Mu opioid-induced desensitization of ORL1 and mu opioid receptors:  

Differential intracellular signaling determines receptor sensitivity

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Abbreviations: DAMGO, D-Ala2,N-Me-Phe4, Gly5-ol enkephalin; ERK1/2, extracellular signal-regulated kinases 1 and 2; GRK, G protein-coupled receptor kinase; HBSS, Hank’s balanced salt solution; IBMX, 3-isobutyl-1-methylxanthine; µ, mu opioid receptor (same as the IUPHAR designated mu opioid peptide receptor, MOP); ORL1, opioid receptor-like1 receptor (same as the IUPHAR designated nociceptin opioid peptide receptor or NOP); OFQ/N, orphanin FQ/nociceptin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PKC, Protein kinase C; SDS, sodium dodecyl sulfate.
Abstract

Mu opioid receptors have been shown to contribute to orphanin FQ/nociceptin (OFQ/N)-mediated analgesia and hyperalgesia, indicating that both pro- and anti-nociceptive actions of OFQ/N are influenced by \( \mu \) opioid receptors. A 60 min activation of \( \mu \) or opioid receptor-like1 (ORL1) opioid receptors natively expressed in BE(2)-C human neuroblastoma cells desensitized both \( \mu \) and ORL1 receptor-mediated inhibition of cAMP accumulation. The mechanism(s) of OFQ/N-mediated \( \mu \) and ORL1 cross-talk involve the conventional protein kinase C isozyme, PKC-\( \alpha \), and G protein-coupled receptor kinases (GRK) 2 and 3 (Mandyam et al., 2002). Unlike OFQ/N-mediated desensitization of ORL1 and \( \mu \) opioid receptors, D-Ala\(^2\), N-Me-Phe\(^4\), Gly\(^5\)-ol enkephalin (DAMGO)-mediated ORL1 desensitization in BE(2)-C cells is PKC-independent. However, DAMGO (1 \( \mu \)M) pretreatment increased membrane levels of GRK2 and GRK3 indicating their translocation to the membrane upon activation. This suggests that DAMGO activation of \( \mu \) opioid receptors results in GRK2 and GRK3 inactivation of ORL1 upon challenge with OFQ/N. Antisense, but not sense, DNA selectively targeting GRK2 or GRK3 blocks DAMGO-mediated \( \mu \) and ORL1 desensitization, respectively. However, in SH-SY5Y neuroblastoma cells, DAMGO failed to desensitize ORL1 or alter membrane PKC-\( \alpha \) or GRK levels. Instead, DAMGO stimulated PKC-\( \varepsilon \) translocation to the cell membrane, and produced \( \mu \) receptor desensitization. These results indicate that acute exposure to \( \mu \) receptor agonists can regulate ORL1 function, but the ability to do so varies from cell type to cell type. These results also confirm the existence of multiple signaling mechanisms for \( \mu \) opioid receptors and the importance of these mechanisms for \( \mu \) receptor mediated-heterologous effects.
Opioid analgesics work in part, by activating opioid receptors in brainstem pain-modulating sites (Heinricher et al., 1997; Pan et al, 2000). Endogenous opioid peptides act via four major types of opioid receptors: μ, δ, κ and opioid receptor-like1 (ORL1). Actions of the endogenous opioid peptide orphanin FQ/nociceptin (OFQ/N) at ORL1 are both opioid-like (analgesic) and anti-opioid in nature (Jhamandas et al., 1998; Lutfy and Maidment, 2000). The dual physiological responses of OFQ/N may be explained by the actions of OFQ/N on ORL1 expressed on two opposing neuronal populations in the midbrain nucleus raphe magnus (Pan et al., 2000); one population expressing both μ and ORL1 (“ON” cells) and the other expressing ORL1 and κ1 receptors (“OFF” cells). Recent in vivo reports also have implicated μ opioid receptor involvement in OFQ/N-mediated analgesia and hyperalgesia. Blockade of spinal OFQ/N analgesia by the irreversible μ receptor antagonist β-funaltrexamine (β-FNA) suggests a role for μ receptors in OFQ/N analgesia (Jhamandas et al., 1998). OFQ/N produces supraspinal hyperalgesia upon blockade of μ opioid receptors by a selective μ opioid receptor antagonist (Lutfy and Maidment, 2000), indicating that both pro- and antinociceptive actions of OFQ/N are influenced by μ opioid receptors. Indeed, OFQ/N synthesis is increased in brain regions involved in morphine antinociception following prolonged morphine administration (Yuan et al., 1999), indicating that OFQ/N levels can be regulated by morphine. In addition, chronic morphine treatment increases ORL1 mRNA and binding sites in the rat and mouse spinal cord (Gouarderes et al., 1999; Ueda et al., 2000). If prolonged morphine treatment can heterologously regulate ORL1 function, it follows that heterologous regulation of ORL1 by μ agonists also occurs following acute exposure in those cell populations expressing both ORL1 and μ opioid receptors.
Acute OFQ/N, but not DAMGO, pretreatment decreased OFQ/N- and DAMGO-mediated stimulation of ERK1/2 activity in Chinese hamster ovary (CHO) cells expressing recombinant μ and ORL1 (Hawes et al., 1998). DAMGO pretreatment desensitized only μ-mediated stimulation of ERK1/2. However, in a human neuroblastoma cell line natively expressing μ and ORL1 subtypes (BE(2)-C), short-term pretreatment with OFQ/N or DAMGO desensitized μ and ORL1 opioid receptor-mediated inhibition of cAMP accumulation (Mandyam et al., 2002; 2000). These data indicate that the ability of μ opioid receptor agonists to heterologously regulate ORL1 function varies between cell types. This is not surprising considering that homologous μ opioid receptor regulation also varies between cell systems. Various kinases have been implicated in the homologous desensitization and/or downregulation of μ opioid receptors including protein kinase C (PKC; Kramer and Simon, 1999), G protein-coupled receptor kinase 2 (GRK2; Zhang et al., 1998; Li and Wang, 2001; Thakker and Standifer, 2002) and GRK3 (Kovoor et al., 1997; Celver et al., 2001), extracellular signal regulated kinase (ERK1/2; Polakiewicz et al., 1998) and tyrosine kinase (Pak et al., 1999). We recently reported that prolonged morphine or DAMGO treatment upregulates GRK2 levels in human neuroblastoma cells, and contributes to ORL1 desensitization (Thakker and Standifer, 2002). Acute heterologous desensitization of ORL1 has been demonstrated (Pu et al., 1999), but the mechanism for this acute desensitization was not determined.

