

## **Orphanin FQ/Nociceptin potentiates DAMGO-induced $\mu$ opioid receptor phosphorylation**

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**Abbreviations:** NOP, nociceptin opioid peptide; OFQ/N, orphanin FQ/nociceptin; DAMGO, [D-Ala<sup>2</sup>, N-Me-Phe<sup>4</sup>, Gly<sup>5</sup>-ol]-enkephalin; GRK, G protein-coupled receptor kinase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TBS/T, Tris-buffered saline/Tween.

## Abstract

The purpose of the present study was to investigate the molecular mechanisms by which acute orphanin FQ/Nociceptin (OFQ/N), acting through the nociceptin opioid peptide (NOP) receptor, desensitizes the  $\mu$  opioid receptor. We previously described the involvement of Protein kinase C and G-protein coupled receptor kinases (GRK) 2 and 3 in OFQ/N-induced  $\mu$  receptor desensitization. Since phosphorylation of the  $\mu$  receptor triggers the successive regulatory mechanisms responsible for desensitization such as receptor uncoupling, internalization and downregulation, we investigated the ability of OFQ/N to modulate DAMGO-induced  $\mu$  receptor phosphorylation in BE(2)-C human neuroblastoma cells transfected with epitope-tagged  $\mu$  receptors. OFQ/N treatment (100 nM, 60 min) potentiated DAMGO-induced  $\mu$  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, consistent with the potentiation being mediated via actions of the NOP receptor. In addition, in cells expressing  $\mu$  receptors in which the GRK-mediated phosphorylation site, Ser<sup>375</sup>, was mutated to Ala, OFQ/N treatment failed to potentiate DAMGO-induced  $\mu$  receptor phosphorylation and failed to desensitize the  $\mu$  receptor. However, DAMGO-induced  $\mu$  receptor phosphorylation and OFQ/N-induced  $\mu$  receptor desensitization occurred in cells expressing  $\mu$  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  $\mu$  receptor, heterologously regulating homologous  $\mu$  receptor desensitization.

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

Like many GPCRs, the  $\mu$  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 downregulation 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 where GRKs can phosphorylate agonist-occupied receptors (Lefkowitz et al., 1998). Several GRK2-mediated phosphorylation sites of the  $\mu$  receptor have been identified, but Ser<sup>375</sup> appears to be the primary site for DAMGO-induced  $\mu$  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, protein kinase C can also phosphorylate and activate GRK2 (Winstel et al., 1996; Chuang et al., 1995). 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  $\mu$  receptor

desensitization are well described, this is not the case for heterologously-mediated  $\mu$  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 CNS, and are colocalized with  $\mu$  receptors in many cells in the descending analgesic pathway (Connor et al., 1996; Heinricher et al., 1997; Pan et al., 2000). OFQ/N binds to NOP receptors and activates protein kinase C, which plays a role in homologous NOP receptor desensitization (Pei et al., 1997; Lou 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  $\mu$  receptor response to DAMGO (Hawes et al., 1998; Mandyam et al., 2000, 2001; Thakker and Standifer, 2002).

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

In the present study, we determined that OFQ/N potentiated DAMGO-induced  $\mu$  receptor phosphorylation via activation of GRK2 in BE(2)-C human neuroblastoma cells transfected with epitope-tagged  $\mu$  receptors. We further tested the role of GRK2 in OFQ/N-mediated potentiation of DAMGO-induced  $\mu$  receptor phosphorylation in cells expressing  $\mu$  receptors in which the GRK phosphorylation site, Ser<sup>375</sup>, 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 NIDA); [<sup>3</sup>H]-cAMP (Amersham Biosciences, Arlington Heights, IL); [<sup>32</sup>P]orthophosphate ([<sup>32</sup>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  $\mu$  receptor antibody (Research & Diagnostic antibodies, Benicia, CA); Chelerythrine (Research Biochemicals International, Natick, MA); phosphodiester oligodeoxynucleotides (Genosys, Inc., Woodlands, TX); Peptide III-BTD (Neosystem, Strasbourg, France); rat hemagglutinin-monoclonal antibody 3F10 (Roche Molecular Biochemicals); PhosphoSer<sup>375</sup>  $\mu$  receptor antibody (Cell Signaling Technology, Beverly, MA); wheat germ lectin-agarose beads (Amersham Pharmacia Biotech, Piscataway, NJ);  $\alpha$ -glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Chemicon International, Inc., Temecula, CA).

