5 ± 514,85 (3)</td>
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<td>96h DOX</td>
<td>3555,1 ± 727,17 (3)</td>
<td>0,2767 ± 0,0306 (3)</td>
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<tr>
<td>24h PTX</td>
<td>898,05 (1)</td>
<td>0,44 (1)</td>
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**Figure 7.** Specific \([^3\text{H}]\text{diprenorphine}\) saturation binding assays. A, performed with membranes from cells non treated (green), treated with DOX for 24 hours (red), with DOX for 96 hours (blue) B, or with DOX plus pertussis toxin for 24 hours (black).
[^35]S\text{GTP}_\gamma S binding experiments were performed in membranes from cells treated with DOX for different times, using DAMGO as agonist at different concentrations (Fig. 8A), in order to stimulate the specific incorporation of[^35S]GTP\_\gamma S in membranes. Despite membranes obtained from cells treated with DOX for 24h or 96h have different baseline values; we obtained similar curves when plotted as fold over basal (Fig. 8A).

Figure 8.[^35S]GTP\_\gamma S binding assays. A, membranes obtained from cells treated with doxycycline (0.01 µg/ml) at different times to induce MOP-Cherry receptor expression were incubated with an agonist (DAMGO) at different concentrations. Results are expressed in cpm and in fold over basal. B, membranes obtained from cells non treated (-DOX), treated with doxycycline for 96h (DOX 96h) or with doxycycline plus pertussis toxin for 24h (PTX) were incubated with DAMGO (10^{-3}M) or an antagonist/inverse agonist (naloxone 10^{-4}M). C, Western Blott analysis performed with membranes used in[^35S]GTP\_\gamma S assays from cells non treated (c), treated with DOX for 24 hours (24h), with DOX for 96 hours (96h) or with DOX plus pertussis toxin for 24 hours (PTX).
As previously shown by microscopy experiments and radioligand bindings, MOP-Cherry receptor expression is undetectable in the absence of DOX, therefore the extent of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in response to DAMGO was greater in the treated than in the untreated cells (Fig. 8B). DAMGO stimulated the specific incorporation of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ in a dose-dependent manner (Fig. 8A) whereas naloxone did not show any effect (Fig. 8B). Membranes from cells treated with pertussis toxin plus DOX for 24h were devoid of interaction between receptors and $G_{\alpha i/o}$ subunits (Fig. 8B). The pharmacological parameters of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ experiments are shown in table 3.

Table 3. Pharmacological parameters of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding assays performed with membranes from cells treated with DOX for 24 hours or with DOX for 96 hours. Parentheses indicate number of independent experiments performed. Values are presented as mean ± S.E.M. The values in parentheses represent $n$.

<table>
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<tr>
<th>Sample</th>
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<td>24h DOX</td>
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<tr>
<td>96h DOX</td>
<td>8,667 ± 0,02 (3)</td>
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Discussion

G protein-coupled receptors are the most common cellular targets for drugs used in clinic (Rosenbaun et al., 2009). Of the four main opioid receptor subtypes, µ-opioid receptors are of the greatest clinical importance, because they mediate the physiological actions of clinically used opioid alkaloid drugs such as morphine.

In this study, we have generated a permanent cell line heterologously expressing MOP-Cherry receptors in an inducible manner to conduct molecular pharmacological studies. This experimental system provides MOP-Cherry expression by using doxycycline as an ON/OFF agent allowing the use of the same host cells as negative control when MOP-Cherry receptor is not induced to express. In this particular expression model, MOP-Cherry receptor distributes both in the cell membrane and as trafficking vesicles. Addition of DOX resulted in a concentration- and time-dependent increase of MOP-Cherry receptor expression that was paralleled by the augmentation of intracellular vesicles.

The origin of these vesicles corresponds to a constitutive endocytosis process since Concanavalin A treatment cause the complete inhibition of vesicle formation. ConA binds terminal sugar residues resulting in the agglutination of cell surface receptors and has been used to block GPCR internalization (Luttrell et al., 1997). As revealed in our results, inhibition of vesicle formation was observed after 4h of ConA treatment. There is no evident effect of vesicle formation inhibition after the first 30 min of ConA treatment; probably because that is due to the previously presence of vesicles before starting treatment.

