Orphanin FQ/Nociceptin-Mediated Desensitization of Opioid Receptor-Like 1 Receptor and μ Opioid Receptors Involves Protein Kinase C: A Molecular Mechanism for Heterologous Cross-Talk

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ABSTRACT

Morphine tolerance in vivo is reduced following blockade of the orphanin FQ/nociceptin (OFQ/N) opioid receptor-like 1 (ORL1) receptor system, suggesting that OFQ/N contributes to the development of morphine tolerance. We previously reported that a 60-min activation of ORL1 receptors natively expressed in BE(2)-C cells desensitized both μ and ORL1 receptor-mediated inhibition of cAMP. Investigating the mechanism(s) of OFQ/N-mediated μ and ORL1 receptor cross-talk, we found that pretreatment with the protein kinase C inhibitor, chelerythrine chloride (1 μM), blocked OFQ/N-mediated homologous desensitization of ORL1 and heterologous desensitization of μ opioid receptors. Furthermore, depletion of PKC by 12-O-tetradecanoylphorbol-13-acetate exposure (48 h, 1 μM) also prevented OFQ/N-mediated μ and ORL1 desensitization. OFQ/N pretreatment resulted in translocation of PKC-α, G protein-coupled receptor kinase 2 (GRK2) and GRK3 from the cytosol to the membrane, and this translocation was also blocked by chelerythrine. Reduction of GRK2 and GRK3 levels by antisense, but not sense DNA treatment blocks ORL1 and μ receptor desensitization. This suggests that PKC-α is required for GRK2 and GRK3 translocation to the membrane, where GRK can inactivate ORL1 and μ opioid receptors upon rechallenge with the appropriate agonist. Our results demonstrate for the first time the involvement of conventional PKC isoforms in OFQ/N-induced μ-ORL1 cross-talk, and represent a possible mechanism for OFQ/N-induced anti-opioid actions.

Orphanin FQ/nociceptin (OFQ/N), an exquisitely selective agonist at the opioid receptor-like 1 (ORL1) receptor, modulates both behavioral (nociception, anxiety, learning, reward) and immune (cell proliferation) responses (Harrison and Grandy, 2000). Although ORL1 and OFQ/N share greater than 40% homology with other opioid receptors and endogenous opioid peptides, many of their actions are anti-opioid in nature (Harrison and Grandy, 2000). One cellular mechanism for their anti-opioid effect is attributed, at least partially, to actions of OFQ/N on different populations of neurons in the brainstem (Pan et al., 2000). The molecular basis of their anti-opioid effects still remains to be determined, but heterologous cross-talk is a possibility since μ and ORL1 receptors are colocalized on several cell populations within the descending analgesic pathway (Connor et al., 1996; Connor and Christie, 1998; Heinricher et al., 1997; Pan et al., 2000).

Several lines of evidence support the functional interaction of ORL1/OFQ/N with μ opioid receptor responses in vivo. The ORL1 antagonist [Nphe1]NC(1–13)NH2 (i.c.v.) potentiates morphine analgesia (Rizzi et al., 2000), suggesting that supraspinal release of OFQ/N following morphine administration has a nociceptive effect. Indeed, OFQ/N synthesis is increased in brain regions involved in morphine antinociception following morphine administration (Yuan et al., 1999), indicating that OFQ/N levels can be regulated by morphine.

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ABBREVIATIONS: OFQ/N, orphanin FQ/nociceptin; ORL1, opioid receptor-like 1; DAMGO, [d-Ala2,N-Me-Phe4,Gly5-ol]-enkephalin; ERK1/2, extracellular-regulated kinases 1 and 2; PKC, protein kinase C; MEK-1, mitogen-activated protein kinase kinase; GRK, G protein-coupled receptor kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; 4αPDD, 4α-phorbol-12,13-didecanoate; HBSS, Hanks’ balanced salt solution; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; ODN, oligodeoxynucleotide; TBS/T, Tris-buffered saline/Tween 20; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ANOVA, analysis of variance; DAG, diacylglycerol; PKA, protein kinase A; PD98059, 4-H-1-benzopyran-4-one 2-[2-(amino-3-methoxyphenyl)]-H-89, N-[2-((p-bromocinnamyl)amino)ethyl]-5-isouquinoline sulfonamide.

