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Orphanin FQ/Nociceptin Potentiates [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-Enkephalin–Induced μ-Opioid Receptor Phosphorylation

Hatice Z. Ozsoy,[1] Deepak R. Thakker,[2] and Kelly M. Standifer

Department of Pharmacological and Pharmaceutical Sciences, University of Houston, College of Pharmacy, Houston, Texas

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ABSTRACT

In this study, we investigate the molecular mechanisms by which acute orphanin FQ/nociceptin (OFQ/N), acting through the nociceptin opioid peptide (NOP) receptor, desensitizes the μ-opioid receptor. We described previously the involvement of protein kinase C and G-protein-coupled receptor kinases (GRK) 2 and 3 in OFQ/N-induced μ receptor desensitization. Because phosphorylation of the μ receptor triggers the successive regulatory mechanisms responsible for desensitization, such as receptor uncoupling, internalization, and down-regulation, we investigated the ability of OFQ/N to modulate [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO)-induced μ receptor phosphorylation in BE(2)-C human neuroblastoma cells transfected with epitope-tagged μ receptors. OFQ/N treatment (100 nM, 60 min) potentiated DAMGO-induced μ receptor phosphorylation; inhibition of GRK2 or protein kinase C concomitant with OFQ/N treatment blocked the OFQ/N-mediated increase in DAMGO-induced phosphorylation. Inclusion of the NOP antagonist peptide III-BTD during OFQ/N pretreatment blocked the potentiation of DAMGO-induced phosphorylation by OFQ/N, which is consistent with the potentiation being mediated via actions of the NOP receptor. In addition, in cells expressing μ receptors in which the GRK-mediated phosphorylation site Ser^{375} was mutated to alanine, OFQ/N treatment failed to potentiate DAMGO-induced μ receptor phosphorylation and failed to desensitize the μ receptor. However, DAMGO-induced μ receptor phosphorylation and OFQ/N-induced μ receptor desensitization occurred in cells expressing μ receptors lacking non-GRK phosphorylation sites. These data suggest that OFQ/N binds to NOP receptors and activates protein kinase C, which then increases the ability of GRK2 to phosphorylate the agonist-occupied μ receptor, heterologously regulating homologous μ receptor desensitization.

μ Receptor agonists acting through μ-opioid receptors are widely used analgesics in the treatment of severe pain, despite the fact that long-term treatment with these drugs results in the development of tolerance and dependence. At the molecular level, μ receptor desensitization or loss of receptor function has been suggested to be the underlying reason for the development of tolerance to μ receptor agonists (Harrison et al., 1998).

Like many GPCRs, the μ receptor can undergo homologous and heterologous receptor desensitization. Homologous receptor desensitization occurs when a cognate agonist leads to a decrease in the receptor responsiveness through the induction of regulatory events such as phosphorylation, internalization, and down-regulation of the receptor, and it often involves G-protein-coupled receptor kinases (GRKs). Agonist binding to receptor stimulates G proteins, and the $\beta\gamma$ subunits of activated G proteins recruit GRKs to the plasma membrane, in which GRKs can phosphorylate agonist-occupied receptors (Lefkowitz et al., 1998). Several GRK2-mediated phosphorylation sites of the μ receptor have been identified, but Ser^{375} seems to be the primary site for DAMGO-induced μ receptor phosphorylation (El Kouhen et al., 2001; Schulz et al., 2004).

Heterologous receptor desensitization occurs when activation of a GPCR blunts the agonist response of other receptors present in the same cell, and it involves second-messenger kinases such as protein kinase C. Besides the involvement of protein kinase C in heterologous receptor desensitization,

[1] Current address: Winter Centers for Heart Failure Research, Baylor College of Medicine, Houston, Texas.
[2] Current address: Neuroscience Research, Novartis Institutes for BioMedical Research, Novartis Pharma AG, Basel, Switzerland.

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ABBREVIATIONS: GPCR, G-protein-coupled receptor; NOP, nociceptin opioid peptide; OFQ/N, orphanin FQ/nociceptin; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; GRK, G-protein-coupled receptor kinase; PBS, phosphate-buffered saline; [^{32}Pi], [^{32}P]orthophosphate; HA, hemagglutinin; RIPA, radioimmunoprecipitation assay; NLX, naloxone; HAμ, hemagglutinin-tagged μ receptor.

protein kinase C can also phosphorylate and activate GRK2 (Chuang et al., 1995; Winstel et al., 1996). Protein kinase C-activated GRKs translocate to the plasma membrane and become readily available to phosphorylate GPCRs upon agonist binding, indicating that homologous desensitization can be heterologously regulated via a protein kinase C-dependent pathway (Chuang et al., 1995). Although mechanisms leading to homologous μ receptor desensitization are well described, this is not the case for heterologously mediated μ receptor desensitization.

OFQ/N is an endogenous ligand for the nociceptin opioid peptide (NOP) receptor. OFQ/N and NOP receptors are widely distributed in the central nervous system and are colocalized with μ receptors in many cells in the descending analgesic pathway (Heinricher et al., 1994; Connor et al., 1996; Pan et al., 2000). OFQ/N binds to NOP receptors and activates protein kinase C, which plays a role in homologous NOP receptor desensitization (Lou et al., 1997; Pei et al., 1997; Pu et al., 1999; Mandyam et al., 2002). Besides homologous NOP receptor desensitization, activation of NOP receptors by OFQ/N heterologously regulates the μ receptor response to DAMGO (Hawes et al., 1998; Mandyam et al., 2000, 2003; Thakker and Standifer, 2002).

We recently reported that 1-h OFQ/N treatment desensitized the inhibitory cAMP response of the μ receptor through a protein kinase C-dependent pathway in BE(2)-C human neuroblastoma cells that natively express μ and NOP receptors (Mandyam et al., 2002). We also found that 1-h OFQ/N treatment induced activation of protein kinase C, GRK2, and GRK3 and that inhibition of those kinases blocked OFQ/N-induced μ receptor desensitization. These results suggest that protein kinase C and GRK2 and/or GRK3 play important roles in OFQ/N regulation of μ receptor signaling.

In the present study, we determined that OFQ/N potentiated DAMGO-induced μ receptor phosphorylation via activation of GRK2 in BE(2)-C human neuroblastoma cells transfected with epitope-tagged μ receptors. We further tested the role of GRK2 in OFQ/N-mediated potentiation of DAMGO-induced μ receptor phosphorylation in cells expressing μ receptors in which the GRK phosphorylation site, Ser^{375}, was mutated to alanine.

Materials and Methods

Materials. The following were purchased from or provided by the sources indicated: OFQ/N, DAMGO, and naloxone (Research Technology Branch of the National Institute on Drug Abuse, Bethesda, MD); [^{3}H]cAMP (Amersham Biosciences Inc., Piscataway, NJ); [^{32}P]orthophosphate ([^{32}Pi]; MP Biomedicals, Irvine, CA); protein-G agarose, protein-A agarose, rabbit GRK2 and GRK3 antibodies, and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-Flag M2 antibody (Sigma-Aldrich, St. Louis, MO); rabbit polyclonal μ receptor antibody (Research and Diagnostic Antibodies, Benicia, CA); chelerythrine (Sigma/RBI, Natick, MA); phosphodiester oligodeoxynucleotides (Sigma-Genosys, The Woodlands, TX); peptide III-BTD (Neosystem, Strasbourg, France); rat hemagglutinin-monoclonal antibody 3F10 (Roche Molecular Biochemicals, Indianapolis, IN); phosphoSer^{375} μ receptor antibody (Cell Signaling Technology, Beverly, MA); wheat germ lectin-agarose beads (Amersham Biosciences); and α-glyceraldehyde-3-phosphate dehydrogenase antibody (Chemicon International, Temecula, CA).