**Cells and Cell Culture:** Flag- or HA-tagged  $\mu$  receptors subcloned into the expression vector pcDNA3 were generous gifts of Dr. Lakshmi Devi (Mount Sinai School of Medicine, NY, NY) and Dr. Ping-Yee Law (University of Minnesota, Minneapolis, MN), respectively. To ensure effective immunoprecipitation of  $\mu$  receptors, BE(2)-C cells were stably transfected with the pcDNA3 vector expressing flag-tagged or HA-tagged  $\mu$  receptors using FuGene reagent. Geneticin (800  $\mu$ g/ml) was used as the selection drug. Clones were screened for  $\mu$  receptor expression levels using 0.01-5 nM [<sup>3</sup>H]-DAMGO as described previously (Mandyam et al., 2000). Clones with approximately 500 fmol/mg  $\mu$  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 ≥70% confluence that were lifted from plates with PBS (phosphate-buffered saline, pH 7.4) containing 1 mM EGTA.

**Pretreatment conditions:** Transfected BE(2)-C cells were treated with 100 nM OFQ/N for 1 hr 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 (1µM) was added to the plates 15 min prior to addition of OFQ/N. Antagonist treatments with Naloxone (100 nM) or Peptide III-BTD (10 µM; Thakker and Standifer, 2002) were performed 15 min prior to and during the OFQ/N treatment. In the experiments involving DAMGO challenge, the treatment media was 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 hr; Aiyar et al., 2000) or GRK3 antisense DNA, 5'-TCC AGT GTC TGC TTT CCT-3', (1µM; 48 hr) as described (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 Hank's 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 13,000g; and the supernatants were used to determine cAMP levels in a [<sup>3</sup>H]-cAMP-binding assay as previously described (Mandyam et al., 2000).

**μ Receptor Phosphorylation:** BE(2)-C cells transfected with flag-tagged μ receptors (Flagμ-BE cells) were grown to 80-90% confluency in 100 mm<sup>2</sup> 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 hr. This was followed by addition of [<sup>32</sup>Pi] (150 μCi/ml) into the culture medium. Cells were labeled with [<sup>32</sup>Pi] for 2 hr at 37°C. After labeling, OFQ/N was added to the plates for 1 hr. 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<sup>+</sup> buffer (1% IGEPAL CA-630, 0.5% Na<sub>2</sub> deoxycholate, 0.1% SDS, 5 mM EDTA, 10 mM NaF, 10 mM Na<sub>2</sub> 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 150,000g for 15 min at 4°C, and the supernatant was used for further analysis. After pre-clearing with protein G agarose, the supernatant was incubated with 10 μg 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<sup>+</sup> 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 electrophoretically transferred onto polyvinylidene fluoride membrane. Autoradiography of the membrane was used for quantifying  $\mu$  receptor phosphorylation. The same membrane was used for immunoblotting the  $\mu$  receptor with mouse monoclonal anti-FLAG M2 HRP-conjugated antibody (1:100) or rabbit polyclonal anti- $\mu$  (1:500) antibody. The ratio of the  $^{32}\text{P}$ - $\mu$  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 were shown in figures.