To delineate the mechanism by which μ receptors regulate ORL1 responsiveness and further understand why μ/ORL1 cross talk may or may not occur in distinct neuronal populations within the CNS, two different human neuronal cell lines in which μ agonist pretreatment differentially affects ORL1 responsiveness were utilized. Here we report that acute (60 min) activation of μ opioid receptors desensitize μ and ORL1 responses in BE(2)-C cells via
activation of GRK. However, μ agonist-mediated activation of PKC-ε in SH-SY5Y cells is not sufficient to produce ORL1 desensitization.
Materials and Methods

Materials: The Research Technology Branch of NIDA provided DAMGO and OFQ/N. [³H]cAMP was obtained from Amersham Life Sciences (Arlington Heights, IL). SDS-PAGE reagents were obtained from Bio-Rad (Hercules, CA). All other chemicals/reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Cell culture: BE(2)-C and SH-SY5Y human neuroblastoma cells were generously provided by Dr. Robert A. Ross (Fordham University; Bronx, NY), and were cultured and maintained as described (Standifer et al., 1994). Studies were performed on cells at >60% confluence from passage 19-45, and were lifted from substrate with phosphate-buffered saline (PBS, pH 7.4) containing 1 mM EGTA.

Pretreatment conditions for agonists and inhibitors: BE(2)-C or SH-SY5Y cells were pretreated with or without 1 µM DAMGO in serum free media for 1 hr at 37°C. After treatment, cells were lifted and washed four times with ice cold phosphate-buffered saline (PBS, pH 7.4) to remove excess drug. Cells were pretreated with 1 µM chelerythrine chloride, an inhibitor of PKC, for 15 min (Kramer and Simon, 1999), or 10 µM PD98059, an inhibitor of MEK-1, for 60 min prior to addition of DAMGO and were subsequently subjected to measurement of OFQ/N and DAMGO-mediated inhibition of forskolin-stimulated cAMP accumulation.

Antisense oligodeoxynucleotide (ODN) treatment: Phosphodiester antisense or sense ODNs (> 99% purity) were dissolved in sterile water to a concentration of 3 mM. The ODN designated GRK2/3 antisense: 5’ ACCGCCTCCAGTGCGCCCAT 3’ or its corresponding sense strand were added to the BE(2)-C (10 µM) or SH-SY5Y (30 µM) cells and incubated for 60 hr in media deprived of serum (Mandyam et al., 2002). To selectively downregulate GRK2 or GRK3, cells received 1 µM GRK2 antisense ODN: 5’-CTC CAG GTC CGC CAT CTT-3’ (72 hr; Aiyar et
al., 2000), GRK3 antisense ODN: 5'-TCC AGT GTC TGC TT-3' (48 hr; Thakker and Standifer et al., 2002), or their corresponding sense ODNs. Cells were treated with several concentrations of each antisense for various lengths of time to determine which concentration and time produced the maximal downregulation of protein (determined by immunoblotting) without causing cell toxicity. The serum free condition itself did not produce any apparent alteration in cell growth or morphology compared to untreated cells, nor did they alter total protein content or levels of the proteins of interest. After pretreatment, cells were washed four times with ice cold PBS, lifted with PBS/EGTA and were subjected to measurement of cAMP accumulation or immunoblotting to confirm that loss of the targeted protein was selective (see below).