Cells and Cell Culture. Flag- or HA-tagged μ receptors subcloned into the expression vector pcDNA3 were generous gifts from Dr. Lakshmi Devi (Mount Sinai School of Medicine, New York, NY) and Dr. Ping-Yee Law (University of Minnesota, Minneapolis, MN), respectively. To ensure effective immunoprecipitation of μ receptors, BE(2)-C cells were stably transfected with the pcDNA3 vector expressing Flag-tagged or HA-tagged μ receptors using FuGene reagent. Geneticin (800 μg/ml) was used as the selection drug. Clones were screened for μ receptor expression levels using 0.01 to 5 nM [^{3}H]DAMGO as described previously (Mandyam et al., 2000). Clones with approximately 500 fmol/mg μ receptor levels were selected and used in all experiments. Transfected BE(2)-C human neuroblastoma cells were cultured and maintained as described previously for untransfected BE(2)-C neuroblastoma cells (Mandyam et al., 2000), except that geneticin (600 μg/ml) was included in the cell-culture media. Studies were performed on cells at $\geq$70% confluence that were lifted from plates with phosphate-buffered saline (PBS), pH 7.4, containing 1 mM EGTA.

Pretreatment Conditions. Transfected BE(2)-C cells were treated with 100 nM OFQ/N for 1 h at 37°C in culture media containing bovine serum albumin (0.1%) and bacitracin (0.25 mg/ml). Vehicle-treated cells received bovine serum albumin and bacitracin in the absence of OFQ/N and served as controls. In the experiments involving the inhibition of protein kinase C, chelerythrine (1 μM) was added to the plates 15 min before the addition of OFQ/N. Antagonist treatments with naloxone (100 nM) or peptide III-BTD (10 μM) (Thakker and Standifer, 2002) were performed 15 min before and during the OFQ/N treatment. In the experiments involving DAMGO challenge, the treatment media were aspirated at the end of the OFQ/N treatment period, and cells were washed with ice-cold PBS or phosphate-free media before stimulating with DAMGO for 10 min at 37°C. Washed cells were either lysed for phosphorylation experiments or lifted using PBS/EGTA for cAMP accumulation assay.

In experiments involving GRK2 or GRK3 antisense/sense treatment, plated cells were washed with serum-free media and treated with phosphodiester antisense oligodeoxynucleotides in serum-free media: GRK2 antisense DNA, 5′-CTC CAG GTC CGC CAT CTT-3′ (1 μM, 72 h) (Aiyar et al., 2000); or GRK3 antisense DNA, 5′-TCC AGT GTC TGC TTT CCT-3′ (1 μM, 48 h), as described previously (Thakker and Standifer, 2002). The sense treatments served as negative controls. In the last hour of the treatment, OFQ/N or vehicle was added to the plates as described above.

Measurement of cAMP Accumulation. Intact cells were preincubated for 5 min in Hanks' balanced salt solution buffer containing 0.5 mM 3-isobutyl-1-methylxanthine at 37°C. Forskolin and/or different concentrations of agonist were then added, and the reaction mixtures were incubated for 10 min at 37°C. The reaction was terminated by boiling the samples for 5 min in a water bath. After boiling, the samples were centrifuged for 5 min at 13,000*g*, and the supernatants were used to determine cAMP levels in a [^{3}H]cAMP-binding assay as described previously (Mandyam et al., 2000).

μ Receptor Phosphorylation. BE(2)-C cells transfected with Flag-tagged μ receptors (Flagμ-BE cells) were grown to 80 to 90% confluence in 100-mm^2 plates. On the day of the assay, they were washed with phosphate-free media and incubated with 4 ml of the same media for 1 h. This was followed by the addition of [^{32}Pi] (150 μCi/ml) into the culture medium. Cells were labeled with [^{32}Pi] for 2 h at 37°C. After labeling, OFQ/N was added to the plates for 1 h. At the end of the OFQ/N pretreatment, cells were washed with phosphate-free media and challenged with DAMGO (1 μM) for an additional 10 min. After 10-min DAMGO treatment, cells were washed with ice-cold PBS and lysed with RIPA$^+$ buffer [1% IGEPAL CA-630 (Stepan Company, Northfield, IL), 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, 1 μM okadaic acid, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml benzamidine, 10 μg/ml leupeptin, and 1 μg/ml pepstatin A in phosphate-buffered saline buffer] (Zhang et al., 1996). The lysates were centrifuged at 150,000*g* for 15 min at 4°C, and the supernatant was used for further analysis. After preclearing with protein-G agarose, the

supernatant was incubated with 10 μg of anti-Flag M2 antibody-preconjugated with protein-G agarose overnight at 4°C. At the end of the incubation period, the protein-antibody-bead complex was washed with RIPA$^+$ buffer and heated for 20 min at 60°C in SDS sample buffer. After centrifugation of samples, the supernatant was resolved on a 10% SDS-polyacrylamide gel and was electrophoretically transferred onto polyvinylidiene fluoride membrane. Autoradiography of the membrane was used for quantifying μ receptor phosphorylation. The same membrane was used for immunoblotting the μ receptor with mouse monoclonal anti-Flag M2 horseradish peroxidase-conjugated antibody (1:100) or rabbit polyclonal anti-μ (1:500) antibody. The ratio of the ^{32}P-μ receptor band to the immunoblotted band provided data about the relative phosphorylation state per receptor and is normalized with respect to basal values (vehicle-treated). Similar results were obtained with both antibodies, but the immunoblots obtained with the anti-Flag M2 antibody are shown in the figures.

GRK2/3 Phosphorylation. Flagμ-BE cells were labeled with [^{32}Pi] (350 μCi/ml) and then treated with vehicle, OFQ/N (100 nM), chelerythrine (1 μM), or the combination of OFQ/N and chelerythrine as described above. After terminating the treatments by washing with ice-cold PBS, cells were lysed with RIPA$^+$ buffer. For immunoprecipitation, an antibody recognizing both GRK2 and GRK3 (1 μg of H-222; Santa Cruz Biotechnology) was preconjugated with protein-A agarose. GRK2 and GRK3 in the lysates were immunoprecipitated with the protein-A agarose-preconjugated GRK2/3 antibody (Horie and Insel, 2000). The immunoprecipitates were run on an SDS gel as described above. For immunoblots, GRK2 (C-15)- and GRK3 (C-14)-specific antibodies (1:1000; Santa Cruz Biotechnology) were used. The ratio of the phosphoprotein band to the immunoblotted band provided data about the relative phosphorylation state of GRK2 and GRK3 and was normalized with respect to basal values (vehicle-treated).

Partial Purification of the HA-Tagged μ Receptor Protein and Western Blotting. After pretreatments, BE(2)-C cells transfected with HA-tagged μ receptors (HAμ-BE cells) were washed three times with PBS and lysed in HEPES buffer [25 mM HEPES, pH 7.4, 1% (v/v) Triton X-100, 5 mM EDTA with 10 μg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml benzamidine, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, and 0.1 mM sodium vanadate] (El Kouhen et al., 2001). Insoluble debris was removed by centrifugation at 14,000*g* for 15 min at 4°C. The supernatant was incubated with 100 μl of wheat germ lectin-agarose beads for 90 min at 4°C (Koch et al., 2001). After the incubation, beads were washed four times with the lysis buffer (with 0.1% Triton X-100); adsorbed glycoproteins were eluted into 100 μl of SDS sample buffer at 60°C for 20 min. Samples were resolved on a 10% SDS-polyacrylamide gel and were electrophoretically transferred onto polyvinylidene difluoride membrane. Membranes were blocked with Tris-buffered saline/Tween 20 (0.05%) containing 5% nonfat dried milk for 1 h and incubated with rabbit phosphoSer^{375} antibody (1:1000) overnight at 4°C, followed by 1-h anti-rabbit secondary antibody (1:2000) incubation. After detection of the Ser^{375}-phosphorylated form of the μ receptor protein band, the same membrane was stripped and reprobed with rat HA-antibody 3F10 (1:500) overnight at 4°C, followed by 1-h anti-rat (1:2000) secondary antibody incubation. The densitometric ratio of the phosphoSer^{375} μ receptor protein band to total μ receptor protein band was calculated to detect changes in DAMGO-induced μ receptor phosphorylation after 1-h OFQ/N pretreatment.