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Activation of μ opioid receptors can also regulate ORL1 levels, as chronic morphine treatment increases ORL1 mRNA and binding sites in the rat and mouse spinal cord (Gouarderes et al., 1999; Ueda et al., 2000). ORL1 knockout mice develop significantly less tolerance to spinal morphine, further supporting the involvement of the OFQ/N system in morphine tolerance (Ueda et al., 2000).

A 10-min pretreatment with OFQ/N, but not DAMGO, decreased both OFQ/N- and DAMGO-mediated stimulation of extracellular signal-regulated kinase (ERK) activity in Chinese hamster ovary cells expressing recombinant μ and ORL1 receptors (Hawes et al., 1998). We recently reported that pretreatment with OFQ/N or DAMGO for 1 h desensitized the inhibited cAMP response of natively expressed ORL1 and μ opioid receptors in BE(2)-C human neuroblastoma cells (Mandyam et al., 2000). Although evidence suggests that the ORL1/OFQ/N system modulates morphine analgesia and tolerance, the underlying molecular mechanism for such an interesting interaction is poorly understood.

μ opioid receptors undergo homologous desensitization and down-regulation upon treatment with DAMGO or morphine, and this cellular mechanism is thought to be responsible for μ receptor tolerance in vivo (Nestler and Aghajanian, 1997). Homologous desensitization of μ opioid receptors in vitro has been blocked by inhibition of protein kinase C (PKC) (Chen and Yu, 1994), mitogen-activated protein kinase kinase (MEK-1) (Polakiewicz et al., 1998), G protein-coupled receptor kinase 2 (GRK2) (Zhang et al., 1998; Li and Wang, 2001), and GRK3 (Kovoor et al., 1997; Celver et al., 2001). μ opioid receptor tolerance in vivo also can be blocked by inhibition or reduction of PKC (Inoue and Ueda, 2000; Narita et al., 2001), MEK-1 (Pearson et al., 2000), and GRK3 levels (Terman et al., 2000). Heterologously produced phosphorylation and desensitization of μ receptors is also blocked by PKC inhibition (Zhang et al., 1996).

OFQ/N activates PKC through ORL1 receptors (Lou et al., 1997), and homologous desensitization of ORL1 is mediated by PKC (Pei et al., 1997; Pu et al., 1999). Since PKC is involved in the regulation of both μ and ORL1 receptors, its role in OFQ/N-induced μ and ORL1 desensitization was investigated. In the present study we demonstrate that OFQ/N-induced μ and ORL1 receptor desensitization is both PKC- and GRK-dependent.

**Experimental Procedures**

**Materials.** DAMGO, OFQ/N, and [3H]OFQ/N were provided by the Research Technology Branch of the National Institute on Drug Abuse. [3H]DAMGO and [3H]cAMP were obtained from Amersham Biosciences (Arlington Heights, IL). 12-O-Tetradecanoylphorbol-13-acetate (TPA) and 4α-phorbol-12,13-didecanoate (4αPDD) were purchased from Calbiochem (San Diego, CA). PD98059 and ERK anti sera were purchased from Cell Signaling Technology Inc. (Beverly, MA). All SDS-PAGE reagents were obtained from Bio-Rad (Hercules, CA). All other chemicals/reagents were purchased from Sigma-Al drich (St. Louis, MO).

**Cell Culture.** BE(2)-C human neuroblastoma cells were generously provided by Dr. Robert A. Ross (Fordham University; Bronx, NY), and were cultured and maintained as described (Mandyam et al., 2000). Studies were performed on cells at >60% confluence from passage 19 to 45, and were lifted from substrate with phosphate-buffered saline (PBS, pH 7.4) containing 1 mM EGTA.

**Measurement of cAMP Accumulation.** Intact BE(2)-C cells (0.09–0.20 mg protein) were incubated in microcentrifuge tubes in duplicate for 5 min at 37°C in 0.5 ml of 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, and 1 mM CaCl2) containing 0.5 mM 3-isobutyl-1-methylxanthine, 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 a 5-min incubation in a boiling water bath. After boiling, the reaction mixture was subjected to centrifugation for 5 min at 13,000g, and cAMP levels from the supernatant were determined in a [3H]cAMP binding assay described below.

