Chapter 30

Molecular Assays for Characterization of Alternatively Spliced Isoforms of the Mu Opioid Receptor (MOR)

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Abstract

Mu-opioid receptor (MOR) belongs to a family of heptahelical G-protein-coupled receptors (GPCRs). Studies in humans and rodents demonstrated that the *OPRM1* gene coding for MOR undergoes extensive alternative splicing afforded by the genetic complexity of *OPRM1*. Evidence from rodent studies also demonstrates an important role of these alternatively spliced forms in mediating opiate analgesia via their differential signaling properties. MOR signaling is predominantly $G_{i/o}$ coupled. Release of the α subunit from G-protein complex results in the inhibition of adenylyl cyclase/cAMP pathway, whereas release of the $\beta\gamma$ subunits activates G-protein-activated inwardly rectifying potassium channels and inhibits voltage-dependent calcium channels. These molecular events result in the suppression of cellular activities that diminish pain sensations. Recently, a new isoform of *OPRM1*, MOR3, has been identified that shows an increase in the production of nitric oxide (NO) upon stimulation with morphine. Hence, there is a need to describe molecular techniques that enable the functional characterization of MOR isoforms. In this review, we describe the methodologies used to assay key mediators of MOR activation including cellular assays for cAMP, free Ca^{2+} , and NO, all of which have been implicated in the pharmacological effects of MOR agonists.

Key words: Alternative splicing, *OPRM1*, Opioid, Calcium, cAMP, Nitric oxide, Fluo-4, Fluo-3, GPCR, FSK, Capsaicin

1. Introduction

The major form of *OPRM1*, called MOR-1, is coded by exons 1, 2, 3, and 4 (1). Exon 1 codes for first transmembrane domain (TMH1) and exons 2 and 3 code for the TMH2 through TMH7. However, many 5'-end and 3'-end alternatively spliced forms of *OPRM1* have also been cloned (2). A common splicing pattern among the 3'-end alternatively spliced forms involves the alternative

C-terminus, coded by various exons, while all seven transmembrane domains that form the binding pocket are coded by exons 1, 2, and 3, stay intact. The structural difference at the C-terminus has been proposed to drive differential cellular signaling in response to receptor activation (1–5). Alternative splicing at the 5'-end results either in an alternative TMH1 domain (via alternative exon 11) or in isoforms that start from exon 2 (6, 7), which lack an amino acid sequence of approximately 90 amino acids (N-terminus, TMH1 and part of the first intracellular loop) (8) but retain the ligand binding pocket distributed across the conserved TMH2, TMH3, and TMH7 domains.

The binding of an agonist to MORs usually results in the inhibition of cellular activity; however, there is a growing body of research demonstrating excitatory events downstream to MOR receptor stimulation (9, 10). Both cell culture and animal experiments suggest that opioid-induced analgesia is a consequence of inhibitory cellular effects resulting from the binding of μ -opioid agonists to MOR (11). Since MOR is predominately coupled to G_i , the resulting molecular cascade leads to decreased levels of cAMP (12) and intracellular calcium (13–15) with a resulting inhibition of neuronal activity. The analgesic effect of MOR activation has also been attributed to the release of the $G_{\beta\gamma}$ dimer from $G_{i/o}$, which activates inwardly rectifying potassium (GIRK) channels (16) and inhibits voltage-dependent calcium channels (VDCCs) (17). The resulting hyperpolarization suppresses neuronal activity. Adenylyl cyclase inhibition may also contribute to opioid analgesia, since its activation has been suggested to elicit analgesic tolerance or tolerance-associated hyperalgesia (18, 19).