**GRK2/3 phosphorylation:** Flag $\mu$ -BE cells were labeled with [ $^{32}\text{P}$ ] (350  $\mu\text{Ci/ml}$ ) and then treated with vehicle, OFQ/N (100 nM), chelerythrine (1 $\mu\text{M}$ ) or the combination of OFQ/N and chelerythrine as described above. After terminating the treatments by washing with cold PBS, cells were lysed with RIPA<sup>+</sup> buffer. For immunoprecipitation, an antibody recognizing both GRK2 and GRK3 (1  $\mu\text{g}$ ; H-222, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was pre-conjugated 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, Inc., Santa Cruz, CA) 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  $\mu$  receptor protein and Western Blotting:** After pretreatments, BE(2)-C cells transfected with HA-tagged  $\mu$  receptors (HA $\mu$ -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 PMSF, 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 X 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 electrophoretically transferred onto PVDF membrane. Membranes were blocked with TBST (Tris-buffered saline/Tween 20, 0.05%) containing 5% non-fat dried milk for 1 hr and incubated with rabbit phosphoSer<sup>375</sup> antibody (1:1000) overnight at 4°C, followed by 1 hr-anti rabbit secondary antibody (1:2000) incubation. After detection of the Ser<sup>375</sup> 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 hr-anti-rat (1:2000) secondary antibody incubation. The densitometric ratio of the phosphoSer<sup>375</sup> µ receptor protein band to total µ receptor protein band was calculated to detect changes in DAMGO-induced µ receptor phosphorylation after 1 hr OFQ/N pretreatment.

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

**Data analysis:** LogEC<sub>50</sub> values were determined using non-linear regression analysis. Data were expressed as 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 GraphPad Prism version 4.00 for Windows 95/98 (GraphPad Software Inc., San Diego, CA). Data were considered significant if p<0.05.

## Results

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

To test the roles of GRK2 and GRK3 in OFQ/N-induced  $\mu$  receptor desensitization in Flag $\mu$ -BE cells, we utilized GRK isoform specific antisense DNA treatment, which we previously reported selectively reduces levels of GRK2 or GRK3 (Thakker and Standifer, 2002; Mandyam et al., 2003). OFQ/N-induced  $\mu$  receptor desensitization was attenuated in cells pretreated with GRK2 antisense, but not GRK2 sense DNA (Fig. 1B). OFQ/N treatment reduced

DAMGO efficacy ( $61.91 \pm 7.9\%$ ) compared to control DAMGO efficacy ( $72.8 \pm 3.9\%$ ;  $n=2$ ,  $*p<0.05$ ). Inhibition of GRK2 blocked OFQ/N-induced reduction in the efficacy of DAMGO to inhibit forskolin-stimulated cAMP accumulation ( $74 \pm 3.0\%$ ;  $n=2$ ,  $\#p<0.05$ ). In contrast, GRK3 antisense DNA treatment had no effect (Fig. 1C). These experiments assured us that the flag $\mu$  receptors in the Flag $\mu$ -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  $\mu$  receptor by OFQ/N.

Expressing flag-epitope-tagged  $\mu$  receptors enabled us to immunoprecipitate the  $\mu$  receptor with an antibody raised against the flag epitope. In immunoblots, flag-tagged  $\mu$  receptors from Flag $\mu$ -BE cells appear as the band at 65-70 Kda as reported in other systems (Zhang et al., 1996); samples from untransfected BE(2)-C cells lack this band (Fig 2A). The  $\mu$  receptor protein shown at this band underwent a robust DAMGO-induced phosphorylation indicating that the 65-70 kDa band, not the other bands in the immunoblots, represents the fully glycosylated and functional  $\mu$  receptor protein (Fig. 2B), as previously shown by many others (e.g., El-Kouhen et al., 1999; Zhang et al., 1996). The same band was detected using a rabbit anti- $\mu$  opioid receptor polyclonal antibody raised against a peptide sequence of the carboxyl terminal of the  $\mu$  receptor as with the mouse Flag M2 monoclonal antibody-peroxidase conjugate raised against the flag epitope (Fig. 2A). Immunoblotting was performed with both antibodies to confirm the effect, but analysis of only the anti-flag M2 antibody data is included.