**[3H]cAMP Binding Assay.** Supernatant fractions were added to duplicate tubes for a total volume of 0.2 ml containing 25 mM Tris-HCl, pH 7.0, 10 mM theophylline, 0.1% BSA, 0.8 μM [3H]cAMP, and 40 to 60 μg of adrenal cortex extract (Norstedt and Fredholm, 1990) for 1 h 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 no. 34 glass-fiber filters and washed three times with 2 ml of ice-cold 10 mM Tris-HCl, pH 7.0. Filters were placed in vials with 5 ml of Liquisint (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.

**Receptor Binding.** Cell membranes were prepared by homogenizing cells in 10 volumes of 50 mM Tris-HCl, pH 7.4 (containing 100 mM NaCl, 1 mM Na2EDTA, and 0.1 mM PMSF) for 3 s at setting 5 with a Polytron homogenizer. Homogenates were incubated for 15 min at 25°C, and crude membranes were sedimented by centrifugation at 50,000g for 30 min at 4°C. Pellets were resuspended in 0.32 M sucrose and stored at −80°C. [3H]DAMGO and [3H]OFQ/N binding (1–2 nM) were performed with 0.4 to 1.0 mg/ml membrane protein as described (Mandyam et al., 2000).

**Pretreatment Conditions for Agonists and Inhibitors.** BE(2)-C cells were pretreated with or without 0.1 nM OFQ/N in serum-free media (containing BSA and bacitracin) for 1 h at 37°C. After treatment, cells were washed and four times with ice-cold PBS (pH 7.4) to remove excess drug. Cells were pretreated with 1 μM chelerythrine chloride (Kramer and Simon, 1999), an inhibitor of PKC, for 15 min prior to addition of OFQ/N. In other experiments, cells were exposed to 1 μM TPA (or its inactive isofom 4αPDD) in dimethyl sulfoxide for 48 h to deplete total PKC (Kramer and Simon, 1999) prior to the 60-min treatment with OFQ/N.

**Antisense Oligodeoxynucleotide (ODN) Treatment.** Phosphodiester antisense or sense ODNs (>98% purity) were dissolved in sterile water to a concentration of 3 mM. The ODN designated GRK2/3 antisense: 5′-ACCGCTCTGGTCCGCGCAT-3′ or its corresponding sense strand (10 μM; Shih and Malbon, 1994) were added to the cells (80–90% confluent) and incubated for 60 h in media deprived of serum (Dautzenberg et al., 2001). This treatment did not produce any visible alteration in cell growth compared with untreated cells. After pretreatment, cells were washed four times with ice-cold PBS, lifted with PBS/EGTA, and subjected to measurement of cAMP accumulation and immunoblotting (see below).

**ERK Activation and Immunoblotting.** Cells were serum-deprived for 24 h, washed free of media with PBS, and stimulated with or without OFQ/N for 5 min at 37°C in HBSS in a total volume of 0.5 ml. At the end of that time, cells were rapidly washed to remove drug and rechallenged with agonist for an additional 5 min. The reactions were stopped with the addition of 75 μl of ice-cold cell-lysis buffer (pH 7.5, containing 50 mM Tris, 500 mM NaCl, 50 mM NaF, 2 mM Na2VO4, 10 μM Na3P2O7, 10 mM EDTA, 0.25 mM PMSF, 2 mM EGTA, 1% Triton X-100, 0.02% NaN3, and 1 μg/ml each of leupeptin, aprotinin, and pepstatin). Cell lysates were solubilized in an equal volume of 2× Laemmli buffer and heated for 5 min at 85°C. Lysates (5–10 μg protein) were resolved on a 10% SDS-polyacrylamide gel,
transferred, and blocked as described below. Membranes were probed with phospho-specific ERK1 and ERK2 antisera (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) and were later stripped, blocked, and reprobed with ERK1/ERK2 antisera (1:1000; total ERK). Phospho/total ERK ratios were calculated for each treatment.