Conversely, the pronociceptive effects of μ -opioid agonists are associated with excitatory cellular events: increase in levels of cAMP (20) and intracellular Ca^{2+} (21). Excitatory cellular events have been implicated in molecular mechanism of opioid receptor-mediated hyperalgesia, tolerance, and dependence (22). Both extremely low and extremely high doses of morphine, as well as chronic administration of opioids, can elicit a hyperalgesia in both human clinical and animal models of pain (22–24). A dual effect of opioids on cAMP and Ca^{2+} levels in vitro has been corroborated by Rubovitch and coworkers (9). It has been proposed that a switch in the G-protein coupling profile of the OPRM1 from G_i to both G_s and G_q as well as adenylyl cyclase (AC) activation by $G_{\beta\gamma}$ explains the phenomenon of opioid induced excitation (9, 25, 26). It has also been established that the activation of cAMP and cGMP cascades through Ca^{2+} -dependent activation of nitric oxide synthase leads to activation of protein kinases A (PKA) and G (PKG) and establishment of a pronociceptive phenotype (27). The increased influx of Ca^{2+} leads to activation of nitric oxide synthase through Ca-calmodulin dependent mechanism resulting in increased production of NO (28). In turn, increased levels of

NO stimulate guanylyl cyclase, leading to the production of cGMP, activation of PKG, and establishment of a pro-nociceptive phenotype (28). In agreement with this, the newly cloned human 5'-end alternatively spliced isoform of MOR-3 that codes for six transmembrane variant rather than the classic seven transmembrane variant, exhibited a dose-dependent release of NO following treatment with morphine (8). The effect was abrogated by administration of naloxone (8).

The present report reviews techniques used to assay the levels of cAMP, Ca^{2+} , and released NO produced by μ -opioid receptor agonists in in vitro cellular assays designed to characterize the functional properties of alternatively-spliced isoforms of MOR.

2. Materials

2.1. Measurement of cAMP Levels in Forskolin (FSK) Stimulated Cells

1. Geneticin and hygromycin (Invitrogen, Carlsbad, CA).
2. Lipofectamine 2000 (Invitrogen).
3. 2-(2-(Bis(carboxymethyl)amino)ethyl)-(carboxymethyl)amino)acetic acid (EDTA).
4. Trypsin (Gibco/BRL, Bethesda, MD).
5. Capsaicin (Sigma St. Louis, MO) is dissolved in 100% ethanol to make 10,000 $\times$ (3 mM) stock solution. Toxic if inhaled, irritant. Store at 4°C. If stock solution is made, store at 4°C as well. Stock solution is stable indefinitely.
6. IBMX3-Isobutyl-1-methyl-xanthine (Sigma).
7. 7 β -Acetoxy-8,13-epoxy-1 α ,6 β ,9 α -trihydroxyabdr-14-en-11-one (Forskolin) (Sigma).
8. Cyclic AMP EIA Kit (Cayman Chemicals Ann Arbor, MI).

2.2. Measurement of cAMP Levels in PGE2 Stimulated Cells

1. Hanks' balanced salt solution (HBSS, pH 7.3 at 4°C): 17 mM NaCl, 6 mM KCl, 1.6 mM NaHPO, 0.5 mM KHPO, 6 mM D-glucose, and 0.01% phenol red.
2. Collagenase (Sigma).
3. Tripsin (Gibco).
4. Dulbecco's Modified Eagle's Medium (DMEM) (Gibco/BRL).
5. Nerve growth factor (NGF) (Harlan, Indianapolis, IN).
6. Fetal bovine serum (FBS) (Gibco/BRL).
7. Penicillin–Streptomycin (PenStrep) 50–100 U of penicillin, 50–100 μ g of streptomycin (Gibco/BRL).
8. Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), DTNB) (Thermo Scientific, Rockford, IL).

9. Uridine 5'-diphosphate disodium salt hydrate.
10. 5-fluoro-2'-deoxyuridine.
11. 4-(3-cyclopentyloxy-4-methoxy-phenyl)pyrrolidin-2-one Rolipram (Peninsula Laboratories, Torrance, CA).
12. (d-Ala², N-MePhe⁴, Gly-ol⁵) enkephalin (DAMGO) (Peninsula Laboratories).
13. Morphine sulfate is dissolved in ddH₂O to make 1,000× (1 mM) stock solution. Store at 4°C.
14. Naloxone hydrochloride dehydrate.
15. Prostaglandin E₂ (PGE₂) (Cayman Chemicals).