**Measurement of cAMP accumulation:** Intact BE(2)-C or SH-SY5Y cells (0.09-0.20 mg protein) were incubated in microfuge tubes in duplicate for 5 min at 37°C in 0.5 ml HBSS (137 mM NaCl, 5 mM KCl, 0.6 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 4 mM NaHCO₃, 6 mM D-glucose, 0.5 mM MgCl₂, 0.4 mM MgSO₄, 1 mM CaCl₂) containing 0.5 mM IBMX, 0.25 mg/ml bacitracin, and 0.1% protease-free bovine serum albumin (BSA). Agonists and/or forskolin (10 µM) were added to cells on ice before incubating for 10 min at 37°C. The reaction was terminated by 5 min incubation in a boiling water bath. After boiling, the reaction mixture was subjected to centrifugation for 5 min at 13000xg, and cAMP levels from the supernatant were determined in a [³H]cAMP binding assay.

**[³H]cAMP binding assay:** Supernatant fractions were added to duplicate tubes for a total volume of 0.2 ml containing 25 mM Tris-Cl pH 7.0, 10 mM theophylline, 0.1% BSA, 0.8 pM [³H]cAMP, and 0.3 mg/ml adrenal cortex extract for 1 hr at 4°C. The reaction was terminated by the addition of 75 µl of hydroxyapatite (50% w/v) for 6 min at 4°C, then filtered onto #34 glass-fiber filters and washed three times with 2 ml ice-cold 10 mM Tris-Cl, pH 7.0. Filters were
placed in vials with 5 ml Liquiscent (National Diagnostics, Atlanta, GA), and levels of radioactivity were determined by scintillation spectroscopy in a Beckman LS 6000 counter. The amount of cAMP in the supernatant was calculated from a standard curve determined with unlabeled cAMP. Data are plotted as the inhibition of forskolin (10 µM)-stimulated cAMP accumulation by each agonist (i.e. in the absence of agonist, there is zero inhibition of forskolin-stimulated cAMP accumulation).

Membrane preparation, immunoblotting and image analysis: BE(2)-C or SH-SY5Y cells plated in six-well plates were pretreated with or without DAMGO for 60 min as described above. Cells were then washed twice with buffer A (20 mM Tris-HCl, 0.15 M NaCl pH 7.5) and were incubated with 300 µl of buffer B (20 mM Tris-HCl, 0.15 M NaCl pH 7.5, 2 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM dithiothreitol and 2 µg/ml leupeptin) for 20 min on ice. To separate the membrane fraction from the cytosol, the preparation was centrifuged at 100,000xg for 20 min at 4°C (Kramer and Simon, 1999). The supernatant that contained the cytosolic fraction was removed; the pellet was incubated in lysis buffer for 1 hr at 4°C, resuspended in an equal volume of 2X Laemmli buffer, boiled for 5 min at 95°C and stored at -70°C.

Cell lysates, or cytosolic and membrane fractions from agonist or ODN treatments (20-30 µg protein) were resolved on a 10% SDS polyacrylamide gel and electrophoretically transferred onto polyvinylidene fluoride membrane (PVDF; Osmonics, Inc., Westborough, MA). PVDF membranes were blocked with Tris-buffered saline/Tween 20 (0.05%; TBS/T) containing 5% non-fat dried milk for 1 hr and incubated overnight at 4°C with PKC-α, PKC-ε, GRK2 (sc-562), or GRK3 (sc-563) antisera (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) diluted in TBS/T containing 2.5% non-fat dried milk. Membranes were then subjected to 4 washes of 10
min with TBS/T before incubating for 1 hr at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA). After washing, immunoreactive bands were visualized by chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA), and densitized with a Nucleovision Imaging Workstation (Nucleotech Corporation, San Carlos, CA). Membranes were stripped and re-probed with mouse anti-GAPDH as a loading control (1:5,000; Research and Diagnostics Antibodies, Berkeley, CA). PKC or GRK to GAPDH ratios were calculated for each treatment and normalized with respect to basal values.

Representative blots were scanned (Hewlett Packard Scanjet 6300C, with 1200 dpi optical resolution); the resulting images were cropped and sized for figures using Adobe Photoshop, version 6.0 for PC.