OFQ/N-induced  $\mu$  receptor desensitization was measured as the ability of the  $\mu$  opioid receptor agonist DAMGO to inhibit adenylyl cyclase activity for a 10 min period following a one hr treatment with OFQ/N. This 10 min exposure to DAMGO during the measurement of  $\mu$  receptor response is enough to activate rapid regulatory mechanisms leading to homologous  $\mu$  receptor desensitization such as activation of GRKs and phosphorylation of the  $\mu$  receptor

(Zhang et al., 1996). To determine whether one hr OFQ/N treatment enhances DAMGO-induced  $\mu$  receptor phosphorylation via this pathway, cells were labeled with [ $^{32}$ Pi] and treated with OFQ/N for 1 hr. After that period, cells were washed and challenged with the  $\mu$  agonist DAMGO for 10 min, then flag $\mu$  receptors were immunoprecipitated as described in Methods. One hr OFQ/N treatment significantly increased DAMGO-induced  $\mu$  receptor phosphorylation (Fig. 3). However, receptor phosphorylation was not noted after 1 hr OFQ/N in the absence of DAMGO challenge. DAMGO treatment alone induced three-fold increase in  $\mu$  receptor phosphorylation compared to vehicle- treated cells. DAMGO challenge in OFQ/N-treated cells increased  $\mu$  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  $\mu$  receptor phosphorylation is mediated directly through  $\mu$  receptors, cells were treated with OFQ/N in the presence and absence of the NOP receptor antagonist, Peptide III-BTD (BTD; 10  $\mu$ M), or the  $\mu$  antagonist Naloxone (NLX; 100 nM) only during the 1 hr OFQ/N preincubation period, not during the DAMGO challenge period. The NOP receptor antagonist completely reversed the enhancement of DAMGO-induced  $\mu$  receptor phosphorylation while NLX had no effect (Fig. 3).

Because protein kinase C is involved in heterologous desensitization of the  $\mu$  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  $\mu$  receptor phosphorylation. Flag $\mu$ -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, Flag $\mu$ -BE cells were washed and challenged with DAMGO. In the presence of Chelerythrine, OFQ/N did not potentiate DAMGO-induced  $\mu$  receptor phosphorylation (Fig. 4). Chelerythrine alone did not significantly alter basal or DAMGO-induced phosphorylation of the  $\mu$  receptor.

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

As direct phosphorylation of GRK2 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 Flag $\mu$  -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 over 2-fold compared to that in vehicle treated cell (Fig. 6; \* $p < 0.05$ ;  $n = 3$ ). OFQ/N-induced increase in GRK2 phosphorylation was blocked by Chelerythrine (Fig. 6; # $p < 0.05$ ;  $n = 3$ ).

Our data suggest that one hr OFQ/N pretreatment leads to an increase in the ability of GRK2 to phosphorylate the agonist bound  $\mu$  receptor. Ser<sup>375</sup> is the primary site for GRK-mediated  $\mu$  receptor phosphorylation because it gets phosphorylated only in the presence of a  $\mu$  agonist (El Kouhen et al., 2001; Schulz et al., 2004). We hypothesized that the Ser<sup>375</sup> residue is responsible for OFQ/N enhancement of DAMGO-induced  $\mu$  receptor phosphorylation. To study this hypothesis, we tested two different mutant  $\mu$  receptors; HA3A $\mu$ , in which three phosphorylation sites (Ser<sup>363</sup>, Thr<sup>370</sup> and Ser<sup>375</sup>) were mutated to Ala, and HA2A $\mu$ , in which two phosphorylation sites (Ser<sup>363</sup> and Thr<sup>370</sup>) were mutated to Ala. We employed these mutants because the mutant HA3A $\mu$  lacks Ser<sup>375</sup>, the putative GRK phosphorylation site, whereas the mutant HA2A $\mu$  retains that site so that we could determine the role of Ser<sup>375</sup>. Both mutants lack putative protein kinase C phosphorylation sites (Ser<sup>363</sup> and Thr<sup>370</sup>) 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 HA3A $\mu$ , HA2A $\mu$  or wild type HA-tagged  $\mu$  receptor (HA $\mu$ ). HA $\mu$ -BE cells, HA3A $\mu$ -BE cells and HA2A $\mu$ -BE cells refer to BE(2)-cells transfected with the HA $\mu$ , HA3A $\mu$  and HA2A $\mu$  receptors, respectively. Clones were selected that expressed the  $\mu$  receptor in levels similar to those in Flag $\mu$ -BE cells. The values of the B<sub>max</sub>, EC<sub>50</sub> and % inhibition of cAMP accumulation induced by 10  $\mu$ M DAMGO for all cell lines are shown in Table 1. No difference was observed among the wild type and mutant  $\mu$  receptors neither in terms of their ability to inhibit forskolin-stimulated cAMP accumulation, nor levels of receptor expression.

