MU OPIOID RECEPTOR-EFFECTOR COUPLING AND TRAFFICKING IN DORSAL ROOT GANGLIA NEURONS

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Abstract—Morphine induces profound analgesic tolerance in vivo despite inducing little internalization of the mu opioid receptor (μOR). Previously proposed explanations suggest that this lack of internalization could either lead to prolonged signaling and associated compensatory changes in downstream signaling systems, or that the receptor is unable to recycle and resensitize and so loses efficacy, either mechanism resulting in tolerance. We therefore examined, in cultured neurons, the relationship between μOR internalization and desensitization in response to two agonists, D-Ala2, N-MePhe4, Gly5-ol-enkephalin (DAMGO) and morphine. In addition, we studied the chimeric mu/delta opioid receptor (μ/δOR) which could affect internalization and desensitization in neurons. Dorsal root ganglia neurons from μOR knockout mice were transduced with an adeno virus expressing either receptor and their respective internalization, desensitization and trafficking profiles determined. Both receptors desensitized equally, measured by Ca2+ current inhibition, during the first 5 min of agonist exposure to DAMGO or morphine treatment, although the μ/δOR desensitized more extensively. Such rapid desensitization was unrelated to internalization as DAMGO, but not morphine, internalized both receptors after 20 min. In response to DAMGO the μ/δOR internalized more rapidly than the μOR and was trafficked through Rab4-positive endosomes and lysosomal-associated membrane protein-1-labeled lysosomes whereas the μOR was trafficked through Rab4 and Rab11-positive endosomes. Chronic desensitization of the Ca2+ current response, after 24 h of morphine or DAMGO incubation, was seen in the DAMGO, but not morphine-treated, μOR-expressing cells. Such persistence of signaling after chronic morphine treatment suggests that compensation of downstream signaling systems, rather than loss of efficacy due to poor receptor recycling, is a more likely mechanism of morphine tolerance in vivo. In contrast to the μOR, the μ/δOR showed equivalent desensitization whether morphine or DAMGO treated, but internalized further with DAMGO than morphine. Such ligand-independent desensitization could be a result of the observed higher rate of synthesis and degradation of this chimeric receptor. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.

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The ability of ligand-activated G-protein coupled receptors (GPCRs) to signal decreases with continued agonist exposure (Harris and Williams, 1991; Fiorillo and Williams, 1996). The underlying mechanisms are complex and involve adaptations at both the level of the receptor and in the downstream signaling pathways. Agonist-induced receptor phosphorylation, which prevents further G-protein coupling, accompanied by internalization to remove the receptor from the cell surface, has long been considered a key component of this adaptive process. However, the role of receptor internalization and desensitization in the development of opiate tolerance in vivo is controversial. The observation that the prototypical mu opioid receptor (μOR) agonist, morphine, causes little or no internalization of the μOR (Keith et al., 1996, 1998; Borgland et al., 2003; Koch et al., 2005), despite inducing tolerance in vivo, led to a model wherein receptor endocytosis, by curtailing receptor signaling, prevents the induction of compensatory downstream events, that themselves may be responsible for tolerance. In this model, morphine induces tolerance precisely because it fails to internalize the receptor and curtail signaling (Whistler et al., 1999; see Kieffer and Evans, 2002). In support of this model, the degree of internalization induced by a series of μOR agonists in HEK 293 cells was positively correlated with their ability to induce rapid desensitization of G-protein-linked inwardly rectifying potassium channels in locus ceruleus neurons (Alvarez et al., 2002). However, if both internalization and desensitization are tested in the same cellular background, the efficacy of opioids to internalize the μOR appears unrelated to their capacity to induce rapid homologous desensitization of Ca2+ channel currents in AtT20 cells (Borgland et al., 2003).

On the other hand if internalization is considered as part of the recycling pathway returning dephosphorylated, re-sensitized receptors back to the cell surface, then internalization may not only reduce signaling within downstream second messenger systems but also, through recycling, replenish the pool of cell surface receptors available.
able for further ligand activation. The endocytosed μOR, similar to several other GPCRs, such as the β2-adrenergic (Pippig et al., 1995; Odley et al., 2004), muscarinic (Edwardson and Szekeres, 1999), CCR5 (Mueller and Strange, 2004) and NK1 (Roosterman et al., 2004) receptors, may use this mechanism to both maintain signaling efficacy as well as an adequate pool of cell surface receptors (Koch et al., 1998, 2001; Law et al., 2000; Finn and Whistler, 2001; see Connor et al., 2004 for review). In this hypothesis internalization is accompanied by enhanced recovery and less desensitization, leading to an inverse relationship between internalization and desensitization (Koch et al., 2005; see Cox, 2005 for review).