2.3. Microplate Reader Measurement of Intracellular Ca²⁺ (Fluo-3)

1. Capsaicin (Sigma).
2. IBMX3-Isobutyl-1-methyl-xanthine (Sigma).
3. NOVOstar fluorescence microplate reader (BMG Offenburg, Germany).
4. Morphine sulfate.
5. Fluo-3 AM (Sigma).
6. PSS buffer (pH 7.4): 5.9 mM KCl, 1.5 mM MgCl₂, 1.2 mM NaH₂PO₄, 5 mM NaHCO₃, 140 mM NaCl, 11.5 mM glucose, 1.8 mM CaCl₂, and 10 mM HEPES.

2.4. Microplate Reader Measurement of Intracellular Ca²⁺ (Fluo-4)

1. Fluo-4 NW Calcium Assay Starter Kit (Invitrogen). Store and desiccate contents (assay buffer, probenecid, Fluo-4 NW dye mix) at -20°C. Protect contents from light.
2. Microplate reader Wallac1420 Victor3 (PerkinElmer Waltham, MA) with 500 nm excitation and 515 nm emission filters.

2.5. Measurement of Nitric Oxide Release from Transfected Cells

1. Clonfectin Transfection Kit (Clontech Palo Alto, CA).
2. Multilineage progenitor cells (MLPC) (St. Paul, MN).
3. Puromycin (Sigma).
4. Fetal Bovine Serum.
5. 96 well plates (Falcon San Jose, CA).
6. DMEM (Invitrogen).
7. Micromanipulator (World Precision Instruments Sarasota, FL).
8. S-nitroso- N-acetyl-D,L-penicillamine (World Precision Instruments).
9. NO-specific amperometric probe (30 μm, 0.5 mm; World Precision Instruments).
10. Inverted microscope (Diaphot Nikon, Tokyo, Japan).
11. 4-channel ESA BioStat with an NO-selective amperometric 600 μm nanoprobe (Innovative Instruments).

3. Methods

Signaling through GPCRs can lead to a variety of cellular responses via changing intracellular levels of second messengers and gene transcription (reviewed by (29)). Activation of MOR that is coupled to G_i inhibits adenylate cyclase (30), decreasing the production of cAMP (31). Due to variable levels of cAMP in cultured cells, the activity of GPCRs coupled to G_i is assayed in cells with artificially elevated cAMP levels. The levels of cAMP are usually stimulated by either PGE2 or FSK. A mu-opioid receptor agonist is then administered at varying concentrations. The extent of decrease in cAMP levels indicates the efficacy of receptor–ligand interaction.

To determine the optimal concentration of the mu-opioid agonist (see Note 1), it is often necessary to assess its effect across a range of the doses. This is especially true when characterizing a newly identified receptor isoform or working with variable tissues and cell types. An in-depth discussion of the construction of concentration curves is beyond the scope of the present paper as excellent reviews on this topic already exist (32). A simple way to prepare a dose response curve for morphine to assay cAMP levels in vitro is provided (see Note 2).

As activation of the opioid receptors decreases Ca^{2+} uptake (36, 37) and inhibits voltage-dependent calcium channels (13), measuring intracellular Ca^{2+} levels is an important assay for characterizing MOR signaling. Fluorescent Ca^{2+} detection was developed in the late eighties (33). This approach allows for optical detection and quantification analysis of intracellular calcium levels. Due to the inhibitory nature of GPCRs coupled to G_i , Ca^{2+} levels are assayed in cells with artificially (capsaicin) elevated Ca^{2+} levels (13, 34, 35). Since not all cell types express capsaicin receptor TRPV1, the cells are transfected with TRPV1 expressing construct. In the experiment described below, the HEK293 cells that do not express either MOR or TRPV1 are transfected with these receptors prior to the experiments.