Protein Estimation. The method described by Lowry et al. (1951) was used to determine protein concentrations as described previously.

Data Analysis. Log EC_{50} values were determined using nonlinear regression analysis. Data were expressed as the mean ± S.E.M. unless otherwise indicated. Statistical comparisons of data were performed with Student's *t* test or one-way analysis of variance followed by Tukey's post hoc test using Prism version 4.0 for Windows 95/98 (GraphPad Software Inc., San Diego, CA). Data were considered significant if $p < 0.05$.

Results

We reported previously that activation of the NOP receptor by OFQ/N desensitized the inhibitory cAMP response of the μ receptor in BE(2)-C cells (Mandyam et al., 2000, 2002; Thakker and Standifer, 2002). Desensitization of the μ receptor is a multistep process like desensitization of all GPCRs. To elaborate the mechanisms by which OFQ/N desensitizes the μ receptor, we wanted to focus on phosphorylation of the receptor, because our previous study indicated that 1-h OFQ/N pretreatment activated protein kinase C, GRK2, and GRK3, all of which are capable of phosphorylating the μ receptor (Zhang et al., 1996, 1998). To confirm that OFQ/N desensitizes the μ receptor in Flagμ-BE cells via protein kinase C- and GRK-dependent pathways as it does in untransfected BE(2)-C cells, Flagμ-BE cells were treated with OFQ/N (100 nM) in the presence and absence of the protein kinase C inhibitor chelerythrine (1 μM) and were assayed for the ability of DAMGO to inhibit forskolin (10 μM)-stimulated cAMP accumulation. One-hour OFQ/N treatment produced μ receptor desensitization, and chelerythrine (1 μM) prevented OFQ/N-induced μ receptor desensitization (Fig. 1A). None of the treatments affected the basal cAMP levels of Flagμ-BE cells. OFQ/N treatment reduced DAMGO efficacy (59.49 ± 1.9%) compared with control DAMGO efficacy (75.86 ± 4.2%; $n = 3$, $\star p < 0.05$). Chelerythrine completely blocked the ability of OFQ/N to desensitize the μ receptor (77.54 ± 0.8%; $n = 3$, $\# p < 0.05$).

To test the roles of GRK2 and GRK3 in OFQ/N-induced μ receptor desensitization in Flagμ-BE cells, we used GRK isoform-specific antisense DNA treatment, which we reported previously selectively reduces levels of GRK2 or GRK3 (Thakker and Standifer, 2002; Mandyam et al., 2003). OFQ/N-induced μ receptor desensitization was attenuated in cells pretreated with GRK2 antisense but not GRK2 sense DNA (Fig. 1B). OFQ/N treatment reduced DAMGO efficacy (61.91 ± 7.9%) compared with control DAMGO efficacy (72.8 ± 3.9%; $n = 2$, $\star p < 0.05$). Inhibition of GRK2 blocked OFQ/N-induced reduction in the efficacy of DAMGO to inhibit forskolin-stimulated cAMP accumulation (74 ± 3.0%; $n = 2$, $\# p < 0.05$). In contrast, GRK3 antisense DNA treatment had no effect (Fig. 1C). These experiments assured us that the Flagμ receptors in the Flagμ-BE cells responded as the native receptors did to OFQ/N and are, therefore, a useful model in which to study heterologous regulation of the μ receptor by OFQ/N.

Expressing Flag-epitope–tagged μ receptors enabled us to immunoprecipitate the μ receptor with an antibody raised against the Flag epitope. In immunoblots, Flag-tagged μ receptors from Flagμ-BE cells appear as the band at 65 to 70 kDa, as reported in other systems (Zhang et al., 1996); samples from untransfected BE(2)-C cells lack this band (Fig. 2, left). The μ receptor protein shown at this band underwent a robust DAMGO-induced phosphorylation, indicating that the 65- to 70-kDa band, not the other bands in the immunoblots, represents the fully glycosylated and functional μ receptor protein (Fig. 2, right) as shown previously by many others (Zhang et al., 1996; El Kouhen et al., 1999). The same band was detected using a rabbit anti-μ-opioid receptor polyclonal

antibody raised against a peptide sequence of the carboxyl terminal of the μ receptor, as with the mouse Flag M2 monoclonal antibody-peroxidase conjugate raised against the Flag epitope (Fig. 2, left). Immunoblotting was performed with both antibodies to confirm the effect, but an analysis of only the anti-Flag M2 antibody data is included.

OFQ/N-induced μ receptor desensitization was measured as the ability of the μ-opioid receptor agonist DAMGO to inhibit adenylyl cyclase activity for a 10-min period after a 1-h treatment with OFQ/N. This 10-min exposure to DAMGO during the measurement of μ receptor response is enough to activate rapid regulatory mechanisms, leading to homologous μ receptor desensitization such as activation of GRKs and phosphorylation of the μ receptor (Zhang et al., 1996). To determine whether 1-h OFQ/N treatment enhances DAMGO-induced μ receptor phosphorylation via this pathway, cells were labeled with [^{32}Pi] and treated with OFQ/N for 1 h. After that period, cells were washed and challenged with the μ agonist DAMGO for 10 min, and then Flagμ receptors were immunoprecipitated as described under *Materials and Methods*. One-hour OFQ/N treatment significantly increased DAMGO-induced μ receptor phosphorylation (Fig. 3). However, receptor phosphorylation was not noted after 1-h OFQ/N in the absence of DAMGO challenge. DAMGO treatment alone induced a 3-fold increase in μ receptor phosphorylation compared with vehicle-treated cells. DAMGO challenge in OFQ/N-treated cells increased μ receptor phosphorylation 50% more than a 10-min DAMGO treatment in vehicle-treated cells (Fig. 3). To rule out the possibility that the OFQ/N-induced increase in μ receptor phosphorylation is mediated directly through μ receptors, cells were treated with OFQ/N in the presence and absence of the NOP receptor antagonist, peptide III-BTD (BTD, 10 μM) or the μ antagonist naloxone (NLX, 100 nM) only during the 1-h OFQ/N preincubation period, not during the DAMGO challenge period. The NOP receptor antagonist completely reversed the enhancement of DAMGO-induced μ receptor phosphorylation, whereas NLX had no effect (Fig. 3).