Different μOR agonists vary in their ability to induce tolerance. For example morphine, a low efficacy or partial μOR agonist, induces greater tolerance than etorphine and fentanyl, high efficacy or full μOR agonists (Duttaroy and Yoburn, 1995). These agonists also differ in their capacity to induce internalization and desensitization (Alvarex et al., 2002; Koch et al., 2005), properties that have been used to provide some insight into the cellular mechanisms leading to morphine-induced tolerance. Since the majority of these studies have been carried out in non-neuronal cell-lines the applicability of these models to neuronal systems is largely unknown. Indeed, recent data suggest that morphine may actually internalize the μOR in striatal neurons (Habberstock-Debic et al., 2005). Dorsal root ganglia (DRG) sensory neurons are a major site of action of opiate drugs in mediating analgesia and are therefore arguably an ideal model neuron in which to study molecular mechanisms of μOR activation, desensitization, internalization and recycling that may have a direct bearing on analgesic tolerance. We utilized primary cultures of DRG neurons from μOR knockout mice transduced with either the μOR or a chimeric receptor (mu/delta opioid receptor, μ/δOR) in which the carboxyl-terminus (CT) of the μOR is replaced with that of the delta opioid receptor (δOR), since this receptor was previously shown to internalize in response to morphine in HEK293 cells and neurons (Whistler et al., 1999; Habberstock-Debic et al., 2003). Associated with such enhanced internalization, this chimeric receptor also showed greater desensitization of the adenyl cyclase response to morphine, forming an integral component of the RAVE hypothesis linking receptor internalization with desensitization (Whistler et al., 1999). The relationship between receptor internalization and desensitization of agonist-induced inhibition of Ca2+ channel currents was examined in these two receptors in response to two ligands, δ-Ala2, N-MePhe4, Gly5-ol-enkephalin (DAMGO) and morphine, recently shown to be at opposite ends of the internalization–desensitization spectrum when tested in cell-lines (Koch et al., 2005).

**EXPERIMENTAL PROCEDURES**

**Primary cultures**

Primary DRG neuronal cultures were made from p0–p3 pups of either wild-type or μOR knockout mice in which exon 2 has been disrupted by the neomycin cassette (Matthes et al., 1996). DRG from all spinal levels were removed and enzymatically dissociated in trypsin (2.5%, Invitrogen, Carlsbad, CA, USA) for 20 min after which they were triturated through a graded series of fire polished Pasteur pipettes. The dissociated cells were plated at a density of 1×10^5 cells/cm^2 onto a polyl-ornithine– (Sigma, St. Louis, MO, USA) and laminin–(Becton Dickinson, Bedford, MA, USA) coated surface. For flow cytometry experiments the cells were plated on a sunken coverslip in the bottom of a 10 cm diameter dish. This forms a well 16 cm^2 in area into which 2×10^5 cells were plated in 1 ml of media and allowed to settle before adding the remaining 9 ml of media. For immunocytochemistry experiments these cells were plated at the same density but on polyl-ornithine-coated 16-well chamber slides (Fisher, Pittsburgh, PA, USA). The cultures were fed with serum free Neurobasal A (Invitrogen) supplemented with B-27, L-glutamxl (Invitrogen), 2.5s nerve growth factor (10 μg/ml, Roche, Indianapolis, IN, USA) and 5-fluoro-2-deoxyuridine (FDU; 20 μg/ml, Sigma) and incubated at 37 °C and 5% CO_2.

**Adenoviral vector production**

The cDNA encoding the flag-tagged mouse μOR has been described and characterized by Keith et al. (1996). The HA-μOR cDNA was kindly donated by Dr. P. Law (University of Minnesota Medical School, Minneapolis, MN, USA) and is described in detail by Alfry et al. (1998). The μOR or δOR cDNAs and enhanced green fluorescent protein (EGFP) were placed behind two cytemagaloviral promoters in tandem orientation and incorporated into the adenoviral genome using the Adeasy system (He et al., 1998). Purified and concentrated stocks were obtained by a discontinuous step gradient and the titers of the Ad-μOR determined to be 1.45×10^10 infectious units (IU)/ml and the Ad-δOR, 2.54×10^10 IU/ml.

**Adenoviral transduction of DRG neurons**

Within 12–24 h after plating, the media was removed, leaving just enough to cover the cells. The virus was dialyzed, diluted appropriately and dropped into each well. After 1–2 h of adsorption the remaining conditioned media was added to the wells and the cultures returned to the incubator. The volume of virus applied per dish depended on the number of cells plated and desired multiplicity of infection (MOI or number of IU applied per cell) which was between 1 and 10 for all experiments.

**Whole cell patch clamp recordings**

The whole-cell patch-clamp technique was used to record voltage-activated Ca2+ channel activity from cultured DRG neurons after 3–4 days in vitro (Axopatch 200A amplifier, Axon Instruments Inc., Sunnyvale, CA, USA). Culture media were replaced with an external solution containing (in mM): 130 TEA-Cl, 10 CaCl2, 5 Heps, 25 α-glucose and 2.5×10^{-4} tetrodotoxin at pH 7.2. Recording electrodes contained (in mM): 105 CsCl, 40 Heps, 5 α-glucose, 2.5 MgCl2, 10 EGTA, 2 Mg-ATP and 0.5 GTP, pH 7.2. The potential difference between the open electrode and the bath ground was zeroed prior to establishing a ±1-GOhm resistance seal. No compensation was made for the cancellation of liquid junction potential. Ca2+ currents were activated by depolarizing neurons from −80 mV to 10 mV for 100 ms at 10 s intervals. Currents were low-pass filtered at 2 kHz and digitized (Digitax, Axon Instruments Inc.) at 10 KHz for storage on the hard drive of a Pentium PC. Leak currents were nulled using the P/4 subtraction method. Opioid agonists were diluted into external solution on the day of the experiment and applied through the perfusion system. Experiments were performed at room temperature (22–24 °C). Rapid desensitization was examined by recording at 20 s intervals during continual perfusion (5 ml/min) of the cells with external solution containing DAMGO or morphine (1 μM) for 5 min. Long-term desensitization was examined in cells that had been pre-treated with DAMGO (1 μM) or morphine (1 μM) for 24 h. The
media were removed and the cells washed extensively with the external solution before testing the Ca\(^{2+}\) current inhibition induced by DAMGO (1 \(\mu\)M).