**Protein determination:** Protein concentrations were determined using BSA as a standard as described previously (Standifer et al., 1994).

**Data analysis:** LogEC$_{50}$ values were determined using non-linear regression analysis. Statistical comparisons of data were performed with Student’s $t$-test or one-way ANOVA using GraphPad Prism version 3.00 for Windows 95/98 (GraphPad Software, San Diego, CA). Data are expressed as mean ± S.E.M. unless otherwise indicated and were considered significant if $p<0.05$. 

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Results

Previously, Kramer and Simon (1999) reported that homologous $\mu$ opioid receptor downregulation in differentiated SH-SY5Y cells was mediated via activation of PKC, and that downregulation could be blocked by PKC inhibition. Pretreatment of SH-SY5Y cells with DAMGO (1 $\mu$M, 60 min) reduced the maximal response of DAMGO by almost 50% (Fig. 1; n=7, *p<0.05) and as expected, inclusion of the PKC inhibitor, chelerythrine (1 $\mu$M) blocked DAMGO-mediated $\mu$ receptor desensitization in SH-SY5Y cells. DAMGO pretreatment failed to desensitize the OFQ/N response in SH-SY5Y cells (Fig. 1). DAMGO pretreatment was previously shown to reduce OFQ/N efficacy in BE(2)-C cells (Mandyam et al., 2000). In the current study as well, pretreatment of intact BE(2)-C cells with DAMGO (1 $\mu$M, 60 min) reduced the maximal inhibition of forskolin-stimulated cAMP accumulation by OFQ/N and DAMGO by 50-60% (Fig. 2; n=5, *p<0.05). However, unlike in SH-SY5Y cells, chelerythrine failed to block the ability of DAMGO to desensitize either the ORL1 or the $\mu$ receptor responses in BE(2)-C cells. Mitogen activated protein kinase inhibitors have previously been demonstrated to block $\mu$ receptor desensitization (Polakiewicz et al., 1998), so the MEK-1 inhibitor (PD98059, 1 $\mu$M) was included in some assays. Instead of blocking DAMGO desensitization, PD98059 significantly increased the extent of DAMGO-mediated desensitization in BE(2)-C cells with no effect on that of ORL1 (Fig. 2).

Since PKC involvement in DAMGO-mediated desensitization was not apparent in BE(2)-C cells, the role of another family of kinases reported to be involved in $\mu$ opioid receptor desensitization in many other cell lines/model systems was investigated. Both GRK2 and GRK3 have been reported to phosphorylate and/or inactivate the $\mu$ receptor, producing desensitization (Zhang et al., 1998; Kovoor et al., 1997; Li and Wang, 2001; Celver et al., 2001; Mandyam et
GRK2 activity also is inhibited by mitogen activated protein kinase (ERK1/2) phosphorylation (Pitcher et al., 1999). The fact that increased µ opioid receptor desensitization was noted when the ERK1/2 pathway was blocked suggested that perhaps GRK2 was involved in µ receptor desensitization. To explore this possibility further, we examined the ability of DAMGO (1 µM, 60 min) to stimulate the translocation of GRK2 and GRK3 to the cell membrane fraction from the cytosol. DAMGO was previously reported to stimulate the translocation of PKC isoforms to the membrane fraction of SH-SY5Y cells (Kramer and Simon, 1999), and this was used as a control for comparison (Fig. 3).

Since PKC inhibition failed to block DAMGO-mediated µ or ORL1 desensitization in BE(2)-C cells, it was not surprising that membrane levels of neither PKC-α nor PKC-ε were elevated following DAMGO pretreatment (Fig. 4). Interestingly, while a 60 min treatment with DAMGO was not sufficient to mobilize GRK2 or GRK3 to the cell membrane fraction in SH-SY5Y cells (Fig. 3), levels of both isoforms were increased in the membrane fraction from BE(2)-C cells. This suggests a role for GRK2 and GRK3 in DAMGO-mediated ORL1 and µ opioid receptor desensitization.

To confirm the involvement of GRK2 or GRK3 in the desensitization of ORL1 and µ opioid receptors, BE(2)-C cells were pretreated with antisense or sense ODNs (10 µM, 60 hr) recognizing a sequence common to both GRK2 and GRK3. As reported previously, antisense, but not sense ODN reduced basal GRK3 levels by 61.5% and basal GRK2 levels by 45.9% in BE(2)-C cells (Mandyam et al., 2002). More importantly, DAMGO-mediated desensitization of ORL1 and µ opioid receptors in BE(2)-C cells was blocked upon exposure to the antisense (Fig. 5A), but not sense, ODN treatment. This suggests that activation of GRKs by DAMGO contributes to DAMGO-mediated heterologous desensitization of ORL1 as well as homologous...
µ receptor desensitization in BE(2)-C cells. The inability of antisense treatment to alter homologous µ receptor desensitization in SH-SY5Y cells (Fig 5B), despite the significant reduction in GRK levels (Fig. 5C), is consistent with the important role of PKC-ε in µ opioid receptor regulation in this cell line (Kramer and Simon, 1999).