First, we tested the ability of OFQ/N to desensitize the wild type HA-tagged  $\mu$  receptor. As shown in Fig. 7A, the ability of DAMGO to inhibit cAMP accumulation was reduced in OFQ/N-treated cells ( $67.39 \pm 5.6\%$ ) compared to vehicle-treated cells ( $85 \pm 2.1\%$ ; n=2, \*p<0.05). This

data demonstrates that OFQ/N induces desensitization of the HA-tagged  $\mu$  receptor in HA $\mu$ -BE cells as it does in BE(2)-C and Flag $\mu$ -BE cells. With the availability of an antibody against the Ser<sup>375</sup> phosphorylated form of the  $\mu$  receptor, we were able to determine whether DAMGO-induced  $\mu$  receptor phosphorylation occurred at that site using immunoblotting. Cells were treated with OFQ/N for 1 hr; at the end of treatment, cells were extensively washed and challenged with DAMGO for 10 min. After DAMGO treatment, cells were lysed and  $\mu$  receptors were partially purified as explained in Methods.

As shown in Fig. 8A, 10 min DAMGO treatment caused phosphorylation of the  $\mu$  receptor at the Ser<sup>375</sup> residue in HA $\mu$ -BE cells expressing wild type HA-tagged  $\mu$  receptors whereas vehicle-treated cells did not show any phosphorylation at this site. Similar to vehicle-treated cells, 1 hr 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  $\mu$  receptor at the Ser<sup>375</sup> residue compared to 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  $\mu$  receptor phosphorylation. This data indicates that Ser<sup>375</sup> is involved in OFQ/N-mediated enhancement of DAMGO-induced  $\mu$  receptor phosphorylation.

To further test the role of the Ser<sup>375</sup> residue, we performed the same experiment in HA3A $\mu$ -BE cells, which express  $\mu$  receptors lacking Ser<sup>375</sup> as well as putative protein kinase C phosphorylation sites Ser<sup>363</sup> and Thr<sup>370</sup>. As shown in Fig. 8C, we did not detect Ser<sup>375</sup>-phosphorylated form of the  $\mu$  receptor in the samples from any treatment group. This indicates that Ser<sup>375</sup> is the primary site for DAMGO-induced  $\mu$  receptor phosphorylation and that OFQ/N-mediated potentiation of DAMGO-induced  $\mu$  receptor phosphorylation is, also, mediated by this



residue. We also performed the same experiment in HA2A $\mu$ -BE cells, which express  $\mu$  receptors lacking putative protein kinase C phosphorylation sites, but retaining the Ser<sup>375</sup> residue. We observed the same pattern of the Ser<sup>375</sup> phosphorylation in HA2A $\mu$ -BE cells as in HA $\mu$ -BE cells expressing the wild type  $\mu$  receptors (Fig. 8B and D) further confirming that the Ser<sup>375</sup> residue is responsible for OFQ/N-mediated increase in DAMGO-induced  $\mu$  receptor phosphorylation.