**Data analysis**

Mean Ca\(^{2+}\) current amplitudes were measured (pCLAMP 9.0, Axon Instruments Inc.) between 5 and 10 ms after initiating the depolarizing step. Mean current amplitudes were then plotted against time. Recordings that exhibited marked rundown were discarded. Stable recordings were fitted by a linear function to compare, by extrapolation, current amplitude with the current amplitude recorded in the presence of DAMGO or morphine. Rapid desensitization was determined by comparing agonist-induced inhibition of current amplitude with that after 5 min of continous agonist application and the difference in inhibition expressed as a percentage of the initial inhibition. Long-term desensitization was determined by comparing agonist-induced inhibition of current amplitude in agonist pre-treated versus untreated cells. Data are expressed as mean\(\pm\)S.E.M and analyzed by one-way analysis of variance (ANOVA).

**Flow cytometry**

**Internalization.** By days 3–4 in vitro or 2–3 days after transduction with the Ad-\(\mu\)OR or Ad-\(\mu\)/\(\delta\)OR the neurons were treated with DAMGO or morphine (1 \(\mu\)M ea), for 20 min or 24 h, or remained untreated, the media replaced with ice-cold phosphate-buffered saline (PBS)/2 mM EDTA and the cells harvested on ice. After pelleting the cells at 160 \(g\) for 5 min at 4 °C, the cells were washed in 2% FBS/0.1% Na\(_2\)PO\(_4\)/PBS (FBS/Na\(_2\)PO\(_4\)/PBS) and incubated in the primary antibody (biotinylated anti-Flag, 10 \(\mu\)g/ml Sigma), biotinylated anti-hemagglutinin (HA), 10 \(\mu\)g/ml Roche, or anti-\(\mu\)OR, Chemicon, Billerica, MA, USA) for 30 min. The cells were incubated in streptavidin–peridinin chlorophyll-a protein (PerCP, BD Immunocytometry, Carlsbad, CA, USA, 1:200) or allophycocyanin (APC) -conjugated IgG (Molecular Probes, Eugene, OR, USA, Invitrogen) for an additional 30–60 min at RT and after a final 30 min wash in FBS/Na\(_2\)PO\(_4\)/PBS the data were acquired on a FACScalibur flow cytometer (CellQuest 3.0.1, BD Immunocytometry Systems, Mountain View, CA, USA).

Cell surface receptor recycling, synthesis and degradation. Monensin, an ionophoric drug that prevents vesicle export from the Golgi apparatus (Mollenhauer et al., 1990) was used to inhibit GPCR recycling at a 300 nM concentration. Receptor turnover was assessed by applying cycloheximide at a dose sufficient to prevent amino acid incorporation (100 mg/ml, Mitchell et al., 2004). DRG neurons were treated with monensin or cycloheximide for 30 min, or remained untreated, harvested with PBS/EDTA, and the \(\mu\)OR or \(\mu\)/\(\delta\)OR was labeled and analyzed by flow cytometry as described for the internalization experiments.

**Data analysis.** The flow cytometry data were analyzed using FlowJo software (FlowJo, Ashland, OR). The neuronal population was initially defined by size and granularity (FSC-H and SSC-H) and selected as Region 1 (R1). The EGFP expressing neurons were then selected by their fluorescence in the first fluorescence channel and defined as Region 2 (R2). The mean fluorescence intensity of the PerCP-labeled cells in R2 was then obtained. After subtracting background fluorescence from the unlabeled and isotype specific controls, the mean fluorescence of the experimental samples was normalized to the untreated sample. All data were analyzed by ANOVA with the LSD as a post hoc test, significance accepted at the 5% confidence interval and are presented as mean\(\pm\)S.E.M.