We decided to investigate MOP-Cherry receptor endocytosis mechanisms and designed experiments to inhibit dynamin-dependent pathways. In the present studies both dynasore and dominant-negative mutant N272 dynamin were unable to inhibit MOP-Cherry receptor internalization, suggesting then that MOP-Cherry receptor internalization is a clathrin-independent endocytosis.

As observed in fluorescent microscopy images, pertussis toxin treatment shows a significant decrease of both intracellular vesicle formation and receptor expression level, suggesting that the constitutive MOP-Cherry receptor endocytosis is a G-protein coupled dependent process. This result was also corroborate by western blot assays, $[^{35}\text{S}]{\text{GTP}\gamma\text{S}}$ and $[^{3}\text{H}]$diprenorphine binding experiments, since membranes from cells treated with pertussis toxin for 24h were devoid of interaction between receptors and Gai/o subunits and showed a significant decrease in receptor expression level.

Constitutive activity has been previously demonstrated for MOP receptor (Burford et al., 2000) and could be defined as the coupling of receptors to G proteins in absence of activating agonists. There are compounds that stabilize poorly coupled state of MOP
receptors and consequently can inhibit the constitutive activity. These compounds are known as inverse agonists. The degree to which these ligands inhibit the basal level of the output being measured is considered to reflect the amount of constitutive activity present in the system. However, intrinsic efficacy is not a fixed characteristic of receptors but it appears to depend on the context of the cells, the state of the receptor, and experimental conditions (Fathy et al. 1999; Yang and Lanier 1999).

Naloxone and naltrexone are compounds which have exhibited properties of inverse agonist at MOP receptor (Wang et al. 1994; Burford et al., 2000). In our experimental model, cells treated with naltrexone or naloxone did not show any evident effect on either constitutive endocytosis or constitutive activity of MOP-Cherry receptors, as observed in fluorescent microscopy images as well as in \(^{[35S]}\)GTP\(_\gamma\)S binding assays. In relation to receptor expression levels any of both compounds affected the number of MOP-Cherry receptors as observed in PTX treatment. Overall and taking together, these results suggest that in this experimental cell model both naloxone and naltrexone do not act as inverse agonists.

There have been previously described two cases of constitutive endocytosis of MOP receptors in the literature. Macé and co-workers established that p38 MAPKs play a key role in the modulation of MOP endocytosis. By investigating the molecular mechanisms that underlie MOP endocytosis, they uncovered an unexpected functional connection between p38 MAPK activity and the endocytic machinery regulated by the small GTPase Rab5. They showed that p38 MAPKs can phosphorylate EEA1, one of the best-characterized Rab5 effectors, and Rabenosyn-5 on Thr-1392 and Ser-215, and that both phosphorylation sites are located within the FYVE finger, which plays an essential role in the recruitment of the proteins to the early endosome membrane by a clathrin-dependent process. Interestingly and supporting their results, a published genome-wide screen uncovered a wider role of protein kinases in the regulation of endocytosis (Pelkmans et al, 2005). They demonstrate that p38 MAPK activation is required for MOP endocytosis in HEK293 cells and sufficient to trigger its constitutive internalization in the absence of agonist (Macé et al., 2005).