Mu-opioids signaling also involves production and release of NO via Ca^{2+} -stimulated activation of constitutive nitric oxide synthase that has been specifically linked to MOR-3 isoform of *OPRM1*. (8) The following technique developed to rapidly evaluate MOR agonist NO stimulating activity in vitro.

3.1. Stimulation of cAMP by FSK

1. Human embryonic kidney cells (*HEK293*) are transfected with full-length MOR expression construct in pcDNA3 tagged with FLAG using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol, and stable FLAG-MOR expressants are selected with 0.5 mg/ml geneticin (see Note 3).

2. Colonies originating from a single cell are selected and FLAG-MOR expression is verified by Western Blotting.
3. Stable FLAG-MOR expressants are split every 4–6 days using trypsin/EDTA and maintained at 37°C in a 5% humidified CO₂ incubator in DMEM medium containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM l-glutamine, and 110 mg pyridoxine.
4. For the cAMP accumulation assay, FLAG-MOR expressants are plated on 12 well plates and grown to 90% confluency.
5. Twenty-four hours prior to sample preparation, media are changed to DMEM supplemented with 0.5% heat-inactivated FBS.
6. On the day of sample preparation, cells are washed with DMEM to remove serum and incubated with serum-free DMEM containing the phosphodiesterase inhibitor IBMX (100 µM) for 30 min, mu-opioid agonist (see Note 1) is then added and cells incubated for a further 15 min.
7. Following this, FSK (50 µM) is added to the wells and the cells are incubated for 15 min to stimulate cAMP production. DMSO alone is used as a vehicle control.
8. After incubation, reactions are terminated by aspiration of the medium and addition of 0.1 M HCl followed by 20 min incubation at room temperature. After centrifugation of the cell samples at 10,000×g for 10 min, the protein content of the supernatant is assessed and the samples are diluted to protein concentrations of 20 µg/ml using *enzyme immunoassay* (EIA) buffer supplied with the cAMP EIA kit.
9. 100 µl of EIA Buffer is added to Nonspecific Binding wells, and 50 µl to Maximum Binding wells.
10. 50 µl from the tubes containing cAMP standard is added into the wells allocated for the 8 point standard curve. Two aliquots of 50 µl each should be taken from each tube, as the standard is being run in duplicate.
11. 50 µl of sample is added per well. Each sample is assayed in at a minimum of two dilutions. Each dilution is assayed in duplicate or triplicate.
12. 50 µl of cAMP Tracer is placed into to each well except the Total Activity and Blank wells.
13. 50 µl of rabbit anti-AMP antibody is added to each well except the Total Activity, Non-specific Binding, and Blank wells.
14. The plate is incubated for 18 h at 4°C after covering each plate with a plastic film.
15. The wells are rinsed five times with Wash Buffer.
16. 200 µl of Ellman's Reagent is added to each well.

17. 5 μ l of tracer is added to the Total Activity wells.
18. The plate is covered with plastic film and placed on orbital shaker in the dark. Allow the plate to develop for 90–120 min.
19. Intracellular cAMP levels are measured with a competitive cAMP EIA kit in triplicate. An example result is shown in Fig. 1 (13).

3.2. Stimulation of cAMP Levels by PGE2

1. Fresh trigeminal ganglion neurons (TG) are washed with HBSS (Ca^{2+} , Mg^{2+} free), digested with 3 mg/ml collagenase for 30 min at 37°C and centrifuged to pellet cells/tissue as described by Vasko and coworkers (36).
2. The pellet is resuspended following further digestion with 0.1% trypsin (15 min) and 167 μ g/ml DNase (10 min) at 37°C in the same solution.
3. Cells are pelleted by centrifugation (5 min at 5000 $\times g$) and resuspended in DMEM (high glucose) containing 250 ng/ml NGF, 10% FBS, 1 $\times$ Pen/Strep, 1 $\times$ l-glutamine and the mitotic inhibitors: 7.5 μ g/ml uridine and 17.5 μ g/ml 5-fluoro-2'-deoxyuridine.