**Immunocytochemistry**

DRG neurons plated in chamber slides, remained untreated, or were treated with DAMGO for 20 min, 1 h or 4 h, fixed in 4% paraformaldehyde for 5 min and washed. After a 20 min wash in PBS containing 1% normal goat serum (NGS) and 0.3% Triton X-100 (TX) at RT, the cultures were incubated overnight at 4 °C in the primary antibody. The cells were then washed in PBS/1% NGS/0.3% TX and incubated in the secondary antibody for 90 min at RT followed by a further three washes in the same solution. If a tertiary antibody was used it followed this same protocol as for the secondary. The primary antibodies used were; anti-Rab4 and 11, anti-CD107a (lysosomal-associated membrane protein-1, LAMP-1, BD Biosciences, San Jose, CA, USA), anti-HA (MBL, Nagoya, Japan), biotinylated anti-HA (Roche), and anti-Flag and biotinylated anti-Flag (Sigma). The secondary antibodies used were: Cy3-conjugated anti-rabbit/rat/mouse IgG (1:200–1:500), biotinylated anti-mouse (1:200), Cy5-conjugated anti-rabbit/rat/mouse (1:200–1:500) and Cy3/Cy5-conjugated streptavidin (1:1000–2000, all from Vector Laboratories, Burlingame, CA, USA). Having identified the EGFP and by extension, HA- or Flag-\(\mu\)OR positive neurons, images were obtained by confocal laser scanning microscopy (Carl Zeiss LSM 310) and are portrayed as maximum intensity projections of five to seven images taken at 1 \(\mu\)m z-scale intervals through the near-equatorial region of each neuron.

**Data analysis**

Confocal laser scanning RGB images were analyzed for co-localization of the blue (Cy5, the \(\mu\)OR or \(\mu\)/\(\delta\)OR) and red (Cy3, Rab4/11 and LAMP-1) channels. The color (purple) of the co-localized pixels was then selected and this selection applied to all neurons labeled with the same antibodies (Rab4, Rab11 or LAMP-1 with the \(\mu\)OR or \(\mu\)/\(\delta\)OR), the images of which were placed in a single file. This color was displaced into the unused channel, green, of the RGB image (green, Photoshop 7, Adobe) and the numbers of pixels in the green channel quantified (Imagequant, Molecular Probes, Invitrogen). All images from one labeled intracellular protein (Rab4, Rab11 or LAMP-1) were compiled into a single file and batch processed to avoid observer bias. The data are expressed as the percentage of green, or co-localized, pixels per unit area of the neuron to account for the different neuronal sizes present. Data were analyzed by the two-tailed Student’s t-test.

**RESULTS**

**Characterization of Ad-\(\mu\)OR and Ad-\(\mu\)/\(\delta\)OR expression in DRG neurons**

All of the experiments described in this paper used primary neuronal cultures harvested from DRG of early postnatal \(\mu\)OR knockout or wild-type mice in the C57BL/6 background (Matthes et al., 1996), the composition of which we have previously analyzed by flow cytometry and shown to contain neurons (76±1.1% of the total cell population), fibroblasts (6.6±2.9%) and Schwann cells (10.0±2.0%) (Walwyn et al., 2004). We have also previously shown how adenoviral-mediated expression of the \(\mu\)OR in DRG neurons from \(\mu\)OR knockout mice recapitulates many of the features of the endogenously expressed receptor, including Ca\(^{2+}\) channel inhibition, desensitization and internalization (Walwyn et al., 2004). Using the same adenoviral expression vector, we replaced the \(\mu\)OR cDNA with that of the chimeric \(\mu\)/\(\delta\)OR. The \(\mu\)/\(\delta\)OR cDNA contains an N-terminal HA tag and the CT has been switched with that of

the /iOR at residue 341 (Afify et al., 1998). Apart from the gene of interest, all other aspects of this /iOR adenovirus (Ad-/iOR) matched the /OR adenovirus (Ad-/OR), expressing both the gene of interest and EGFP under the control of two independent cytomembral promoters (He et al., 1998). We characterized the chimeric /iOR as we had done for the /OR (Walwyn et al., 2004) and found that increasing the viral load or MOI from 1 to 10 increased the number of neurons expressing EGFP and the /OR or /iOR but at MOIs higher than 10 no further increase occurred. Again similar to the data obtained from analysis of /OR expression, /iOR expression levels, as indicated by relative fluorescence intensity of the N-terminal-tag, remained constant over the range of MOIs (1–100) used. Simultaneous analysis of neurons transduced with the Ad-/OR and Ad-/iOR viruses showed similar expression levels; /iOR; 8.8 × 10^5; /OR; 8.6 × 10^5 receptors/cell (n = 6).

Rapid desensitization of Ca^{2+} current inhibition

Ligand-induced phosphorylation of GPCRs prevents further G-protein coupling with the receptor, initiating desensitization. Such acute or rapid desensitization of the endogenous /OR, occurring within minutes of continual agonist exposure, has been previously observed in DRG neurons by measuring the Ca^{2+} currents during continuous agonist perfusion (Samoriski and Gross, 2000; Tan et al., 2003). Similar rapid desensitization of agonist-induced Ca^{2+} current inhibition was observed in the current study in DRG neurons of /OR knockout mice expressing the /OR or /iOR, examples of the effect of DAMGO are shown as the change in the calcium current as a result of the depolarizing stimulus (Fig. 1A) and of the peak current amplitude (Fig. 1B). We found that DAMGO and morphine did not differ in their ability to rapidly desensitize either the /OR (DAMGO: 9 ± 3%; morphine: 10.9 ± 3.9%); or the /iOR (DAMGO: 20 ± 3%; morphine, 30.8 ± 4.9%; Fig. 1C) at the 1 μM concentration used. However, irrespective of which ligand was applied, more extensive desensitization was seen with the /iOR than the /OR (P < 0.05).