Membrane Preparation, Immunoblotting, and Image Analysis. BE(2)-C cells plated in six-well plates were pretreated with or without 0.1 nM OFQ/N for 60 min in the presence or absence of 1 μM chelerythrine 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 100,000g 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 h at 4°C, resuspended in an equal volume of 2× Laemml 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 a polyvinylidene fluoride membrane (Osmonics, Inc., Westborough, MA). Polyvinylidene fluoride membranes were blocked with Tris-buffered saline/Tween-20 (0.05%; TBS/T) containing 5% nonfat dried milk for 1 h and incubated overnight at 4°C with PKC-α, PKC-ε, GRK2 (sc-562), or GRK3 (sc-563) antisera (1:1000; Santa Cruz Biotechnology) diluted in TBS/T containing 2.5% nonfat dried milk. Membranes were then subjected to four washes of 10 min with TBS/T before incubating for 1 h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (1:2000; Santa Cruz Biotechnology). After washing, immunoreactive bands were densitized with a Nucleovision Imaging Workstation (Nucleotech Products, Inc., San Carlos, CA). Membranes were stripped and reprobed with mouse anti-GAPDH as a loading control (1:5000; Research and Diagnostics Antibodies, Berkeley, CA). PKC/GAPDH or GRK/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 (Lowry et al., 1951).

Data Analysis. log EC50 values were determined using nonlinear 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 are considered significant if p ≤ 0.05.

Results

The PKC Inhibitor, Chelerythrine, Blocks OFQ/N-Mediated μ and ORL1 Opioid Receptor Desensitization. As reported previously, OFQ/N pretreatment (0.1 nM, 60 min) desensitized both OFQ/N- and DAMGO-mediated inhibition of cAMP accumulation (Fig. 1), reducing both agonist potency (log EC50 OFQ/N, >−10; DAMGO, −7.8 ± 0.43) and efficacy (OFQ/N, 9.5 ± 4.3%; DAMGO: 26.5 ± 8.2%) compared with controls (Fig. 1, p < 0.05; n = 3–8; OFQ/N, −12.44 ± 0.45 and 49.1 ± 5.6%; DAMGO, −8.44 ± 0.26 and 72 ± 7.8%). OFQ/N pretreatment did not alter basal (57.8 ± 10.6 pmol/mg) or forskolin-stimulated (98.5 ± 16.2 pmol/mg) levels of cAMP compared with vehicle-treated controls (basal, 64.7 ± 16; forskolin, 135.6 ± 29 pmol/mg). Since previous reports linked ORL1 activation and desensitization with PKC (Lou et al., 1997; Pei et al., 1997; Pu et al., 1999), we explored the possibility that OFQ/N-mediated heterologous desensitization of μ opioid receptors also involved PKC. Cells were pretreated with the PKC inhibitor, chelerythrine (1 μM), 15 min before addition of OFQ/N. Chelerythrine pretreatment completely blocked the ability of OFQ/N to desensitize ORL1 and μ receptor responses, and returned the potency (OFQ/N, −11.87 ± 0.73; DAMGO, −9.18 ± 0.34) and efficacy of OFQ/N and DAMGO to their control values (Fig. 1, p < 0.05 when compared with OFQ/N pretreatment alone). Chelerythrine pretreatment alone did not alter OFQ/N (−12.42 ± 0.42 and 50.6 ± 7.1%) or DAMGO (−8.14 ± 0.27 and 64.2 ± 2.6%) responses.

In addition to the cross-talk involving cAMP accumulation, OFQ/N also produces a similar heterologous desensitization of μ opioid receptor-mediated ERK stimulation (Hawes et al., 1998). OFQ/N and DAMGO stimulate phospho ERK 2- to 3-fold over basal levels in BE(2)-C cells (Thakker et al., 2000; Fig. 2). OFQ/N pretreatment (0.1 nM, 5 min) desensitized ORL1 and μ opioid receptor-mediated phospho ERK stimulation (∗, p < 0.05; n = 3–4; Fig. 2). Chelerythrine (1 μM, 10 min) also blocks this desensitization by OFQ/N (p < 0.05), further supporting a role for PKC in this process. Chelerythrine alone did not alter basal responses (Fig. 2).