Fig. 1. Inhibition of FSK-stimulated cAMP accumulation by morphine (1 μ M). cAMP levels in FLAG-MOP stable colonies were measured using an enzyme-immunoassay kit either in unstimulated cells (no FSK), or in cells treated with FSK (50 μ M) and IBMX (100 μ M). Morphine (1 μ M) significantly reduced cAMP levels in FSK-stimulated cells (morphine) compared to stimulated cells treated with water (vehicle). Morphine inhibited cAMP production by $67.0 \pm 4.6\%$ for FLAG-MOP/TRPV1 colony 13 (dotted bars) and $79.2 \pm 7.4\%$ for colony 21 (cross-hatched bars). Data are presented as mean $\pm$ SEM from $n=3$ samples read in triplicate. ** $p<0.01$ compared to vehicle. (Vetter et al. Molecular Pain 2006 2:22)

4. After triturating to disrupt tissue, the cell suspension is seeded on poly-d-lysine-coated 0.17 mm glass inserts in 24-well plates or polylysine-coated 48-well plates (3 TG per plate).
5. Media are changed at 24 h and then every 48 h after plating. Cells are used after 5 or 6 days in culture and serum and NGF are removed from the media 24 h before experimentation.
6. TG cultures are washed twice with HBSS containing 10 mM Hepes and 4 mM sodium bicarbonate, pH 7.4 wash buffer.
7. Cells are preequilibrated in 250–500 μ l wash buffer per well of 48 well plate for 30 min at 37°C in 5% CO₂. Where indicated, inhibitors are added during the preequilibration period.
8. To determine MOR-mediated effects, cells are incubated with the phosphodiesterase inhibitor, rolipram (10 μ M), along with mu-opioid agonist (see Note 1) for 15 min at 37°C.
9. PGE₂ (1 μ M) is added, followed by incubation for a further 15 min.
10. Incubations are terminated by aspiration of the wash buffer and addition of 500 μ l ice cold absolute ethanol.
11. The ethanol extracts from individual wells are dried under a gentle air stream and reconstituted in 100 μ l of 50 mM sodium acetate, pH 6.2.
12. The cAMP content of each 100 μ l sample is determined by radioactive immune assay RIA or EIA. (37, 38)

3.3. Measuring of Intracellular Ca²⁺ Levels with Fluo-3

1. FLAG-MOR/TRPV1 expressants are plated on poly-D-lysine-coated 96-well plates at a cell density of approximately $3\text{--}4 \times 10^5$ cells/ml 3–5 days prior to experiments and used at 70–90% confluency (see Note 4).
2. Cells are loaded with Fluo-3 AM (6 μ M) in loading buffer (pH 7.4, composition as PSS plus 3 mg/ml bovine serum albumin (BSA)) for 20–30 min at 37°C.
3. Wells containing FLAG-MOR/TRPV1 double expressants are then washed three times with PSS buffer and incubated for 15 min with PSS or mu-opioid agonist followed by 15 min of incubation with PSS or kinases activators with mu-opioid agonist as appropriate.
4. Preincubation steps and Ca²⁺ imaging are carried out at 29°C in order to avoid subcellular dye compartmentalization while maintaining constant experimental conditions.
5. Fluo-3 loaded cells are excited at 420 nm and emission is recorded at 510 nm using a NOVOstar fluorescence microplate reader.
6. Fluorescent emission readings are recorded every 0.5 s. Raw fluorescence data are normalized by subtracting the average