Internalization of the /OR and /iOR

Following agonist activation, phosphorylation and G-protein uncoupling, the /OR may be internalized, albeit by different amounts depending on the agonist applied (Alvarez et al., 2002; Borgland et al., 2003; Koch et al., 2005). Such differences in internalization efficacy also depend on the receptor. For example, the /iOR shows greater internalization than the /OR in cell lines and in nucleus accumbens neurons (Afify et al., 1998; Whistler et al., 1999; Haberstock-Debic et al., 2003). We quantified ligand-induced internalization of these receptors in virally transduced DRG neurons by flow cytometry (Fig. 2). We gated on the larger and less granular, therefore neuronal (Walwyn et al., 2004) EGFP-receptor positive population, and found that a 20 min exposure to the /OR agonist, DAMGO (1 μM), internalized both receptors (P < 0.05) although the /iOR had internalized by almost twice as much as the /OR (P < 0.05). Longer exposure to DAMGO (24 h, 1 μM) increased the loss of cell surface /ORs and /iORs compared with the 20 min exposure (P < 0.05). Morphine (1 μM), on the other hand, did not internalize either receptor after 20 min, but after 24 h of morphine treatment, both receptors showed an equivalent loss of cell surface receptor number (P < 0.05 vs. untreated), albeit to a lesser extent than induced by DAMGO (P < 0.05).

Long-term desensitization of Ca^{2+} current inhibition

The /OR desensitizes after prolonged agonist incubation as a result of adaptations occurring at the level of the receptor, and also within second messenger signaling systems and associated cellular processes. Although not equivalent to tolerance, a behavioral response, chronic receptor desensitization has long been considered an important cellular component of this phenomenon. We therefore treated /OR- and /iOR-expressing cells with DAMGO or morphine (1 μM), for 24 h, washed the cells extensively, and determined the extent of Ca^{2+} current inhibition induced by a 1 μM DAMGO test concentration (Fig. 3). A comparison of these data to the inhibition from cells that had not been pre-treated (/OR: 46.5 ± 2.8, μiOR: 53.1 ± 3.09, n.s.) showed that 24 h of DAMGO, but not morphine (both at 1 μM) attenuated the /OR-mediated Ca^{2+} current inhibition compared with untreated cells (P < 0.001, Fig. 3). In comparison, the /iOR desensitized less than the /OR following 24 h DAMGO pre-treatment and showed a similar degree of desensitization whether DAMGO or morphine treated (P < 0.05, Fig. 3).

If receptor internalization is primarily a means of curtailing signaling and preventing compensatory intracellular downstream events that would otherwise produce tolerance in vivo, then internalization and desensitization of Ca^{2+} current inhibition would be expected to be positively related (Alvarez et al., 2002). The present data support a positive relationship in the case of the /OR, which both desensitized (DAMGO: 44 ± 2% vs. morphine −17 ± 10%) and internalized more (20 min: DAMGO: 17 ± 2% vs. morphine 5 ± 5% and 24 h: DAMGO: 39 ± 2% vs. morphine: 25 ± 7%), when treated with DAMGO than morphine. The endogenous /OR showed a similar positive relationship between internalization, measured at 20 min (DAMGO: 13 ± 4 vs. morphine: 4 ± 1%), or 24 h (DAMGO: 22 ± 4 vs. morphine: 4 ± 4%); and desensitization (DAMGO: 96 ± 1% vs. morphine: 62 ± 3%) although desensitization was greater than the viral system, presumably a result of higher levels of receptor expression (Walwyn et al., 2004). In contrast, the /iOR, which desensitized equally whether DAMGO (25 ± 5%) or morphine (21 ± 8%) treated, exhibited no such positive or negative relationship.

For both receptors this desensitization was heterologous in nature, as shown by DAMGO or morphine desensitization of the inhibitory effect of baclofen, a GABA_b agonist, on the Ca^{2+} currents in μOR or μiOR expressing neurons (Fig. 4).
Intracellular trafficking pathways of the μOR and μδOR

Compared with the μOR, the chimeric μδOR showed greater internalization during the first 20 min of DAMGO application (Fig. 2) and less chronic desensitization (Fig. 3) when treated with DAMGO. This could be a result of different intracellular trafficking of these internalized receptors. We investigated this by treating DRG neurons with...
DAMGO (1 μM) for increasing times, fixing and labeling the cells with antibodies to the two receptors and different endosomal/lysosomal organelles and used confocal laser scanning microscopy to acquire and subsequently quantify receptor co-localization with these different organelles.

Recycling. There are at least two recycling pathways by which GPCRs can return to the cell membrane: a fast/early sorting pathway occurring within minutes and a slow/late sorting pathway taking several hours (Sheff et al., 1999). Two of the small Rab GTPases have been used to label these pathways; Rab4 is expressed on the early sorting endosomes regulating exit to early recycling endosomes (Li and Stahl, 1993) and is used to label the fast/early pathway, and Rab11, associated with the trans-Golgi network in the peri-nuclear region, mediates the exit of receptors from early/sorting to late recycling endosomes and has been used as a marker of the slow/late recycling pathway (Ren et al., 1998).