DAMGO recruited both GRK2 and GRK3 to the plasma membrane of BE(2)-C cells (Fig. 4), and reducing levels of both GRK isoforms blocked µ-mediated ORL1 and µ receptor desensitization in that cell line (Fig. 5A). However, it remains unclear which GRK isoform is responsible for the desensitization. The fact that inclusion of PD98059 increases µ receptor homologous desensitization without altering ORL1 desensitization (Fig. 2) suggests that µ receptor desensitization is mediated via GRK2, while ORL1 desensitization occurs through GRK3. To test this hypothesis, BE(2)-C cells were treated with antisense DNA selectively targeting either GRK2 (Fig. 6) or GRK3 (Fig. 7) prior to 60 min DAMGO pretreatment.

GRK2 antisense DNA selectively reduced GRK2 levels by 53 ± 8.8%, with no effect on GRK3 levels (6.2 ± 5.9% reduction; Fig. 6C). DAMGO pretreatment desensitized both µ and ORL1 responses, but GRK2 antisense treatment reversed only µ receptor desensitization (Fig. 6A). ORL1 desensitization was unchanged (Fig. 6B). We recently reported that homologous ORL1 desensitization in BE(2)-C cells was mediated through GRK3 (Thakker and Standifer, 2002). Since DAMGO also recruits GRK3 to the cell membrane, it was possible that DAMGO-mediated ORL1 desensitization was mediated by GRK3. When GRK3 levels were reduced by antisense DNA treatment, DAMGO-mediated ORL1, but not µ receptor desensitization, was prevented (Fig. 7).
Discussion

The ability of µ agonist pretreatment to alter ORL1 responsiveness varies between cell lines (Hawes et al., 1998; Mandyam et al., 2000) and brain regions (Connor et al., 1996; Hao et al., 1997; Jhamandas et al., 1998). Similarly, µ receptor regulation differs between cell populations (Zhang et al., 1996; Kramer and Simon, 1999; Li and Wang, 2001) and supraspinal and spinal sites (Bohn et al., 2002). Therefore, the mechanism(s) contributing to DAMGO-mediated ORL1 desensitization was investigated in two cell lines with different mechanisms of µ receptor regulation to determine the signaling pathways that were required to produce heterologous ORL1 desensitization.

In undifferentiated SH-SY5Y cells, acute µ receptor activation recruits PKC-ε to the cell membrane and produces homologous desensitization and downregulation that are blocked by chelerythrine (Kramer and Simon, 1999). It is not clear whether PKC directly phosphorylates the µ receptor (Law et al., 2000) or G_1α_1 or G_1α_2 (Murthy et al., 2000) to produce the desensitization.

In contrast, PKC appears to play no role in acute µ receptor regulation in BE(2)-C cells. Pretreatment with DAMGO plus the MEK1/2 inhibitor, PD98059, increased the extent of homologous µ receptor desensitization in BE(2)-C cells compared to pretreatment with DAMGO alone. This effect likely results from the inhibitory actions of ERK1/2 on GRK2 (Pitcher et al., 1999), since inhibition of ERK potentiates GRK2 activity. DAMGO pretreatment increases membrane GRK2 and GRK3 levels, making them more readily available to phosphorylate the receptor upon addition of agonist. Indeed, selectively reducing GRK2 levels with isoform-specific antisense DNA blocked DAMGO-induced desensitization, indicating that GRK2 is responsible for µ receptor desensitization. In fact, GRK3 did not substitute for GRK2 when
GRK2 levels were reduced more than 50% as evidenced by the complete reversal of μ receptor desensitization with GRK2 antisense treatment, indicating that GRK2 is the “preferred” kinase.

Heterologous regulation of ORL1 also differs in the two cell lines. DAMGO desensitizes the OFQ/N response in BE(2)-C cells, but fails to do so in SH-SY5Y cells. GRK3 appears to be the “preferred” kinase for ORL1, as GRK2 antisense treatment did not prevent ORL1 desensitization by DAMGO. These results suggest that ORL1 must be in close proximity to the μ opioid receptors in BE(2)-C cells such that when DAMGO mobilizes GRK3 to the plasma membrane (a “primed” state), ORL1 becomes more sensitive to desensitization upon subsequent challenge with its own agonist, OFQ/N. This also was found to be the case for OFQ/N-mediated μ receptor desensitization in BE(2)-C cells (Mandyam et al., 2002), as well as for other receptor systems (Chuang et al., 1996). Homologous desensitization of ORL1 also involves GRK3 (Mandyam et al., 2002; Thakker and Standifer, 2002). Though ORL1 phosphorylation has yet to be demonstrated, ORL1 contains a putative GRK3 phosphorylation site in its second intracellular loop (Mollereau et al., 1994) that corresponds to the GRK3 phosphorylation site on the μ receptor (T180; Celver et al., 2001).