It is crucial to test whether there is a correlation between  $\mu$  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 hr OFQ/N treatment induces  $\mu$  receptor desensitization in HA3A $\mu$ -BE cells. We measured the ability of DAMGO to inhibit forskolin-stimulated cAMP accumulation in cells treated with or without OFQ/N for 1 hr. As shown in Fig. 7B, OFQ/N did not induce  $\mu$  receptor desensitization in HA3A $\mu$  cells. Dose response curves from vehicle and OFQ/N-treated cells were superimposable indicating that OFQ/N pretreatment in HA3A $\mu$ -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  $\mu$  receptor phosphorylation, HA2A $\mu$ -BE cells, which express  $\mu$  receptors lacking Ser<sup>363</sup> and Thr<sup>370</sup> sites and retaining the Ser<sup>375</sup> site, were tested after OFQ/N treatment. As shown in Fig. 7C, OFQ/N was able to desensitize the  $\mu$  receptor in HA2A $\mu$ -BE cells. This data indicates that phosphorylation of the Ser<sup>375</sup> site plays an important role in OFQ/N-induced  $\mu$  receptor desensitization.

## Discussion

In this study, we examined the ability of OFQ/N to modulate DAMGO-induced  $\mu$  receptor phosphorylation. Our major finding is that activation of endogenously expressed NOP receptors by OFQ/N potentiated DAMGO-induced  $\mu$  receptor phosphorylation via protein kinase C- and GRK2-dependent pathways. The effect of OFQ/N was mediated through the NOP receptor since the NOP receptor antagonist, Peptide III-BTD, but not the  $\mu$  receptor antagonist NLX, blocked OFQ/N-mediated increase in DAMGO-induced  $\mu$  receptor phosphorylation. Inhibition of protein kinase C as well as GRK2 prevented OFQ/N from exerting this effect on DAMGO-induced phosphorylation of the  $\mu$  receptor, but inhibition of GRK3 did not. In addition,  $\mu$  receptors lacking the Ser<sup>375</sup> residue neither displayed OFQ/N-mediated enhancement of DAMGO-induced  $\mu$  receptor phosphorylation nor OFQ/N-induced  $\mu$  receptor desensitization. These findings support the idea that homologous  $\mu$  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  $\beta_2$  adrenergic receptor. The role of receptor phosphorylation in  $\mu$  receptor desensitization is not firmly established since some studies reported that rapid phosphorylation of the  $\mu$  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  $\mu$  receptor desensitization is determined not only by receptor phosphorylation and internalization, but also by recycling and resensitization of the receptor (Law et al., 2000).  $\mu$  receptor dephosphorylation and recycling offset phosphorylation and internalization, which results in the slow-paced desensitization of the  $\mu$  receptor. Blocking  $\mu$  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  $\mu$  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 hr desensitizes the inhibitory cAMP response of the  $\mu$  receptor by DAMGO in these cells. In addition, GRK2-mediated phosphorylation of the  $\mu$  receptor contributes to OFQ/N-induced  $\mu$  receptor desensitization since depletion of GRK2 with antisense DNA treatment blocked OFQ/N-induced  $\mu$  receptor desensitization. Furthermore, the  $\mu$  receptor mutant (HA3A $\mu$ ) lacking the GRK2-mediated phosphorylation site (S375A) did not display OFQ/N-mediated enhancement of DAMGO-induced  $\mu$  receptor phosphorylation. The same mutant also failed to undergo OFQ/N-induced  $\mu$  receptor desensitization indicating  $\mu$  receptor phosphorylation and desensitization are very well correlated in our model.

The mutant HA3A $\mu$  receptors lack Ser<sup>363</sup> and Thr<sup>370</sup> residues as well as the Ser<sup>375</sup> residue. The Ser<sup>363</sup> site is responsible for basal  $\mu$  receptor phosphorylation whereas Thr<sup>370</sup> can get phosphorylated in the presence and absence of the  $\mu$  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<sup>375</sup> residue since this site is the primary agonist-mediated phosphorylation site of the  $\mu$  receptor (El Kouhen et al., 2001, Schulz et al., 2004). Our data suggests that OFQ/N enhancement of DAMGO-induced  $\mu$  receptor phosphorylation at this site clearly contributes to OFQ/N-induced  $\mu$  receptor desensitization.