Because protein kinase C is involved in heterologous desensitization of the μ receptor by OFQ/N (Mandyam et al., 2002), we wanted to determine whether protein kinase C is responsible for the OFQ/N-mediated increase in DAMGO-induced μ receptor phosphorylation. Flagμ-BE cells were treated with OFQ/N in the presence and absence of the protein kinase C inhibitor chelerythrine. At the end of the treatment period, Flagμ-BE cells were washed and challenged with DAMGO. In the presence of chelerythrine,

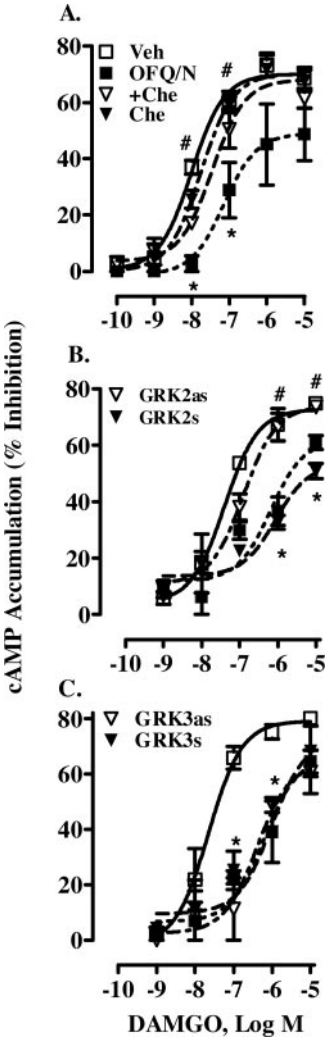

Fig. 1. Protein kinase C and GRK2 inhibition in Flagμ-BE cells blocks OFQ/N-mediated heterologous desensitization of the μ receptor. Intact Flagμ-BE cells were incubated with or without OFQ/N (100 nM) for 1 h and were assayed for the ability of DAMGO to inhibit forskolin (10 μM)-stimulated cAMP accumulation. Cells were exposed to chelerythrine (1 μM) for 15 min (A) or were treated with GRK2 (B) or GRK3 (C) antisense or sense in serum-deprived media as described under *Materials and Methods* before the addition of OFQ/N. The treatments did not affect basal cAMP levels. OFQ/N treatment reduced DAMGO potency (log EC_{50} $-6.9 \pm 0.2\star$) compared with vehicle treatment (log EC_{50} -8.0 ± 0.1). Chelerythrine cotreatment with OFQ/N returned DAMGO potency (log EC_{50} -7.7 ± 0.2#). Chelerythrine treatment alone did not alter DAMGO potency (log EC_{50} -7.7 ± 0.1; A). GRK2 antisense treatment also attenuated the ability of OFQ/N (OFQ/N log EC_{50} $-6.1 \pm 0.3\star$ versus vehicle log EC_{50} -7.4 ± 0.2) to reduce DAMGO potency (OFQ + GRK2as log EC_{50} -6.9 ± 0.1#). GRK2 sense treatment did not return OFQ/N-induced decrease in DAMGO potency (log EC_{50} -6.0 ± 0.2; B). GRK3 antisense or sense treatments did not change the effect of OFQ/N on DAMGO potency (vehicle log EC_{50} -7.6 ± 0.1; OFQ/N log EC_{50} -6.2 ± 0.2; OFQ/N + GRK3as log EC_{50} -6.3 ± 0.2; OFQ/N + GRK3s log EC_{50} -6.0 ± 0.3; C). Data are expressed as mean $\pm$ S.E.M. of two to three experiments. $\star$, $p < 0.05$ significantly different from vehicle-treated cells, #, $p < 0.05$ significantly different from OFQ/N-treated cells.

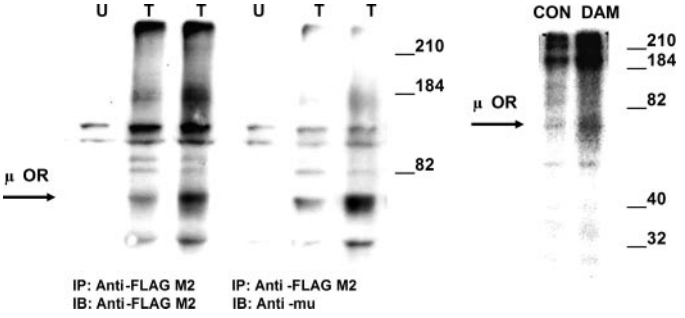

Fig. 2. Characterization of Flag-tagged μ receptor protein from Flagμ-BE cells by immunoprecipitation. Flagμ-BE cells were lysed and immunoprecipitated using anti-Flag M2 antibody. The immunoprecipitates were resolved through SDS-polyacrylamide gel electrophoresis gels and immunoblotted using anti-Flag M2 horseradish peroxidase-conjugated antibody (left, lanes 1–3). The membrane then was stripped and reprobed with the rabbit μ receptor antibody (left, lanes 4–6). Lanes 2 and 4 were loaded with 10 μl of immunoprecipitate samples, and lanes 3 and 6 were loaded with 20 μl. U, untransfected BE(2)-C cells; T, Flagμ-BE cells; μOR, μ-opioid receptor. The μ receptor protein appears as the band at 65 to 70 kDa, untransfected BE(2)-C cells lack this band (left, lanes 1 and 4), and DAMGO-induced phosphorylation significantly occurred at this band only (right). Con, control; DAM, DAMGO treatment (10 min).

OFQ/N did not potentiate DAMGO-induced μ receptor phosphorylation (Fig. 4). Chelerythrine alone did not significantly alter basal or DAMGO-induced phosphorylation of the μ receptor.

The role of GRK2 in OFQ/N-mediated increase in μ receptor phosphorylation in Flagμ-BE cells was tested by depleting GRK2 levels with GRK2 antisense DNA treatment, as described previously (Aiyar et al., 2000; Thakker and Standifer, 2002; Mandyam et al., 2003). This GRK2 antisense DNA treatment reduced GRK2 levels more than 50% (Fig. 5A, right). The 50% increase in DAMGO-induced phosphorylation in OFQ/N-treated cells was blocked in cells cotreated with GRK2 antisense, but not sense, DNA and OFQ/N (Fig. 5A). These data suggest that GRK2 is involved in OFQ/N-mediated heterologous regulation of the μ receptor. Although GRK2 seems to be responsible for desensitizing the μ receptor more than GRK3 in our cell line (Mandyam et al., 2003), we wanted to determine whether this also held true for μ receptor phosphorylation. In GRK3 antisense DNA-treated cells, in which GRK3 levels were reduced more than 70%, OFQ/N was still able to enhance DAMGO-induced μ receptor phosphorylation (Fig. 5B). GRK3 antisense DNA treatment did not affect the basal phosphorylation of the μ receptor or OFQ/N-mediated increase in DAMGO-induced μ receptor phosphorylation, supporting our previous studies showing that GRK2, not GRK3, regulates μ receptor signaling.

Because direct phosphorylation of GRK2 by protein kinase C was shown to increase GRK2/3 activity (Chuang et al., 1995; Winstel et al., 1996), we next investigated whether OFQ/N induces GRK2 phosphorylation via a protein kinase C-dependent pathway in Flagμ-BE cells preloaded with [^{32}Pi]. The preloaded cells were treated with OFQ/N in the presence and absence of the protein kinase C inhibitor chelerythrine. In OFQ/N-treated cells, the level of phosphorylation of GRK2 was increased more than 2-fold compared with that in vehicle-treated cells (Fig. 6; $\star p < 0.05$, $n = 3$). OFQ/N-induced increase in GRK2 phosphorylation was blocked by chelerythrine (Fig. 6; $\# p < 0.05$, $n = 3$).