ORL1 desensitization following acute agonist treatment is mediated via conventional PKC isoforms (α, δ, γ; Lou et al., 1997; Pei et al., 1997; Mandyam et al., 2002; Narita et al., 2002) and GRK (Mandyam et al., 2002). GRK is activated only by conventional PKC isoforms (Krasel et al., 2001), therefore the atypical isoform, PKC-ε, cannot activate GRK. These results suggest that DAMGO fails to desensitize ORL1 in SH-SY5Y cells because the signaling pathway is unable to mobilize GRK during the 1 hr time period tested.

Previous reports of apparent failures of μ agonist pretreatment to alter OFQ/N activity were based upon the unchanging activity of a single, maximal, concentration of OFQ/N (e.g.,
Connor et al., 1996; Hao et al., 1997; Hawes et al., 1998). It is quite possible in those studies that pretreatment with the \(\mu\) agonist reduced the potency of OFQ/N without altering its efficacy, as we recently noted (Thakker and Standifer, 2002). The loss of potency of an analgesic agent in vivo could have dramatic effects, as serious side effects appear as doses are increased to compensate for loss of potency.

There are several possible explanations for activation of differential signaling pathways in the two cell lines upon addition of the \(\mu\) agonist: the \(\mu\) receptors differ, G protein-\(\mu\) receptor coupling differs, or the signaling components and/or recycling processes differ between the cell lines. First, despite the fact that there are no obvious differences in \(\mu\) agonist binding affinity or potency in the two cell lines (Standifer et al., 1994; Cheng et al., 1995), the presence of human \(\mu\) receptor splice variants in BE(2)-C cells was recently reported (Pan et al., 2003). When these splice variants were expressed in CHO cells, they did not exhibit differential affinity for DAMGO. However, the ability of each splice variant to activate downstream effectors and its sensitivity to various kinases are not yet known. Thus, this possibility cannot be ruled out.

A second explanation for the differential results in the two cell lines could be based upon the \(\beta\gamma\) complexes released upon receptor activation. Both PKC and GRK isoforms can be differentially activated by \(\beta\gamma\) subunits (Pitcher et al., 1998). While \(\beta_1\) and \(\beta_2\) bind equally well to both GRK2 and GRK3, \(\beta_3\) prefers GRK3 and has no affinity for GRK2 (Pitcher et al., 1998). Therefore, perhaps \(\beta_3\) is released from an \(\alpha\) subunit activated by DAMGO in BE(2)-C cells, but not in SH-SY5Y cells, thereby not activating GRK3 and not facilitating the desensitization of ORL1 by OFQ/N.

Though we have not determined whether G\(\beta_3\) is present in SH-SY5Y cells (it is expressed in BE(2)-C cells; unpublished observations), there seems to be little difference in
levels of expression of PKC-α, -ε, GRK2 or GRK3 between the two cell lines. However, those are only a few of the proteins that could be involved in the process of receptor signaling or recycling, and does not take into account differences in other families of proteins such as β-arrestin, RGS and rab. Mu receptor recycling and resensitization limits the rate and extent of DAMGO-induced μ receptor desensitization in SH-SY5Y cells; μ receptor activity depends on the presence of functional cell surface receptors and disruption of the recycling process promotes μ receptor desensitization (Law et al., 2000). Like μ receptors, ORL1 appears to internalize through a clathrin-coated pit pathway; recycling and resensitization of the receptor requires phosphatase activity (Spampinato et al., 2001). Further, ORL1 internalization is increased in the presence of elevated levels of β-arrestin 2 (Spampinato et al., 2001). Desensitization and internalization of μ opioid receptors is differentially affected by the level and combination of GRK and β-arrestin present in the cell (Kovoor et al., 1997; Celver et al., 2001). Both cell lines express equivalent levels of β-arrestin 1 (Thakker and Standifer, 2002), but levels of β-arrestin 2 are unknown.