We found that both receptors co-localized with Rab4 after 20 min, 1 h and 4 h of DAMGO treatment indicating no difference between the receptors at this early endosomal recycling/sorting stage (Fig. 5A). However, the images show a different cellular distribution of these Rab4-co-localized receptors. The μOR-Rab4-co-localized pixels were close to the cell surface after 20 min whereas the μ/δOR-Rab4 pixels were found within the cytosol at these time-points.

The internalized μOR also co-localized with Rab11, again within the cytosol in peri-nuclear regions of the cell. However, the chimeric μ/δOR did not co-localize with Rab11 (Fig. 5B) suggesting that the μOR, but not the μ/δOR, is trafficked through the slow, Rab11-mediated recycling pathway in these neurons.

Fig. 2. Internalization of the μOR and χ/δOR. (A) DRG neurons expressing the μOR or μ/δOR either remained untreated or were treated with DAMGO or morphine (1 μM for 20 min or 24 h), labeled with antibodies to their N-terminal tags and the fluorescent intensity of the labeled cell surface receptors analyzed by flow cytometry (see Walwyn et al., 2004 for further details). A decrease in this intensity, compared with the control, untreated cells, was interpreted as internalization. Neurons expressing the μ/δOR internalized further when treated with DAMGO for 20 min (a; P<0.05 vs. μOR) than those expressing the μOR and both showed a further increase in internalization/downregulation after 24 h (b and c; P<0.05 vs. the same receptor treated with DAMGO for 20 min). Although morphine did not result in internalization of either receptor after 20 min, after 24 h of morphine, both receptors had internalized, albeit to a lesser extent than after 24 h DAMGO. (* P<0.05 vs. untreated, same receptor, d and e; P<0.05 vs. the same receptor treated with DAMGO for 24 h, n=3–4 experiments of ~10,000 ea.) (B) Examples of the DRG neurons used for flow cytometry analysis in A, imaged by confocal laser scanning microscopy (shown as maximum intensity projections) to visualize the effect of DAMGO or morphine on μOR distribution. Scale bar=10 μm.
Degradation. Using an antibody to LAMP-1, we found that the internalized chimeric μ/δOR co-localized with this lysosomal marker in DRG neurons, whereas the μOR did not (Fig. 5C).

Although both the μOR and the μ/δOR co-localized with Rab4, the μOR co-localized with Rab11 and the μ/δOR co-localized with LAMP-1, suggesting that the μ/δOR follows both a recycling and degradative pathway whereas the μOR is mostly recycled.

Receptor synthesis, degradation and recycling

The μ/δOR, but not the μOR, desensitized equally whether treated with DAMGO or morphine for 24 h, suggesting that such chronic desensitization was unrelated to the applied ligand but was affected by the CT of this receptor. Suspecting that the δOR CT changed the ligand-independent trafficking profile of the μOR, we looked at how monensin, a recycling inhibitor (Mitchell et al., 2004), affected the basal levels of these receptors on the cell surface. We found that after a 30

Fig. 3. Chronic desensitization. Prolonged DAMGO, but not morphine, pre-treatment (24 h, 1 μM) decreased DAMGO-mediated inhibition of the Ca\textsuperscript{2+} currents in μOR expressing cells. In contrast the μ/δOR desensitized equally whether DAMGO or morphine treated and * P<0.05, *** P<0.001 vs. untreated of the same receptor, n=7–28.

Fig. 4. Heterologous desensitization. Prolonged treatment of the μOR and μ/δOR with DAMGO or morphine (24 h, 1 μM) similarly desensitized the baclofen (50 μM) -mediated Ca\textsuperscript{2+} current inhibition showing heterologous desensitization of GABA\textsubscript{A} receptors. * P<0.05, *** P<0.001 vs. untreated of the same receptor, n=6–11.
**Fig. 5.** The recycling and lysosomal fate of the μOR and μ/δOR in neurons. (A) The internalized μ/δOR and μOR (blue) are trafficked through Rab4-positive early sorting/recycling endosomes (red) after 20 min, 1 h and 4 h DAMGO. Maximum intensity projections of six to seven near-equatorial serial sections were taken, at 0.5 μm z-scale intervals, of neurons expressing the μOR or μ/δOR and the number of co-localized green pixels from three to five such neurons quantified and expressed as a percentage of the total neuronal area. The left panel shows these results from untreated neurons and after 20 min, 1 h and 4 h DAMGO (1 μM) and the right panel representative neurons from each time-point for each receptor. Although there both the μOR and μ/δOR showed the same amount of co-localization, these images show green μOR-Rab4 pixels within the cell bodies whereas the green μ/δOR-Rab4 pixels appeared closer to the cell membrane. (B) The internalized μOR, but not the μ/δOR, traffics through Rab11 containing recycling endosomes whereas the internalized μ/δOR rarely co-localizes with Rab11. This is shown by the co-localization of Rab11, shown in red,
min, 300 nM, monensin treatment, the $\mu$OR decreased to $57\pm5\%$, and the $\mu$OR to $80\pm6\%$ of control levels, $P<0.05$. However, monensin, in disrupting protein exit from the Golgi apparatus (Mollenhauer et al., 1990), also disrupts the appearance of newly synthesized receptors at the cell surface. Cycloheximide, a protein synthesis inhibitor, was therefore used to discern how much receptor was being degraded rather than recycled, and was found to decrease cell surface $\mu$ORs by $15.8\pm0.9\%$ ($P<0.05$ vs. untreated), but had no effect on the $\mu$OR (1.3±2.0%) suggesting that the $\mu$OR is synthesized and degraded more rapidly under basal conditions. Subtracting the effect of cycloheximide from that of monensin to discern the percentage of receptors that are recycled showed that the two receptors undergo equal constitutive recycling ($\mu$OR: 19.2±5.9%, $\mu$OR, 27.1±4.7%).