Chronic Phorbol Ester Treatment Prevents OFQ/N-Mediated ORL1 and μ Opioid Receptor Desensitization. Depletion of PKC by chronic phorbol ester treatment is another method to examine the role of PKC in agonist-mediated receptor desensitization. TPA treatment (1 μM, 48 h) effectively down-regulated membrane and cytosolic PKC content in SH-SY5Y human neuroblastoma cells (Kramer and Simon, 1999), and these conditions were employed to deplete PKC in BE(2)-C cells as well. OFQ/N pretreatment (0.1 nM, 60 min) desensitized ORL1 and μ opioid receptor responses, reducing OFQ/N- and DAMGO-mediated inhibition of cAMP accumulation by 90% and 30%, respectively (∗, p < 0.05 compared with controls, Fig. 3A). Exposure to TPA prior to the addition of OFQ/N blocked the ability of OFQ/N to desensitize ORL1 and μ opioid receptors (#, p < 0.05 compared with OFQ/N treatment, Fig. 3A), whereas pretreatment with
Chelerythrine Blocks OFQ/N-Mediated Loss of \( \mu \) Opioid Receptor Agonist Binding. To determine whether a loss of agonist binding contributed to reduced \( \mu \) and ORL1 responses following OFQ/N pretreatment, single-point binding assays of crude cell membrane homogenates were employed to measure levels of \( \mu \) and ORL1 receptors (Mandyam et al., 2000). Similar to previously reported results, levels of ORL1 within the cell were not reduced by OFQ/N pretreatment (Fig. 4; Spampinato et al., 2001). However, \[^3H\]DAMGO binding to the \( \mu \) opioid receptor was reduced 25% by OFQ/N pretreatment (*, \( p < 0.05 \), Fig. 4). Inclusion of chelerythrine (1 \( \mu \)M, 15 min) blocked the OFQ/N-induced loss of \( \mu \) opioid receptor agonist binding (#, \( p < 0.05 \), Fig. 4). The loss of agonist binding is consonant with either \( \mu \) receptor down-regulation or loss of DAMGO affinity, both of which would contribute to \( \mu \) receptor desensitization. The ability of chelerythrine to block the loss of agonist binding further supports the role of PKC in heterologous desensitization. Chelerythrine alone did not alter \[^3H\]DAMGO binding.

**Chelerythrine Blocks OFQ/N-Mediated Increase in Membrane PKC-\( \alpha \), GRK2, and GRK3 Levels.** TPA treatment depletes conventional and novel, but not atypical, PKC isoforms. The fact that TPA pretreatment blocked receptor desensitization indicates that atypical PKC isoforms are not involved in that process. One approach to determining which conventional or novel PKC isoform is activated by agonist treatment is to follow the membrane translocation of each isoform of interest. OFQ/N pretreatment (0.1 nM, 60 min) increased membrane PKC-\( \alpha \) but not PKC-\( \epsilon \) levels, and that increase was blocked by chelerythrine (Fig. 5), indicating activation of the protein kinase and further supporting the role of PKC in the OFQ/N-mediated ORL1 and \( \mu \) opioid receptor desensitization. Since opioid receptors are substrates for GRK2 and GRK3 phosphorylation (Zhang et al., 1998; Celver et al., 2001; Li and Wang, 2001), and PKC can also increase the membrane association of GRK2 (Chuang et al., 1995; De Blasi et al., 1995) and GRK3 (Krasel et al., 2001), we next determined whether OFQ/N pretreatment altered membrane levels of GRK2 and GRK3. In addition to increasing membrane levels of PKC-\( \alpha \), OFQ/N pretreatment (0.1 nM, 60 min) also increased levels of GRK2 and GRK3 in membrane fractions of BE(2)-C cells. Chelerythrine blocks GRK2 and GRK3 translocation (Fig. 5), suggesting that PKC-\( \alpha \) is involved in GRK translocation. Chelerythrine alone did not alter either PKC or GRK membrane levels.