Our data suggest that 1-h OFQ/N pretreatment leads to an increase in the ability of GRK2 to phosphorylate the agonist-bound μ receptor. Ser^{375} is the primary site for GRK-mediated μ receptor phosphorylation because it gets phosphorylated only in the presence of a μ agonist (El Kouhen et al., 2001; Schulz et al., 2004). We hypothesized that the Ser^{375} residue is responsible for OFQ/N enhancement of DAMGO-induced μ receptor phosphorylation. To study this hypothesis, we tested two different mutant μ receptors: HA3Aμ, in which three phosphorylation sites (Ser^{363}, Thr^{370}, and Ser^{375}) were mutated to alanine; and HA2Aμ, in which two phosphorylation sites (Ser^{363} and Thr^{370}) were mutated to alanine. We used these mutants because the mutant HA3Aμ lacks Ser^{375}, the putative GRK phosphorylation site, whereas the mutant HA2Aμ retains that site so that we could determine the role of Ser^{375}. Both mutants lack putative protein kinase C phosphorylation sites (Ser^{363} and Thr^{370}) that would play a role in purely heterologous receptor desensitization and phosphorylation (El Kouhen et al., 2001). BE(2)-C cells were stably transfected with the mutants HA3Aμ, HA2Aμ, or wild-type HA-tagged μ receptor (HAμ). HAμ-BE, HA3Aμ-BE, and HA2Aμ-BE cells refer to BE(2)-cells transfected with the HAμ, HA3Aμ, and HA2Aμ receptors, respectively. Clones were selected that expressed the μ receptor in levels similar to those in Flagμ-BE cells. The B_{max}, EC_{50}, and

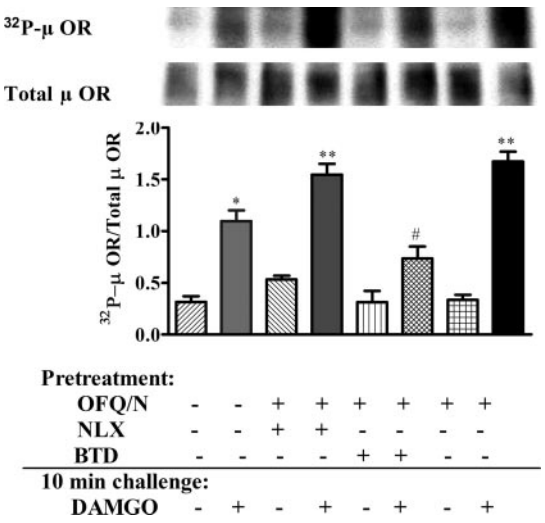

Fig. 3. OFQ/N pretreatment enhances DAMGO-induced μ receptor phosphorylation, which is selectively blocked by the NOP receptor antagonist peptide III-BTD (BTD). Flagμ-BE cells were labeled with [^{32}Pi] and then treated with OFQ/N (100 nM, 1 h) in the absence or presence of BTD (1 μM) or the μ antagonist NLX (100 nM). After treatment, cells were washed with phosphate-free medium and challenged with DAMGO (1 μM) for 10 min. Cell lysates were subjected to immunoprecipitation with anti-Flag M2 antibody as described under *Materials and Methods*. Data from immunoblots and autoradiography were quantified by densitometric analysis, and the ratio of the ^{32}P-Flagμ receptor band to total Flagμ receptor immunoreactivity was presented as the mean ± S.E.M. of four experiments. μ OR, μ-opioid receptor; *, $p < 0.05$ significantly different from vehicle-treated cells; **, $p < 0.05$ significantly different from DAMGO-challenged cells; #, $p < 0.05$ significantly different from OFQ/N-treated + DAMGO-challenged cells.

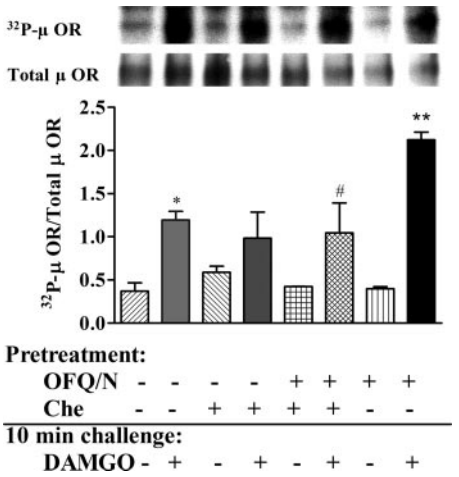

Fig. 4. OFQ/N-mediated increase in DAMGO-induced μ receptor phosphorylation was blocked with protein kinase C inhibition. Flagμ-BE cells were labeled with [^{32}Pi] and then exposed to chelerythrine (1 μM) for 15 min before the addition of OFQ/N (100 nM) as described under *Materials and Methods*. After 1-h OFQ/N treatment, cells were washed with phosphate-free medium and challenged with DAMGO (1 μM) for 10 min. Cell lysates were subjected to immunoprecipitation with anti-Flag M2 antibody as described under *Materials and Methods*. Data from immunoblots and autoradiography were quantified by densitometric analysis, and the ratio of the ^{32}P-Flagμ receptor band to total Flagμ receptor immunoreactivity was presented as mean ± S.E.M. of four experiments. The top and bottom blots of each are representative autoradiography and immunoblots of the μ receptor, respectively. μ OR, μ-opioid receptor; *, $p < 0.05$ significantly different from vehicle-treated cells, ⋆⋆, $p < 0.05$ significantly different from DAMGO-challenged cells; #, $p < 0.05$ significantly different from OFQ/N-treated + DAMGO-challenged cells.

percentage of inhibition values of cAMP accumulation induced by 10 μM DAMGO for all cell lines are shown in Table 1. No difference was observed between the wild-type and mutant μ receptors, in terms of neither their ability to inhibit forskolin-stimulated cAMP accumulation nor their levels of receptor expression.

First, we tested the ability of OFQ/N to desensitize the wild-type HA-tagged μ receptor. As shown in Fig. 7A, the ability of DAMGO to inhibit cAMP accumulation was reduced in OFQ/N-treated cells (67.39 ± 5.6%) compared with vehicle-treated cells (85 ± 2.1%; $n = 2$; ⋆, $p < 0.05$). These data demonstrate that OFQ/N induces desensitization of the HA-tagged μ receptor in HAμ-BE cells as it does in BE(2)-C and Flagμ-BE cells. With the availability of an antibody against the Ser^{375} phosphorylated form of the μ receptor, we were able to determine whether DAMGO-induced μ receptor phosphorylation occurred at that site using immunoblotting. Cells were treated with OFQ/N for 1 h; at the end of treatment, cells were extensively washed and challenged with DAMGO for 10 min. After DAMGO treatment, cells were lysed, and μ receptors were partially purified as described under *Materials and Methods*.

As shown in Fig. 8A, 10-min DAMGO treatment caused phosphorylation of the μ receptor at the Ser^{375} residue in HAμ-BE cells expressing wild-type HA-tagged μ receptors, whereas vehicle-treated cells did not show any phosphorylation at this site. Similar to vehicle-treated cells, 1-h OFQ/N treatment alone did not stimulate any phosphorylation at this residue. In OFQ/N-treated cells, 10-min DAM challenge enhanced phosphorylation of the μ receptor at the Ser^{375}