Fig. 2. Capsaicin dose-response curves from two independent FLAG-MOP/TRPV1 expressants. Ca^{2+} responses of Fluo-3-loaded cells to injection of capsaicin were measured using a fluorescent Microplate reader and maximum change in fluorescence, expressed as $\Delta F/F$, was plotted as a function of capsaicin concentration. A 4-parameter Hill function was fitted to the data using GraphPad Prism. (a) Capsaicin dose-response from FLAG-MOP/TRPV1 double expressant colony 13. (b) Capsaicin dose-response from FLAG-MOP/TRPV1 double expressant colony 21. (Data are expressed as mean $\pm$ SEM with $n=8$.) Vetter et al. *Molecular Pain* 2006 2:22

fluorescence from 4 recordings just prior to addition of agonist from all subsequent time points. The data are analyzed using a 4-parameter logistic Hill equation (see Note 8) and plotted using GraphPad Prism. An example result is shown in Fig. 2 (13).

3.4. Microplate Reader Measurement of Intracellular Ca^{2+} with Fluo-4 Reagent

1. BE2C human neuroblastoma cells are grown to near confluence in black 96-well poly-d-lysine coated plates. The cell cultures are grown in DMEM/F12 media (see Note 3).
2. The indicator Fluo-4 NW dye is prepared as outlined in manufacturer instructions (see Notes 4 and 5).
3. Cell culture medium is removed from all wells except three, which will serve as controls, 100 μL of capsaicin then is added to the wells (see Note 5)
4. After 20 s the treatments are removed from all wells as well as the DMEM/F12 medium from the control wells (see Note 6).
5. Quickly, but carefully, 100 μL of Fluo-4 NW dye is added to each well.
6. The plate is then incubated with the lid on at 37°C for 30 min, then at room temperature for an additional 30 min (see Note 6).
7. The fluorescence is measured using a microplate reader with settings for emission at 515 nm and excitation at 500 nm (see Notes 7 and 9). An example result is shown in Fig. 3.

Fig. 3. Effect of the different doses of morphine on the Ca^{2+} levels in BE(2)C cells. The low doses (L) of morphine (10^{-3} μ M) and high (H) doses (10^2 μ M) result in increase in Ca^{2+} levels in BE2C cells. The 1 μ M dose (M) of morphine caused a small decrease in Ca^{2+} levels. Data are presented as mean $\pm$ SEM from $n=8$ * $p<0.01$ compared to vehicle

3.5. Measuring of NO Production

1. COS-1 cells are stably transfected (see Note 2) with an expression vector (see Note 10) with cloned MOR-3 cDNA using the Clonfectin Transfection Kit.
2. Stable cell lines are selected with puromycin at a concentration of 7.5 μ g/ml. The stable transfectants are grown in DMEM supplemented with 10% FBS at 37°C and then screened by RT-PCR for the MOR-3 opiate receptor variant transcript. The cells are subsequently used for functional analysis. For NO determination, 2.5×10^5 of COS1 cells are placed in each well of a 96-well plate and allowed to adhere overnight in 200 μ l of DMEM supplemented with 2% FBS.
3. Before NO determination, the medium is removed and replaced with 200 μ l of PBS solution. Transfected COS-1 cells as well as nontransfected cells are plated and tested (see Fig. 4).
4. NO release from the transfected and untransfected cell lines is directly measured using an NO-specific amperometric probe.
5. A micromanipulator, which is attached to the stage of an inverted microscope, is used to position the amperometric probe 15 μ m above the cells (see Note 11).

Fig. 4. (a) Real-time NO production measurements in μ 3-transfected COS-1 cells after addition of the opiate alkaloid, morphine, or the opioid peptides, Met- and Leu-enkephalin. The control represents the addition of PBS to the cells. Each experiment was replicated four times $\pm$ SEM. (b), Peak concentration-dependent morphine-stimulated NO release from COS-1 cells and μ 3-transfected COS-1 cells. Each experiment was replicated four times; results shown are the mean $\pm$ SEM. (Copyright 2003 The American Association of Immunologists, Inc.)