**DISCUSSION**

Tolerance readily develops to many of the $\mu$OR-mediated effects of morphine in vivo despite the apparent reluctance of the $\mu$OR to internalize upon exposure to this drug, suggesting that the lack of internalization may be associated with tolerance. Two models have been put forward to explain this association. The first model proposes that failure of an agonist to induce internalization promotes long-term signaling of the receptor resulting in adaptive changes in downstream signaling pathways that are critical components of tolerance (Whistler et al., 1999). The second model considers internalization to be primarily a means of removing uncoupled receptors from the cell surface enabling them to be recycled to the membrane in a coupled state. In the latter case, agonists that fail to internalize the receptor may promote tolerance in vivo because uncoupled receptors are not recycled and signaling is curtailed (Koch et al., 2005). A clear distinction between the two models is that the former model predicts continual receptor–effector coupling during chronic morphine exposure, and thus a positive correlation between internalization and desensitization (Alvarez et al., 2002), while the latter model predicts uncoupling of the receptor and a negative correlation between the two phenomena (Koch et al., 2005). Using DAMGO and morphine, two agonists at opposite ends of the internalization/desensitization spectrum at the $\mu$OR (Koch et al., 2005), we find evidence of a positive relationship between internalization and chronic desensitization in DRG neurons, refuting the concept that internalization is protective against desensitization in this neuronal background.

The global definition of $\mu$OR desensitization, often considered a cellular correlate of tolerance, has typically been measured at different time-points, in different second messenger systems, and in different cellular backgrounds, leading to considerable confusion in this field. We have begun to address this by measuring both types of desensitization.

Rapid desensitization of $\mu$OR-mediated $Ca^{2+}$ channel inhibition, a rapid, membrane-delimited, but heterologous response, possibly due to desensitization of the $G_{0/5}$ interaction with the N-type calcium channel in sensory neurons (Samoriski and Gross, 2000), can be seen during the first 5 min of agonist exposure. This contrasts with the homologous nature of such rapid desensitization in AT20 cells and locus coeruleus neurons, perhaps a result of the different calcium channels present within these different cellular backgrounds (Samoriski and Gross, 2000; Borgland et al., 2003). In our system this type of desensitization occurred equally in cells that were morphine or DAMGO treated suggesting that internalization is not a critical component of rapid desensitization in neurons, and furthermore that receptor uncoupling and internalization are separable events, as suggested from studies of AT20 cells (Celver et al., 2004) and locus coeruleus neurons (Arttamangkul et al., 2006). Although morphine and DAMGO both resulted in equal rapid desensitization, the recovery process may differ between these ligands, as shown by the slower recovery of the morphine- than DAMGO-treated $\mu$ORs (Dang and Williams, 2004). This suggests that internalization may enhance recovery from rapid desensitization but does not affect the initial desensitization response.

After 24 h agonist exposure, a time-point arguably of greater significance to in vivo measures of tolerance, morphine, while promoting less internalization of the $\mu$OR than DAMGO, also induced less desensitization of DAMGO-induced $Ca^{2+}$ current inhibition. Importantly, this positive relationship was also observed for the endogenously expressed $\mu$OR in wild-type DRG neurons indicating that this is not an artifact of receptor over-expression. Our data are not consistent with the concept, most recently espoused by Koch et al. (2005) of internalization as a means to return dephosphorylated receptors back to the surface membrane. The apparent incongruence in the two data sets may be explained by several factors. Most importantly, the two experiments utilized different measures of receptor–effector coupling; adenylyl cyclase versus $Ca^{2+}$ channel inhibition. Coupling between opioid receptors and $Ca^{2+}$ channels is less efficient than coupling between opioid receptors and adenylyl cyclase (Prather et al., 2000) which could contribute to apparent differences in the mechanisms of desensitization. In addition the cyclase assay often uses the same ligand as both the pre-treatment and test ligand. If we used this ‘one ligand’ approach to both induce and measure chronic desensitization of the $\mu$OR-mediated $Ca^{2+}$ channel inhibition we find a greater apparent desensitization induced by morphine and an inverse relationship between internalization and desensitization.

with the $\mu$OR or $\mu$OR, shown in blue. The number of green (red + blue) co-localized pixels was quantified and expressed as a percentage of the total neuronal area in untreated neurons and after 20 min, 1 h and 4 h DAMGO, shown in the left panel. Representative neurons are shown in the right panel. * $P<0.05$ vs. $\mu$OR at the same time-point. (C) The internalized $\mu$OR (shown in blue) co-localized with LAMP-1 labeled lysosomes (shown in red) whereas the $\mu$OR (shown in blue) shows little such co-localization. The number of co-localized green pixels was quantified and expressed as a percentage of the total neuronal area in untreated neurons and after 20 min, 1 h and 4 h DAMGO, shown in the left panel, and representative neurons in the right panel. * $P<0.05$ vs. $\mu$OR at the same time-point, scale bar=20 $\mu$M, $n=4–8$. 