GRK2 plays an important role in desensitization of the  $\mu$  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 hr OFQ/N

treatment induced translocation of GRK2 to the plasma membrane (Mandyam et al., 2002). Our previous study also revealed that acute DAMGO-induced  $\mu$  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  $\mu$  receptor function (Zhang et al., 1996; Zhang et al., 1998; Li & Wang 2001). The Ser<sup>375</sup> residue is the likeliest phosphorylation target for GRK2 in our model because HA3A $\mu$ -BE cells responded to one hr OFQ/N treatment in a similar way to GRK2-depleted Flag $\mu$ -BE cells (Fig. 5A and 8C). Depletion of GRK3 did not affect OFQ/N-induced  $\mu$  receptor desensitization or phosphorylation (Fig. 1C and 5B). These results indicate that GRK3 is not involved in OFQ/N-induced  $\mu$  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 classical, novel or atypical isoforms. Phorbol esters can activate both classical and novel protein kinase C isoforms, and prolonged exposure to phorbol esters results in depletion of these protein kinase C isoforms. We previously reported that protein kinase C-depletion following phorbol ester treatment blocked OFQ/N-induced  $\mu$  receptor desensitization, indicating that classical and/or novel protein kinase C isoforms are involved in OFQ/N-induced  $\mu$  receptor desensitization in BE(2)-C cells (Mandyam et al., 2002). These cells have been reported to express protein kinase C $\alpha$ ,  $\beta$ II,  $\delta$  and  $\epsilon$  (Zeidman et al., 1999), but we detected translocation of only protein kinase C $\alpha$  after OFQ/N treatment in our cells (Mandyam et al., 2002).

Protein kinase C seems to play a major role in heterologous desensitization of  $\mu$  receptors in general, not only in this system. PKC indirectly enhanced morphine- or met enkephalin-induced desensitization through endogenous Gq-coupled M<sub>3</sub> muscarinic receptors in locus

ceruleus neurons (Bailey et al., 2004). Activation of NMDA receptors desensitized the inhibitory cAMP response of  $\mu$  receptors in primary cultures of mouse cortical neurons, which was blocked by inhibition of protein kinase C (Fan et al., 1998). Blockade of NMDA 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 upregulated (Ohsawa et al., 1999). The same study reported that inhibition of protein kinase C by Calphostin C significantly enhanced DAMGO-induced antinociception in diabetic mice. The authors suggested that increased phosphorylation of the  $\mu$  receptor by protein kinase C in diabetic mice leads to a blunted response to DAMGO (Ohsawa et al., 1999). Similarly, pretreatment with chemokines that are known protein kinase C activators (Mueller et al., 1995) blunted the chemotactic response of the  $\mu$  receptor in human monocytes by inducing heterologous  $\mu$  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  $\mu$  receptor in a dose- and time-dependent manner (Zhang et al., 1996). However, agonist-induced  $\mu$  receptor phosphorylation was not blocked by protein kinase C inhibitors (Zhang et al., 1996), indicating that protein kinase C was not involved in homologous  $\mu$  receptor phosphorylation. Instead, it plays a major role in heterologous  $\mu$  receptor phosphorylation (Zhang et al., 1996). Our previous work indicated that OFQ/N-induced GRK2 translocation and  $\mu$  receptor desensitization was blocked with PKC 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  $\mu$  receptor phosphorylation as well as OFQ/N-mediated GRK2 phosphorylation (Fig. 6).