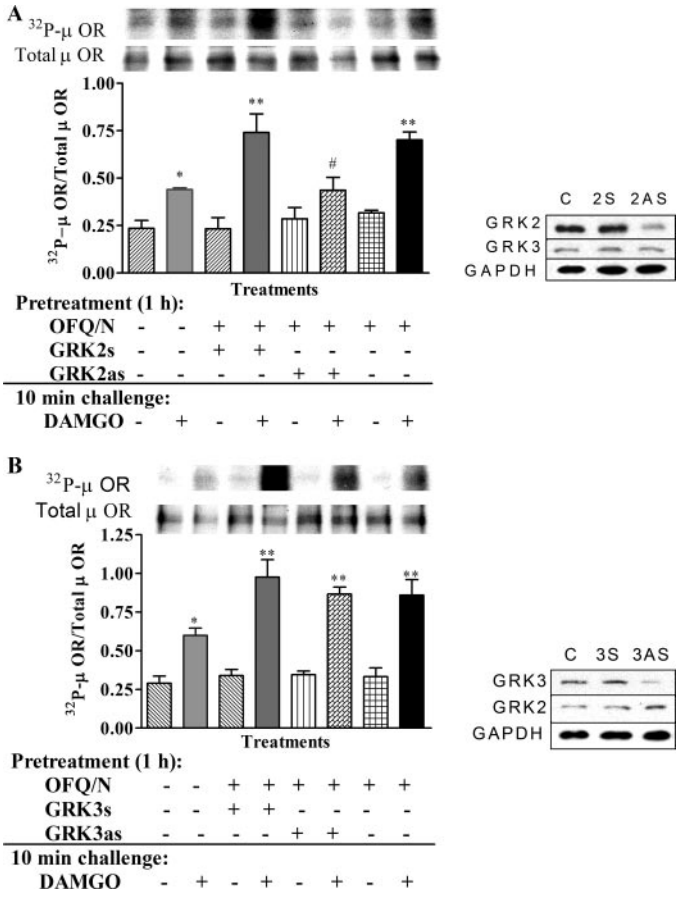

Fig. 5. OFQ/N-mediated enhancement of μ receptor phosphorylation involves GRK2 but not GRK3. Flagμ-BE cells were treated with GRK2 (A) or GRK3 (B) antisense or sense DNA in serum-free media before labeling with [^{32}Pi] and the addition of OFQ/N (100 nM) as described under *Materials and Methods*. After 1-h OFQ/N treatment, cells were washed with phosphate-free media and challenged with DAMGO (1 μM) for 10 min. Cell lysates were subjected to immunoprecipitation with anti-Flag M2 antibody as described under *Materials and Methods*. Left, top and bottom blots of each are representative autoradiography and immunoblots of the μ receptor, respectively. Data from immunoblots and autoradiography were quantified by densitometric analysis, and the ratio of the ^{32}P-Flagμ receptor band to total Flagμ receptor immunoreactivity was presented as the mean ± S.E.M. of four experiments. Right, representative blots showing depletion of GRK2 or GRK3 protein levels after GRK2- or GRK3-specific antisense DNA (2AS, 3AS), respectively, but not sense (2S, 3S) treatments. GRK2 antisense DNA treatment reduced GRK2 levels by 55 ± 8% without changing GRK3 levels, whereas GRK3 antisense DNA treatment reduced GRK3 levels by 73 ± 4% without changing GRK2 levels. α-Glyceraldehyde-3-phosphate dehydrogenase served as loading control. μ OR, μ-opioid receptor; *, $p < 0.05$ significantly different from vehicle-treated cells; **, $p < 0.05$ significantly different from DAMGO-challenged cells; #, $p < 0.05$ significantly different from OFQ/N-treated + DAMGO-challenged cells.

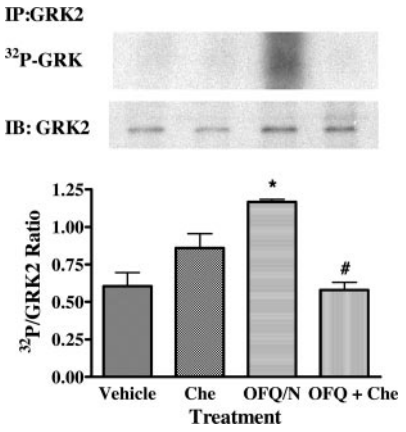

Fig. 6. OFQ/N-activated protein kinase C induces GRK2 phosphorylation in Flagμ-BE cells. Cells were preloaded with [^{32}Pi] for 2 h and then treated with OFQ/N (100 nM) in the absence and presence of chelerythrine (Che) (1 μM). GRK2 in preloaded and treated cells were immunoprecipitated with GRK2/3 common antibody preconjugated with protein-A agarose. The top blot is an autoradiogram showing phosphorylation levels of GRK2 after treatments. The bottom blot is representative of GRK2 from three independent experiments. Data from immunoblots and autoradiography were quantified by densitometric analysis, and the ratio of the phospho-protein to total GRK2 immunoreactivity was presented as mean ± S.E.M. of three experiments in the graph. OFQ/N treatment significantly increased GRK2 phosphorylation compared with vehicle-treated cells (⋆, $p < 0.05$), whereas inclusion of chelerythrine blocked the increase (#, $p < 0.05$).

TABLE 1

Characterization of the epitope-tagged wild-type (Flagμ and HAμ) and mutant (HA2Aμ and HA3Aμ) μ -opioid receptors stably expressed in BE(2)-C cells

Radioligand binding studies were performed on membrane preparations as explained under *Materials and Methods*. B_{max} values were determined by saturation binding with [^{3}H]DAMGO. The functional coupling of the receptors was determined by measuring the ability of DAMGO to inhibit forskolin-stimulated cAMP accumulation in intact cells. Data are expressed as the receptor number (B_{max}), DAMGO concentration producing 50% of the maximal response (EC_{50}), and the maximal response induced by 10 μ M DAMGO (% maximal inhibition). The values shown are means ± S.E.M. from at least two independent experiments.

μ Receptor-Expressing Cell Line	B_{max}	DAMGO EC_{50}	Maximal Inhibition
	fmol/mg protein	*log M*	*%*
Native BE(2)-C	33 ± 2.4	−7.6 ± 0.2	70 ± 6.4
Flagμ	560 ± 31	−8.0 ± 0.1	76 ± 4.2
HAμ	577 ± 19	−7.9 ± 0.1	86 ± 2.1
HA2Aμ	522 ± 32	−7.3 ± 0.3	94 ± 7.4
HA3Aμ	487 ± 40	−7.3 ± 0.1	95 ± 3.3

residue compared with DAMGO challenge in vehicle-treated cells. This is consistent with our previous results showing that depletion of GRK2 blocked OFQ/N-mediated enhancement of DAMGO-induced μ receptor phosphorylation. These data indicate that Ser^{375} is involved in OFQ/N-mediated enhancement of DAMGO-induced μ receptor phosphorylation.

To further test the role of the Ser^{375} residue, we performed the same experiment in HA3Aμ-BE cells, which express μ receptors lacking Ser^{375} as well as putative protein kinase C phosphorylation sites Ser^{363} and Thr^{370}. As shown in Fig. 8C, we did not detect the Ser^{375}-phosphorylated form of the μ receptor in the samples from any treatment group. This indicates that Ser^{375} is the primary site for DAMGO-induced μ receptor phosphorylation and that OFQ/N-mediated potentiation of DAMGO-induced μ receptor phosphorylation is, also, mediated by this residue. We also performed the same experiment in HA2Aμ-BE cells, which express μ receptors lacking putative protein kinase C phosphorylation sites but retaining the Ser^{375} residue. We observed the same pattern of the Ser^{375} phosphorylation in HA2Aμ-BE cells as in HAμ-BE cells expressing the wild-type μ receptors (Fig. 8, B and D), further confirming that the Ser^{375} residue is responsible for OFQ/N-mediated increase in DAMGO-induced μ receptor phosphorylation.