6. The amperometric probe is allowed to equilibrate for at least 10 min before being transferred to the well containing the cells.
7. Morphine-stimulated NO release is evaluated in response to increasing morphine concentrations, such as in the range of 10^{-5} – 10^{-9} M (see Note 1). Each experiment is repeated four times along with a control (cells transfected with vector alone).
8. Real-time release of NO from multilineage progenitor cells (MLPC) is quantified using a 4-channel ESA BioStat with an NO-selective amperometric 600 μ m nanoprobe (see Note 14). MLPCs are used in this assay because they demonstrate high endogenous level of MOR-3 activity (39).
9. The amperometric probe is allowed to equilibrate for at least 10 min before being transferred to the well containing the cells.
10. Baseline levels of NO release are determined by evaluation of real-time NO concentration in PBS. Evoked release of NO from MLPC is evaluated at final concentrations of 10^{-5} M to 10^{-7} M morphine (see Note 13). Using μ opioid receptor antagonist to confirm specificity of agonist binding is recommended (see Note 12) (39).

4. Notes

1. Mu-opioid agonists, such as morphine or DAMGO (d-Ala², N-MePhe⁴, Gly-ol⁵) enkephalin should be used to test for dose-dependent responses. Importantly, MOR isoforms demonstrate a range of both specificity and selectivity towards different mu-opioid agonists. For instance, MOR-3 was shown to be insensitive to opioid peptides (8, 39).
2. A simple way to prepare a dose response curve for morphine to assay cAMP levels in vitro is presented here as an example. Initially, 100 μ L of a 1,000 $\times$ stock solution of agonist (1 mM morphine) is added to a test tube containing 900 μ L of cell culture media. The resulting 100 μ M preparation is vortexed for 15 s to ensure complete mixing. 100 μ L of the 100 μ M solution is pipetted into another test tube containing 900 μ L of media. Repetition of this step yields a serial dilution of the ligand. To demonstrate the receptor specificity of agonist-stimulation, one arm of the experimental protocol should include pretreatment of the cell culture with a selective mu-receptor antagonist such as naloxone.

3. Transient transfection can also be used.
4. Black 96-well plates are used because the fluorescence measurements made by the microplate reader are taken from the top of the wells, and if the plates are not black, they will reflect light and ruin the measurements.
5. It is best to add the capsaicin as quickly, but carefully, as possible as the exposure time of the cells to capsaicin should only be 20–45 s. Be ready to remove the capsaicin from the wells as soon as the 20 s has passed.
6. It is important to remove as much of the medium as possible to eliminate sources of background fluorescence.
7. It is assumed that fluorescence measurements will be done at room temperature. If you plan on doing fluorescence measurements at 37°C, you only need to incubate the plate for 30–45 min at 37°C before taking fluorescence measurements.
8. A 488 nm argon laser line works as well as the microplate reader if you want to use that instead.
9. $\frac{\Delta F}{F} = \frac{(F - F_{\text{base}})}{(F_{\text{base}} - B)}$ Where F is the measured fluorescence of the samples exposed to capsaicin F_{base} is the measured fluorescence of the controls, and B is the background signal. The ratio $\frac{\Delta F}{F}$ is thought to approximately reflect calcium concentration levels if there is no change in dye concentration, intracellular environment, or path length (40).
10. The optimal concentration of capsaicin to use will fall within the linear range of the drug–response curve. We found that range to be approximately 100–500 nM, and settled on 300 nM as an optimal concentration (7).
11. The authors used vector pExP1 (Clontech, Palo Alto, CA).
12. The well-established NO donor *S*-nitroso-*N*-acetyl-dl-penicillamine is used to calibrate the system daily.
13. The authors used naloxone or d-Phe-Cys-Tyr-Trp-Orn-Thr-Pen-Thr-NH₂) at 10^{−7} M. Other antagonists (naltrexone) can also be used.
14. Although different opioid agonists can be used in these types of experiments, it is important to bear in mind that not all MOR isoforms are equally responsive to opioid peptides. For instance, MOR-3 (μ-3 as it is named by the group that reported it) was shown to be insensitive to opioid peptides (8, 39).

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