**Treatment with an Antisense, But Not Sense, ODN Common to Both GRK2 and GRK3 Blocks OFQ/N-Mediated Desensitization of ORL1 and \( \mu \) Receptors in BE(2)-C Cells.** To confirm the role of GRK in OFQ/N-induced ORL1 and \( \mu \) receptor desensitization, cells were pretreated with GRK antisense or sense ODN (10 \( \mu \)M, 60 h) that recognizes a sequence common to GRK2 and GRK3 (Shih and Malbon, 1994). Antisense, but not sense, DNA treatment reduced basal ORL3 levels by 61.5 ± 7%, slightly more (\( p < 0.05 \)) than the 45.9 ± 3% it reduced GRK2 levels (Fig. 6). More importantly, OFQ/N-mediated desensitization of ORL1 and \( \mu \) opioid receptors also was blocked after antisense DNA treatment (Fig. 6). This suggests that activation of GRKs by OFQ/N was PKC-dependent, and that GRK contributed to OFQ/N-mediated homologous and heterologous desensitization of ORL1 and \( \mu \) opioid receptors.

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**Fig. 2.** Chelerythrine (Che) blocks OFQ/N-induced desensitization of ORL1- and \( \mu \) opioid receptor-mediated ERK stimulation. Intact cells were pretreated with or without chelerythrine (1 \( \mu \)M) for 10 min, after which OFQ/N (0.1 nM) was added in the last 5 min of the incubation period. Cells were then washed, stimulated with DAMGO (1 \( \mu \)M) or OFQ/N (1 pM) for 5 min, and lysed, and the lysates were subjected to SDS-PAGE and probed with isoform-selective anti-ORL1 and anti-\( \mu \) opioid receptor sera as described under **Experimental Procedures**. Data were quantified by densitometric analysis and presented as mean ± S.E.M. of three independent experiments (A); a representative immunoblot is shown in B. Solid arrow points to phospho ERK1 (top band) and phospho ERK2 (bottom band), and dashed-line arrow points to total ERK1 (top band) and ERK2 (bottom band). OFQ/N-treated groups differed from controls (*, \( p < 0.05 \)), and chelerythrine treatment blocked the difference (#, \( p < 0.05 \)). Comparisons were made by one-way ANOVA with Tukey’s post hoc test.

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**Fig. 3.** Common to Both GRK2 and GRK3 Blocks OFQ/N-Mediated Desensitization of ORL1- and \( \mu \) Opioid Receptor Agonist Binding. To determine whether a loss of agonist binding contributed to reduced \( \mu \) and ORL1 responses following OFQ/N pretreatment, single-point binding assays of crude cell membrane homogenates were employed to measure levels of \( \mu \) and ORL1 receptors (Mandyam et al., 2000). Similar to previously reported results, levels of ORL1 within the cell were not reduced by OFQ/N pretreatment (Fig. 4; Spampinato et al., 2001). However, \[^3H\]DAMGO binding to the \( \mu \) opioid receptor was reduced 25% by OFQ/N pretreatment (*, \( p < 0.05 \), Fig. 4). Inclusion of chelerythrine (1 \( \mu \)M, 15 min) blocked the OFQ/N-induced loss of \( \mu \) opioid receptor agonist binding (#, \( p < 0.05 \), Fig. 4). The loss of agonist binding is consonant with either \( \mu \) receptor down-regulation or loss of DAMGO affinity, both of which would contribute to \( \mu \) receptor desensitization. The ability of chelerythrine to block the loss of agonist binding further supports the role of PKC in heterologous desensitization. Chelerythrine alone did not alter \[^3H\]DAMGO binding.

4aPDD (an inactive isomer of TPA) did not block OFQ/N-induced desensitization of ORL1 or \( \mu \) receptors (Fig. 3A). This also indicates that TPA-sensitive isoforms of PKC are involved in OFQ/N-mediated desensitization. TPA alone did not alter \( \mu \) opioid receptor response (\( \log EC_{50} = -7.33 ± 0.43; n = 3 \)) compared with control (−7.9 ± 0.26; \( n = 7 \)) but reduced OFQ/N potency (\( \log EC_{50} = -9.9 ± 0.6; n = 2 \)) compared with control (−12.0 ± 0.36; \( n = 8 \)), indicating that conventional or novel PKC isoforms are important for ORL1 receptor signaling in general. TPA treatment did not alter basal or forskolin-stimulated cAMP accumulation, arguing against the existence of constitutively active ORL1 receptors in these cells. Together, this supports our previous results that PKC is involved in OFQ/N-mediated homologous and heterologous desensitization.