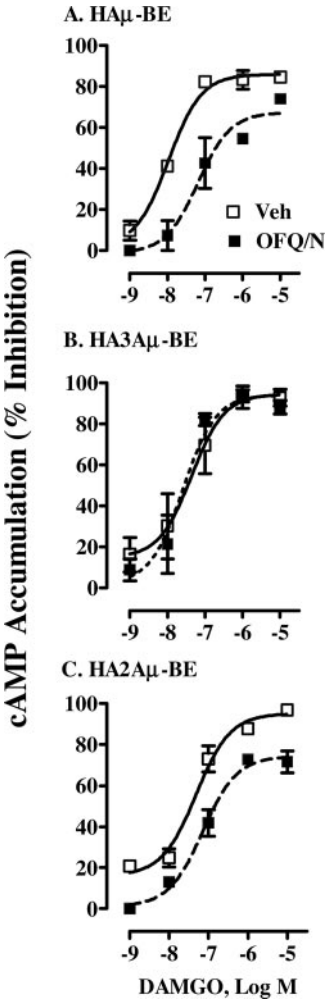

Fig. 7. The μ-opioid receptor lacking the Ser^{375} residue (HA3Aμ) does not undergo OFQ/N-induced heterologous μ receptor desensitization. Intact HAμ-BE (A), HA3Aμ-BE (B), and HA2Aμ-BE (C) cells were incubated with or without OFQ/N (100 nM) for 1 h. After pretreatment, cells were assayed for the ability of increased concentrations of DAMGO to inhibit forskolin (10 μM)-stimulated cAMP accumulation. At some points, error bars are smaller than symbols. Note that OFQ/N pretreatment reduced agonist potency (A; OFQ/N log EC_{50} −7.1 ± 0.2* versus vehicle log EC_{50} −7.9 ± 0.1) and efficacy in HAμ-BE cells. The same treatment did not change the agonist potency in HA2Aμ-BE (B; OFQ/N log EC_{50} −7.1 ± 0.1 versus vehicle log EC_{50} −7.3 ± 0.1) cells but did significantly reduce agonist efficacy. However, OFQ/N reduced neither potency (C; OFQ/N log EC_{50} −7.5 ± 0.2 versus vehicle log EC_{50} −7.3 ± 0.2) nor efficacy in HA3Aμ-BE cells. Data are expressed as mean ± S.E.M. of two to three experiments. *, $p < 0.05$ significantly different from vehicle-treated cells.

It is crucial to test whether there is a correlation between μ receptor phosphorylation and desensitization and whether the loss of the phosphorylation site causes a gain of function after OFQ/N treatment. To test these questions, we looked at whether 1-h OFQ/N treatment induces μ receptor desensitization in HA3Aμ-BE cells. We measured the ability of DAMGO to inhibit forskolin-stimulated cAMP accumulation in cells treated with or without OFQ/N for 1 h. As shown in Fig. 7B, OFQ/N did not induce μ receptor desensitization in HA3Aμcells. Dose-response curves from vehicle and OFQ/N-treated cells were superimposable, indicating that OFQ/N pretreatment in HA3Aμ-BE cells did not reduce the ability of DAMGO to inhibit forskolin-stimulated cAMP accumulation. To further explore the functional consequence of OFQ/N-mediated potentiation of DAMGO-induced μ receptor phosphorylation, HA2Aμ-BE cells, which express μ receptors lacking Ser^{363} and Thr^{370} sites and retaining the Ser^{375} site, were tested after OFQ/N treatment. As shown in Fig. 7C, OFQ/N was able to desensitize the μ receptor in HA2Aμ-BE cells. These data indicate that phosphorylation of the Ser^{375} site plays an important role in OFQ/N-induced μ receptor desensitization.

Discussion

In this study, we examined the ability of OFQ/N to modulate DAMGO-induced μ receptor phosphorylation. Our major finding is that activation of endogenously expressed NOP receptors by OFQ/N potentiated DAMGO-induced μ receptor phosphorylation via protein kinase C- and GRK2-dependent pathways. The effect of OFQ/N was mediated through the NOP receptor, because the NOP receptor antagonist peptide III-BTD but not the μ receptor antagonist NLX blocked OFQ/N-mediated increase in DAMGO-induced μ receptor phosphorylation. Inhibition of protein kinase C and GRK2 prevented OFQ/N from exerting this effect on DAMGO-induced phosphorylation of the μ receptor, but inhibition of GRK3 did not. In addition, μ receptors lacking the Ser^{375} residue displayed neither OFQ/N-mediated enhancement of DAMGO-induced μ receptor phosphorylation nor OFQ/N-induced μ receptor desensitization. These findings support the idea that homologous μ receptor desensitization can be regulated heterologously by OFQ/N via protein kinase C and GRK2.

Phosphorylation of GPCRs upon agonist activation is the initiation of the cascade leading to receptor desensitization, as modeled in the regulation of the β_2 adrenergic receptor. The role of receptor phosphorylation in μ receptor desensitization is not firmly established because some studies reported that rapid phosphorylation of the μ receptor (in minutes) did not correlate well with slow-paced desensitization (in hours) in some overexpression systems (El Kouhen et al., 1999). However, recent studies revealed that μ receptor de-

sensitization is determined by not only receptor phosphorylation and internalization but also recycling and resensitization of the receptor (Law et al., 2000). μ Receptor dephosphorylation and recycling offset phosphorylation and internalization, which results in the slow-paced desensitization of the μ receptor. Blocking μ receptor recycling or decreasing receptor number by an irreversible antagonist improves the correlation between receptor phosphorylation and desensitization in these systems (Law et al., 2000). In our cell lines, epitope-tagged μ receptors are expressed at the level of 500 fmol/mg (Table 1), which approximates the physiological ranges described for midbrain regions (Gomes et al., 2002). OFQ/N treatment for 1 h desensitizes the inhibitory cAMP response of the μ receptor by DAMGO in these cells. In addition, GRK2-mediated phosphorylation of the μ receptor contributes to OFQ/N-induced μ receptor desensitization, because depletion of GRK2 with antisense DNA treatment blocked OFQ/N-induced μ receptor desensitization. Furthermore, the μ receptor mutant (HA3Aμ) lacking the GRK2-mediated phosphorylation site (S375A) did not display OFQ/N-mediated enhancement of DAMGO-induced μ receptor phosphorylation. The same mutant also failed to undergo OFQ/N-induced μ receptor desensitization, indicating that μ receptor phosphorylation and desensitization are very well correlated in our model.

The mutant HA3Aμ receptors lack Ser^{363} and Thr^{370} residues as well as the Ser^{375} residue. The Ser^{363} site is responsible for basal μ receptor phosphorylation, whereas Thr^{370} can get phosphorylated in the presence and absence of the μ agonist DAMGO (El Kouhen et al., 2001). In our study, we looked specifically at OFQ/N-induced changes in DAMGO-induced phosphorylation at the Ser^{375} residue, because this site is the primary agonist-mediated phosphorylation site of the μ receptor (El Kouhen et al., 2001, Schulz et al., 2004). Our data suggest that OFQ/N enhancement of DAMGO-induced μ receptor phosphorylation at this site clearly contributes to OFQ/N-induced μ receptor desensitization.

GRK2 plays an important role in desensitization of the μ-opioid receptor. The present study demonstrates that depletion of GRK2 with GRK2-specific antisense DNA treatment blocked OFQ/N-induced heterologous desensitization as well as OFQ/N-mediated increase in DAMGO-induced phosphorylation. This is consistent with our previous study showing that 1-h OFQ/N treatment induced the translocation of GRK2 to the plasma membrane (Mandyam et al., 2002). Our previous study also revealed that acute DAMGO-induced μ receptor desensitization was blocked by inhibition of GRK2 in BE(2)-C cells (Mandyam et al., 2003). These results are consistent with studies showing that GRK2 is a major homologous regulator of μ receptor function (Zhang et al., 1996, 1998; Li and Wang, 2001). The Ser^{375} residue is the most likely phosphorylation target for GRK2 in our model, because HA3Aμ-BE cells responded to 1-h OFQ/N treatment in a way similar to that of GRK2-depleted Flagμ-BE cells (Figs. 5A and 8C). Depletion of GRK3 did not affect OFQ/N-induced μ receptor desensitization or phosphorylation (Figs. 1C and 5B). These results indicate that GRK3 is not involved in acute OFQ/N-induced μ receptor desensitization in BE(2)-C neuroblastoma cells.