Other research published by Koch and collaborators (Koch et al., 2006) showed that MOP receptor is associated with the phospholipase D2 (PLD2), a phospholipid-specific phosphodiesterase located in the plasma membrane. PLD is a widely distributed enzyme, its principle substrate is phosphatidylincholine and its product, phosphatidic acid, is believed to have many different functions in signal transduction, vesicle formation and cytoskeletal dynamics (Liscovitch and Cantley 1995). They demonstrate in human embryonic kidney 293 cells, that the mouse C-terminal splice variant of the \(\mu\)-opioid receptor (MOR1D) exhibited robust endocytosis in response to both DAMGO and morphine treatment. Moreover, they report that MOR1D also mediates an agonist-independent (constitutive) PLD2-activation facilitating agonist-induced and constitutive receptor endocytosis. It has been previously described that PLD2 might be involved in
vesicle formation from the plasma membrane (Colley et al. 1997). Furthermore, PLD-activity has been shown to be regulated by a number of GPCRs including MOP receptor (Koch et al. 2003). This group provides evidence for an essential role of PLD2 in the agonist-induced and constitutive endocytosis of the µ-opioid receptor splice variant MOR1D. They presume that the ARF6 protein, which has been demonstrated to selectively activate PLD2 in vivo (Hiroyama and Exton 2005), might be involved in the GPCR-mediated PLD2 activation. This assumption was supported by findings that ARF6 protein plays an essential role in the endocytosis regulation of GPCRs sequestered via the clathrin-coated pathway (Claing 2004; Houndolo et al. 2005). Therefore they suggest that is possible that the GPCR mediated PLD2 stimulation may involve a direct interaction of receptors with ARF protein. Furthermore, it has been elucidated that β-arrestins also function as endocytic adapters, linking receptors to the clathrin-coated pit machinery (Shenoy and Lefkowitz 2003; Claing 2004) and that the interaction of β-arrestins with endocytotic elements including clathrin and the adapter protein 2 (AP-2) are important for induction of clathrin-dependent GPCR endocytosis (Laporte et al. 2002). Thus they concluded that receptor endocytosis might be regulated by both GPCR binding to β-arrestin and GPCR-mediated PLD2 activation resulting in the subsequent recruitment and/or interaction with components of the AP-2 complex. In fact, this study revealed that PLD2 activity is essential for both constitutive and agonist dependent MOR1D receptor endocytosis. They concluded that agonist-independent PLD2 activation provides an important alternative mechanism to β-arrestin for the regulation of constitutive endocytosis of GPCRs.

Our results show that MOP-Cherry receptor internalization is a clathrin-independent endocytosis process, since the inhibition of dynamin through different experiments does not promote the inhibition of vesicle formation. Recently, we have made considerable use of Flp-In T-Rex HEK293 cells in order to express and study different GPCRs, and we have not seen before constitutive internalization of other forms of MOP receptors heterologously expressed in this system (Lopez-Gimenez et al., 2008), excluding therefore an artifactual effect of the host cells on this constitutive endocytosis. However further investigations to determine the implication of phospholipase D2 and p38MAPKs on constitutive MOP-Cherry endocytosis our required.

The pharmacological characterization shows that the expressed MOP-Cherry construct is functional. The highly MOP-selective enkephalin analog DAMGO stimulated binding of [35S]GTPγS in membranes from cells expressing MOP-Cherry receptors in a concentration-dependent manner. As suggested by the higher levels of MOP-Cherry receptor expression in cells treated with DOX for 96h, previously observed in microscopy images, the extent of [35S]GTPγS in response to DAMGO was greater in cells treated with DOX for longer periods, an effect achieved without a significant alteration in the potency of DAMGO. These effects of DAMGO were lacking in pertussis toxin-treated cells, indicating that [35S]GTPγS binding via MOP-Cherry was to pertussis toxin-sensitive members of the Ga1 subgroup. Moreover, specific
$[^3]H$ diprenorphine binding experiments showed affinity values (Kd) in agreement with the previously described for this antagonist (Raynor et al., 1995) and corroborate the significant increase in receptor expression levels observed in samples from cells treated with DOX for 96h by fluorescent microscopy and western blot experiments.

Further investigations to determine whether MOP-Cherry receptor constitutive endocytosis concurs with receptor functional constitutive activity will be conducted. If this is the case, this experimental model would represent a model of choice to pharmacologically characterize potential inverse agonist compounds.

**Conclusions**

✓ We have generated a permanent cell line heterologously expressing MOP-Cherry receptors in an inducible manner. This experimental system provides MOP-Cherry receptor expression by using doxycycline as an ON/OFF agent.

✓ The expression level of our gene of interest might be regulated depending on the DOX dose and period of treatment.

✓ In this particular expression model, MOP-Cherry receptor distributes in trafficking vesicles as a result of constitutive endocytosis that is a G-protein coupling dependent process.

✓ Further investigations to determine whether MOP-Cherry constitutive endocytosis concurs with receptor functional constitutive activity will be conducted. If this is the case, this experimental model would represent a model of choice to pharmacologically characterize potential inverse agonist compounds.

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References