The idea that opioid receptors activate different signaling pathways in different cell populations is not new (Shapira et al., 2001), but the role of differential signaling in regulation of other receptors in different cell populations has not been fully examined. ORL1 and μ opioid receptors are co-expressed on cells in many different locations within the descending analgesic pathway (e.g., Connor et al., 1996; Heinricher et al., 1997; Pan et al., 2000). Biochemical evidence for direct μ/ORL1 interactions includes β-FNA-induced blockade of the ability of OFQ/N to inhibit cAMP accumulation (Mandyam et al., 2000) and μ and ORL1 heterodimer formation (Pan et al., 2002). The β-FNA effect is significant because it persisted after washout while a competitive μ opioid antagonist had no effect. The cell specific differences noted in
BE(2)-C and SH-SY5Y cells may be representative of some of these different cell populations and/or receptor confirmations within the CNS through which ORL1 and µ receptors are differentially modulated by µ and ORL1 agonists. By understanding the mechanisms required for receptor cross-talk, it may be possible to predict where in the brain, and under what conditions, receptor cross-talk may occur, as well as its effect on the descending analgesic pathway.
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References


Footnotes

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Figure Legends

Figure 1. DAMGO pretreatment desensitizes µ, but not ORL1, receptors in SH-SY5Y cells: Role of PKC. In order to confirm the role of PKC in DAMGO-mediated desensitization of µ receptors in SH-SY5Y cells, DAMGO pretreatment (1 µM, 60 min) was performed in the presence and absence of chelerythrine (Che, 1 µM) as described in methods. Desensitization was determined by measuring the loss of DAMGO or OFQ/N inhibition of forskolin (1 µM)-stimulated cAMP accumulation in intact cells. Che blocked DAMGO mediated-desensitization of µ opioid receptors in SH-SY5Y cells (*p<0.05 compared to control, # p<0.05 compared to DAMGO pretreatment by ANOVA and Tukey’s post-hoc test). The data are representative of 3-8 experiments; each performed in duplicate and expressed as mean ± S.E.M. DAMGO pretreatment did not alter basal (57.8 ± 16.8 pmol/mg) or forskolin-stimulated (135.1 ± 33 pmol/mg) levels of cAMP compared to vehicle-treated controls (basal: 64.7 ± 16 and forskolin: 135.6 ± 29 pmol/mg) in SH-SY5Y or BE(2)-C cells.

Figure 2. Effect of PKC and MEK1/2 inhibition on 1 µM DAMGO-mediated desensitization of ORL1 and µ-receptors in BE(2)-C cells. In order to elucidate the possible protein kinases involved in DAMGO-mediated desensitization of ORL1 and µ receptors, BE(2)-C cells were treated with DAMGO in the presence or absence of the protein kinase C inhibitor, Che (1 µM) or the MEK1/2 inhibitor, PD98059 (10 µM) as described in methods. Neither Che nor PD98059 blocked DAMGO mediated-desensitization of ORL1 and µ opioid receptors. However, inclusion of PD98059 increased the µ receptor desensitization produced by DAMGO (*p<0.05 when compared
to control, \(^{#}p<0.05\) compared to DAMGO alone, by ANOVA with Tukey’s post-hoc test). The above data are representative of 5-8 experiments; each performed in duplicate, and expressed as mean ± S.E.M.

**Figure 3. DAMGO pretreatment increased membrane PKC-ε levels in SH-SY5Y cells.**

Intact SH-SY5Y cells were treated with DAMGO (1 µM) for 1 hr, after which cells were washed, membrane and cytosolic fractions were separated as described in methods, and membrane fractions were subjected to SDS-PAGE and immunoblotting. PKC/GAPDH and GRK/GAPDH ratios were calculated for each treatment and normalized with respect to basal values. Data from immunoblots were quantified by densitometric analysis and presented as mean ± S.E.M. of 6-8 independent experiments (A). Only PKC-ε exhibited translocation following DAMGO treatment (*p<0.05 compared to control when analyzed by unpaired \(t\)-test). (B) Representative immunoblots.

**Figure 4. DAMGO pretreatment increased membrane GRK2 and GRK3 levels in BE(2)-C cells.** Intact BE(2)-C cells were treated with DAMGO (1 µM) for 1 hr, after which cells were washed, membrane and cytosolic fractions were separated as described in methods. Membrane fractions were subjected to SDS-PAGE and immunoblotting. PKC/GAPDH and GRK/GAPDH ratios of densitometric values of immunoblots were calculated for each treatment, normalized with respect to basal values, and represent the mean ± S.E.M. of 4-6 independent experiments (A). Representative immunoblots of GRK2 and GRK3 with GAPDH as the loading control are shown in (B). DAMGO significantly increased the translocation of GRK2 and GRK3 to the
membrane (*p<0.05 compared to controls when analyzed by unpaired t-test), but failed to stimulate the translocation of PKC-α or -ε.