Protein kinase C is a diacylglycerol-dependent kinase that phosphorylates its substrates at serine and threonine residues. There are 12 different isoforms of protein kinase C, which can be classified as conventional, novel, or atypical isoforms. Phorbol esters can activate both conventional and novel protein kinase C isoforms, and prolonged exposure to phorbol esters results in depletion of these protein kinase C isoforms. We reported previously that protein kinase C depletion after phorbol ester treatment blocked OFQ/N-induced μ receptor desensitization, indicating that conventional and/or novel protein kinase C isoforms are involved in OFQ/N-induced μ receptor desensitization in BE(2)-C cells (Mandyam et al., 2002). These cells have been reported to express protein kinase Cα, βII, δ, and ϵ (Zeidman et al., 1999), but we detected the translocation of only protein kinase Cα after OFQ/N treatment in our cells (Mandyam et al., 2002).

Protein kinase C seems to play a major role in heterologous desensitization of μ receptors in general, not only in this system. Protein kinase C indirectly enhanced morphine- or methionine-enkaphalin–induced desensitization through en-

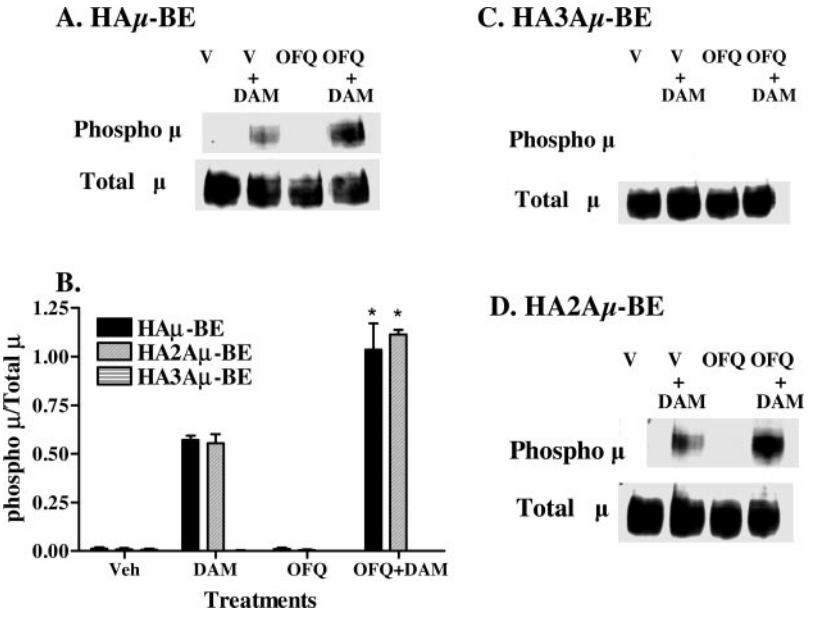

Fig. 8. Ser^{375} is responsible for OFQ/N-mediated enhancement in DAMGO-induced μ receptor phosphorylation. Immunoblots display the Ser^{375} phosphorylated form of the μ receptor protein (top blots) and total μ receptor protein (bottom blots) in HAμ-BE (A), HA3Aμ-BE (C), and HA2Aμ-BE cells (D). μ Receptor proteins in cells were partially purified with wheat germ lectin agarose and immunoblotted using phosphoSer^{375} antibody as described under *Materials and Methods*. After immunoblotting, the membrane was stripped and reprobed with anti-HA antibody. B, graphical representation of the immunoblots. Data from immunoblots were quantified by densitometric analysis, and the densitometric ratios of the phospho-μ receptor band to total μ receptor band were presented as mean ± S.E.M. of three experiments. ⋆, $p < 0.05$ significantly different from DAMGO challenge in vehicle-treated cells.

dogenous Gq-coupled M_3 muscarinic receptors in locus ceruleus neurons (Bailey et al., 2004). Activation of *N*-methyl-D-aspartate receptors desensitized the inhibitory cAMP response of μ receptors in primary cultures of mouse cortical neurons, which was blocked by the inhibition of protein kinase C (Fan et al., 1998). The blockade of *N*-methyl-D-aspartate receptor signaling also blocks the development of morphine tolerance (Pasternak et al., 1995). DAMGO-induced antinociception was attenuated in diabetic mice, in which the activity of protein kinase C was shown to be up-regulated (Ohsawa et al., 1999). The same study reported that the inhibition of protein kinase C by calphostin C significantly enhanced DAMGO-induced antinociception in diabetic mice. The authors suggested that increased phosphorylation of the μ receptor by protein kinase C in diabetic mice leads to a blunted response to DAMGO (Ohsawa et al., 1999). Likewise, pretreatment with chemokines that are known protein kinase C activators (Mueller et al., 1995) blunted the chemotactic response of the μ receptor in human monocytes by inducing heterologous μ receptor phosphorylation (Szabo et al., 2002); in vivo, pretreatment with chemokines blunted the analgesic effect of DAMGO administered into the periaqueductal gray of rats (Szabo et al., 2002).

Phorbol esters, activators of protein kinase C, phosphorylated the μ receptor in a dose- and time-dependent manner (Zhang et al., 1996). However, agonist-induced μ receptor phosphorylation was not blocked by protein kinase C inhibitors (Zhang et al., 1996), indicating that protein kinase C was not involved in homologous μ receptor phosphorylation. Instead, it plays a major role in heterologous μ receptor phosphorylation (Zhang et al., 1996). Our previous work indicated that OFQ/N-induced GRK2 translocation and μ receptor desensitization were blocked with protein kinase C inhibition (Mandyam et al., 2002). In our current study, treatment with the protein kinase C inhibitor chelerythrine blocked the OFQ/N-mediated increase in DAMGO-induced μ receptor phosphorylation and OFQ/N-mediated GRK2 phosphorylation (Fig. 6). Modulation of GRK2 activity by protein kinase C is a unique mechanism in heterologous GPCR regulation in which both homologous and heterologous regulatory mechanisms converge. For instance, phorbol ester treatment enhanced isoproterenol-induced β_2-adrenoceptor phosphorylation, which was mediated by GRK2, in human mononuclear leukocytes (Chuang et al., 1995). Activation of α_{1B} receptors in Chinese hamster ovary cells that are coupled to the G_q-phospholipase C pathway increased the content of GRK2 at the plasma membrane, indicating that protein kinase C phosphorylates GRK2 and translocates it to the plasma membrane (Winstel et al., 1996). Therefore, protein kinase C is able to enhance the phosphorylation of agonist-occupied receptors by increasing the GRK2 activity; our data indicate that this is the mechanism by which OFQ/N produces μ receptor desensitization. It may also explain M_3 muscarinic receptor-mediated heterologous enhancement of morphine and methionine-enkephalin–induced μ receptor desensitization observed by Bailey et al. (2004).

OFQ/N is well known for its opioid-opposing actions in the brain (Mogil et al., 1996; Calo et al., 1998). Animal studies revealed that OFQ/N not only opposes the analgesic actions of opioids but also contributes to the development of morphine tolerance and dependence (Tian and Han, 2000; Ueda et al., 2000). Increased OFQ/N synthesis and release is one of the counteradaptations in response to long-term morphine treatment (Yuan et al., 1999). Although OFQ/N actions in the neuronal circuitry of the descending analgesic pathway offset some of the analgesic effects of μ opioids, colocalization of μ and NOP receptors in these cells also seems to blunt μ receptor signaling at the molecular level. Perhaps the use of a NOP receptor antagonist during morphine administration may prevent or reduce the development of morphine tolerance and dependence and provide a novel therapeutic approach for pain management.

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Address correspondence to: Dr. Kelly M. Standifer, Department of Pharmacological and Pharmaceutical Sciences, University of Houston, 4800 Calhoun Road, 521 Science and Research Building 2, Houston, TX 77204-5037. E-mail: standifer@uh.edu