(\(y = -2.2016x + 88.658, r^2 = 0.9411, P < 0.001\). Spearman’s rank correlation). This may be explained by a differential decrease in efficacy of partial agonists, i.e.; morphine, compared with full agonists, such as DAMGO, during prolonged application. The two experiments also differed in the pre-treatment protocol, measuring desensitization after 1 h (Koch et al., 2005) or 24 h (our data). Although 24 h is more reflective of the changes mediated by tolerance, this time-point may have introduced a receptor down-regulation component that might similarly mask potential re-sensitizing effects of receptor recycling. Recycling may indeed occur and affect desensitization but the overriding effect is of enhanced desensitization occurring together with enhanced internalization.

In a neuronal background we found evidence of a major heterologous component to both DAMGO- and morphine-induced desensitization of Ca\(^{2+}\)-current inhibition at 24 h in that the response to bacoafen was also severely attenuated by opioid pre-treatment of either receptor. Such heterologous desensitization in DRG neurons, presumably occurring at the level of the Ca\(^{2+}\)-channel and possibly mediated by MAP kinase, has been reported previously (Nomura et al., 1994; Samoriski and Gross, 2000; Tan et al., 2003) and may effectively override potentially more subtle effects of receptor recycling in this neuronal setting. This type of desensitization may be mediated by changes in G-protein dissociation (Woolf and Linderman, 2003) and receptor coupling (Tan et al., 2003) and compensatory adaptations within other second messenger systems that affect long-term desensitization such as the MAP kinase cascade. This cascade is an important mediator of long-term desensitization of the \(\mu\)OR in cell lines and DRG neurons (Polakiewicz et al., 1998; Tan et al., 2003).

Although the \(\mu\)i\(\delta\)OR is not found endogenously, we chose to study this chimeric receptor as previous reports had shown greater morphine-induced internalization of the \(\mu\)i\(\delta\)OR than \(\mu\)OR (Whistler et al., 1999; Haberstock-Debic et al., 2003), possibly affecting desensitization in a neuronal background. Indeed rapid desensitization was enhanced by the \(\delta\)OR CT when treated with DAMGO or morphine, perhaps a result of different GPCR kinase phosphorylation sites in the \(\delta\)OR CT (Schulz et al., 2002). Although greater morphine-induced internalization was not apparent in DRG neurons, we did find that the chimeric receptor internalized more than the \(\mu\)OR in response to DAMGO at 20 min (but not at 24 h). \(\beta\)-Arrestin has greater affinity for the \(\delta\)OR than the \(\mu\)OR in response to DAMGO (24 h). \(\beta\)-Arrestin has greater affinity for the \(\delta\)OR than the \(\mu\)OR in response to DAMGO (24 h). Although both receptors co-localized with Rab4, the Rab4 colocalized \(\mu\)i\(\delta\)ORs were found closer to the cell surface perhaps reflecting a more efficient return of the \(\mu\)i\(\delta\)OR to the cell surface.

The \(\mu\)i\(\delta\)OR desensitizes equally whether morphine or DAMGO treated suggesting a ligand, or internalization, independent mechanism of desensitization, such as \(\delta\)OR CT-specific changes in the rate of constitutive recycling or receptor synthesis. As both receptors showed an equal rate of constitutive recycling, this type of trafficking is an unlikely candidate. However, the \(\delta\)OR CT enhanced the rate of synthesis of the chimeric receptor increasing the number of newly synthesized receptors on the cell surface available for ligand activation. Such rapid receptor turnover may provide a mechanism, in addition to receptor internalization and recycling, of regulating cell surface receptor levels and desensitization.

In a complex neuronal background we have found that morphine, a partial \(\mu\)OR agonist that causes analgesic tolerance, results in less internalization and less chronic desensitization of the Ca\(^{2+}\) channel response, than the full agonist, DAMGO. This suggests that the lack of both internalization and desensitization by morphine results in tolerance in vivo by inducing long-term cellular adaptations in downstream signaling systems rather than simply preventing the return of de-phosphorylated receptors back to the cell surface. Although these data suggest that internalization may indeed be related to desensitization in the case of the \(\mu\)OR, the greater synthesis of the \(\mu\)i\(\delta\)OR and equivalent desensitization in spite of differential amounts of internalization suggest that yet another aspect of receptor trafficking, the rate of receptor synthesis and degradation, may also affect receptor function.

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REFERENCES