Figure 5. GRK2/3 common antisense DNA blocked DAMGO-mediated ORL1 and µ receptor desensitization in BE(2)-C, but not SH-SY5Y, cells. Intact BE(2)-C cells (A) were treated with or without GRK2/3 antisense or sense (10 µM) for 60 hr in serum free media after which DAMGO (1 µM, 1 hr) was added and cells were assayed for inhibition of 10 µM forskolin-stimulated cAMP accumulation. Data are expressed as mean ± S.E.M. of 3-4 independent experiments, each performed in duplicate. DAMGO pretreatment desensitized both OFQ/N and DAMGO responses (*p<0.05 compared to vehicle controls), which were blocked with prior exposure to the antisense (#p<0.05 compared to DAMGO pretreatment by ANOVA and Tukey’s post-hoc test). Sense treatment did not alter DAMGO-induced desensitization of µ or ORL1. SH-SY5Y cells (B, C) were treated with or without GRK2/3 antisense or sense (30 µM) for 60 hr in serum free media prior to DAMGO pretreatment (1 µM, 1 hr). Data are expressed as mean ± S.E.M. of 3-4 independent experiments, each performed in duplicate. DAMGO pretreatment desensitized the DAMGO response (*p<0.05 compared to vehicle controls). Antisense or sense treatments were without effect, despite the fact that the antisense treatment reduced GRK2 and GRK3 levels over 50% (C).

Figure 6. GRK2 mediates homologous µ receptor desensitization in BE(2)-C cells. Intact BE(2)-C cells were treated with or without antisense or sense (1 µM) selective for GRK2 for 72 hr in serum free media after which DAMGO (1 µM, 1 hr) was added and cells were assayed for DAMGO (A) and OFQ/N (B) inhibition of 10 µM forskolin-
stimulated cAMP accumulation. DAMGO pretreatment desensitized both OFQ/N and DAMGO responses. μ opioid receptor desensitization was completely prevented in those cells exposed to GRK2 antisense. However, GRK2 antisense treatment did not prevent ORL1 desensitization. Sense treatment did not alter DAMGO-induced desensitization of μ or ORL1. Data are expressed as mean ± S.E.M. of 3 independent experiments, each performed in duplicate. GRK antisense treatment selectively reduced GRK2 levels 53 ± 8.8%, without altering levels of GRK3 (6.2 ± 5.9% inhibition), as indicated by the representative immunoblot (C).

**Figure 7. GRK3 mediates μ receptor mediated heterologous regulation of homologous ORL1 desensitization.** Intact BE(2)-C cells were treated with or without antisense or sense (1 μM) selective for GRK3 for 48 hr in serum free media after which DAMGO (1 μM, 1 hr) was added and cells were assayed for DAMGO (A) and OFQ/N (B) inhibition of 10 μM forskolin-stimulated cAMP accumulation. DAMGO pretreatment desensitized both OFQ/N and DAMGO responses. μ opioid receptor desensitization was unaffected by GRK3 antisense treatment. In contrast, GRK3 antisense treatment fully prevented ORL1 desensitization. Sense treatment did not alter DAMGO-induced desensitization of μ or ORL1. Data are expressed as mean ± S.E.M. of 3 independent experiments, each performed in duplicate.
Figure 1
Figure 2

Forskolin-stimulated cAMP Accumulation (% Inhibition)

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Control</th>
<th>DAMGO</th>
<th>+ Che</th>
<th>+ PD98059</th>
</tr>
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<tr>
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<td><img src="image" alt="Graph of OFQ/N" /></td>
</tr>
</tbody>
</table>

* indicates statistical significance.
Figure 3

A

Membrane PKC GRK/GAPDH Ratio (% Control)

PKCα  PKCε  GRK2  GRK3

B

Vehicle  DAMGO

PKCα  PKCε  GRK2  GRK3  GAPDH
Figure 4
Figure 5

A) Pretreatment:
- None
- DAMGO

B) OFQ/N, DAMGO

C) DAMGO, Log M

DAMGO, Log M
Figure 6

A

Pretreatment:

- Vehicle
- DAMGO
- + GRK2 A.S.
- + GRK2 Sense

DAMGO, Log M

cAMP accumulation (% inhibition)

B

OFQ/N, Log M

cAMP accumulation (% inhibition)

C

Vehicle
GRK2 Sense
GRK2 A.S.
GRK2
GRK3
GAPDH
Figure 7

Pretreatment:

A. 
- Vehicle
- DAMGO
- + GRK3 A.S.
- + GRK3 Sense

B. 
- DAMGO, Log M
- OFQ/N, Log M

Vehicle DAMGO + GRK3 A.S. + GRK3 Sense

Pretreatment:

A. 
- Vehicle
- DAMGO
- + GRK3 A.S.
- + GRK3 Sense

B. 
- DAMGO, Log M
- OFQ/N, Log M

Vehicle DAMGO + GRK3 A.S. + GRK3 Sense