BE(2)-C cells express the conventional (\( \alpha \)) and the novel (\( \epsilon \)) PKC isoforms (Fig. 3C). To confirm that TPA treatment reduced total levels of PKC-\( \alpha \) and -\( \epsilon \), cell lysates were subjected to SDS-PAGE and probed with isoform-selective antisera as described under **Experimental Procedures**. Both PKC-\( \alpha \) and -\( \epsilon \) isoforms were significantly reduced after treatment with TPA, but not 4aPDD (Fig. 3B).
ORL1, a recent addition to the opioid receptor family, is the only opioid receptor subtype selectively activated by OFQ/N, an endogenous opioid ligand (Meunier et al., 1995; Reinscheid et al., 1995). Although OFQ/N and the ORL1 couple to the Gi/Go class of inhibitory G-proteins, inhibit cAMP production, and modulate K_{Ca} and Ca^{2+} channels in a manner similar to /mu-, /delta-, and /kappa-opioid receptors, they produce anti-opioid as well as analgesic effects (for review see Harrison and Grandy, 2000). The anti-opioid effect of OFQ/N is evident when OFQ/N antagonizes /mu-opioid-mediated analgesia, although this may result, in part, from differential activity of OFQ/N on different classes of neurons involved in the descending analgesic pathway (Pan et al., 2000) and the heterologous effect of OFQ/N on /mu-opioid-mediated signaling (Hawes et al., 1998; Yuan et al., 1999; Mandyam et al., 2000; Ueda et al., 2000). Although it appears that the OFQ/N system contributes to /mu-opioid (morphine) tolerance, the mechanism underlying this effect is poorly defined. In the present study we show a potential mechanism for OFQ/N-induced /mu-ORL1 cross-talk.

The involvement of PKC in both ORL1 and /mu-opioid receptor signaling is well documented (Chen and Yu, 1994; Lou et al., 1997; Pei et al., 1997; Pu et al., 1999), and makes PKC a likely participant in any /mu-opioid-mediated analgesia, although this may result, in part, from differential activity of OFQ/N on different classes of neurons involved in the descending analgesic pathway (Pan et al., 2000) and the heterologous effect of OFQ/N on /mu-opioid-mediated signaling (Hawes et al., 1998; Yuan et al., 1999; Mandyam et al., 2000; Ueda et al., 2000). Although it appears that the OFQ/N system contributes to /mu-opioid (morphine) tolerance, the mechanism underlying this effect is poorly defined. In the present study we show a potential mechanism for OFQ/N-induced /mu-ORL1 cross-talk.

The involvement of PKC in both ORL1 and /mu-opioid receptor signaling is well documented (Chen and Yu, 1994; Lou et al., 1997; Pei et al., 1997; Pu et al., 1999), and makes PKC a likely participant in any /mu-ORL1 receptor interaction. PKC is a serine/threonine kinase that regulates a multitude of cellular functions and is a downstream target for a plethora of agonist-mediated signal transduction events (for review see...
PKC isozymes are divided into three categories: the conventional or the classic isozymes (PKC-α, PKC-β, PKC-γ, and PKC-δ) that are diacylglycerol (DAG)- and Ca^2+/-dependent and respond to phorbol esters; the novel isozymes (PKC-ε, PKC-ζ, PKC-η, and PKC-θ) that are DAG-dependent and respond to phorbol esters, but are insensitive to Ca^2+; and atypical isozymes (PKC-ι and PKC-λ) that do not require Ca^2+ and respond to neither DAG nor phorbol esters (Black, 2000).

Conventional and novel PKC isozymes translocate to the membrane from the cytosol when activated and phosphorylate their targets. PKC can produce desensitization by directly phosphorylating the receptor (Zhang et al., 1996; Black, 2000) or, indirectly, via GRK2 and GRK3 (Chuang et al., 1995; De Blasi et al., 1995; Krasel et al., 2001). PKC activation of GRK2 is an isoform-dependent process, preferring to phosphorylate PKC-δ > PKC-α > PKC-γ (Krasel et al., 2001). The activated GRK2 is then translocated to the cell membrane and is thus more readily available to phosphorylate the receptor upon addition of agonist: isoproterenol-stimulated cAMP accumulation through the β-adrenoceptor plateaued more quickly in lymphocytes in which membrane GRK activity was increased by TPA treatment than in untreated lymphocytes (De Blasi et al., 1995). Since the β-adrenoceptor did not desensitize until it was exposed to its own agonist, this mechanism explains how one agonist or treatment can heterologously regulate the homologous desensitization of a second receptor. BE(2)-C cells express detectable levels of PKC-α, which can phosphorylate and activate GRK2; they also express PKC-ε, which does not phosphorylate GRK2 (Krasel et al., 2001). It is not known whether PKC
phosphorylates GRK3 in the same manner, but it has been shown to increase GRK3 expression (De Blasi et al., 1995).

Three approaches were employed to determine the involvement of PKC in OFQ/N-mediated homologous and heterologous desensitization of ORL1 and mu opioid receptors. Chelerythrine chloride, a nonspecific inhibitor of all PKC isoforms, was used to block PKC activity to determine whether PKC played a role in OFQ/N-mediated cross-talk. Pretreatment with chelerythrine prevented OFQ/N-mediated desensitization of mu and ORL1 inhibition of cAMP (Fig. 1) and activation of ERK1/2 (Fig. 2), indicating that the cross-talk was PKC-dependent. Chelerythrine also blocked the OFQ/N-mediated reduction in [3H]DAMGO binding to mu opioid receptors (Fig. 4), further supporting the role for PKC in OFQ/N-mediated receptor tolerance. PKC was previously shown to contribute to mu opioid receptor homologous down-regulation (Kramer and Simon, 1999); therefore, its role in heterologously mediated loss of mu opioid receptor agonist binding is not surprising. To investigate any contribution of PKA or ERK1/2, cells were also pretreated with H-89 (1 μM) or PD98059 (10 μM) to block PKA or ERK1/2, respectively (data not shown). Neither of the inhibitors blocked OFQ/N-induced desensitization of ORL1 or mu receptors, ruling out a role for PKA or mitogen-activated protein kinase in the process.

Previous PKC translocation studies showed that PKC-γ (Narita et al., 2001) and PKC-ζ (Kramer and Simon, 1999) isoforms are involved in homologous mu opioid receptor desensitization and down-regulation, respectively. Since it was evident that PKC was involved in the OFQ/N-mediated cross-talk, chronic phorbol ester treatment was performed to determine which group of isoforms could be involved in the interaction. PKC down-regulation by TPA blocked the subsequent OFQ/N-mediated desensitization (Fig. 3A), indicating a role for conventional and/or novel PKC isoforms in the cross-talk. Probing membrane fractions from OFQ/N-treated cells indicated that PKC-α, but not PKC-ε, was translocated to the membrane following OFQ/N treatment. Furthermore, chelerythrine blocked the translocation (Fig. 5). In addition, OFQ/N treatment increased membrane levels of GRK2 and GRK3 (Fig. 5); this increase was also blocked by chelerythrine. These results strongly suggest that the GRK2 and GRK3 translocation is mediated via PKC activation and that GRK2 and GRK3 may contribute to the OFQ/N-induced cross-talk. The role of GRK2 and GRK3 in OFQ/N-mediated mu desensitization and ORL1 desensitization in BE(2)-C cells was confirmed by incubating cells with GRK2/3 antisense to reduce levels of the proteins (Fig. 6B) prior to pretreatment with OFQ/N. Antisense, but not sense, treatment blocked desensitization and ORL1 inhibition of cAMP (Fig. 5); this increase was also blocked by chelerythrine chloride, a nonspecific inhibitor of all PKC isoforms: heterologous regulation of homologous desensitization and its implications. Trends Pharmacol Sci 17:416–421.

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References


Kramer KH and Simon ED (1999) Role of protein kinase C (PKC) in agonist-induced
Mechanism of ORL1 and μ Opioid Receptor Cross-Talk