Modulation of GRK2 activity by protein kinase C is a very unique mechanism in heterologous GPCR regulation, in which both homologous and heterologous regulatory mechanisms converge. For instance, phorbol ester treatment enhanced isoproterenol-induced  $\beta_2$  adrenoceptor phosphorylation, which was mediated by GRK2, in human mononuclear leukocytes (Chuang et al., 1995). Activation of  $\alpha_{1B}$  receptors in CHO cells that are coupled to the  $G_q$ -PLC 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 phosphorylation of agonist-occupied receptors by increasing the GRK2 activity; our data indicate that this is the mechanism by which OFQ/N produces  $\mu$  receptor desensitization. It may also explain  $M_3$  muscarinic receptor-mediated heterologous enhancement of morphine and met-enkephalin induced  $\mu$  receptor desensitization 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 counter-adaptations in response to chronic morphine treatment (Yuan et al., 1999). While OFQ/N actions in the neuronal circuitry of the descending analgesic pathway offset some of the analgesic effects of  $\mu$  opioids, colocalization of  $\mu$  and NOP receptors in these cells also appear to blunt  $\mu$  receptor signaling at the molecular level. Perhaps 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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## References

- Aiyar N, Disa J, Dang K, Pronin AN, Benovic JL and Nambi P (2000) Involvement of G protein-coupled receptor kinase-6 in desensitization of CGRP receptors. *Eur J Pharmacol* 403:1-7.
- Bailey CP, Kelly E and Henderson G (2004) Protein Kinase C activation enhances morphine-induced rapid desensitization of  $\mu$ -opioid receptors in mature rat locus ceruleus neurons. *Mol Pharmacol* 66:1592-1598.
- Calo' G, Rizzi A, Marzola G, Guerrini R, Salvadori S, Beani L, Regoli D and Bianchi C (1998) Pharmacological characterization of the nociceptin receptor mediating hyperalgesia in the mouse tail withdrawal assay. *Br J Pharmacol* 125:373-378.
- Chuang TT, LeVine H and De Blasi (1995) Phosphorylation and activation of  $\beta$ -adrenergic receptor kinase by protein kinase C. *J Biol Chem* 270:18660-18665.
- Connor M, Vaughan CW, Chieng B and Christie MJ.(1996) Nociceptin receptor coupling to a potassium conductance in rat locus coeruleus neurons in vitro. *Br J Pharmacol* 119:1614-1618.
- El Kouhen R, Maestri-El Kouhen O, Law PY and Loh HH (1999) The absence of a direct correlation between the loss of DAMGO inhibition of adenylyl cyclase activity and agonist-induced  $\mu$ -opioid receptor phosphorylation. *J Biol Chem* 274: 9207-9215.
- El Kouhen R, Burd AL, Erickson-Herbrandson LJ, Chang CY, Law PY and Loh HH (2001) Phosphorylation of Ser<sup>363</sup>, Thr<sup>370</sup>, and Ser<sup>375</sup> residues within the Carboxyl tail differentially regulates  $\mu$  opioid receptor internalization. *J Biol Chem* 276:12774-12780.
- Fan GH, Zhao J, Wu YL, Lou LG, Zhang Z, Jing Q, Ma L and Pei G (1998) N-Methyl-D-Aspartate attenuates opioid receptor-mediated G protein activation and this process involves protein kinase C. *Mol Pharmacol* 53:684-690.



- Gomes BA, Shen J, Stafford K, Patel M and Yoburn BC (2002)  $\mu$ -opioid receptor down-regulation and tolerance are not equally dependent upon G-protein signaling. *Pharmacol Biochem Behav* 69:233-237.
- Harrison LM, Kastin AJ and Zadina JE (1998) Opiate tolerance and dependence: Receptors, G-proteins and antiopiates. *Peptides* 19:1603-1630.
- Hawes BE, Fried S, Yao X, Weig B and Graziano MP (1998) Nociceptin (ORL-1) and  $\mu$ -opioid receptors mediate mitogen-activated protein kinase activation in CHO cells through a  $G_i$  coupled signaling pathway: evidence for distinct mechanisms of agonist-mediated desensitization. *J Neurochem* 71:1024-1033.
- Heinricher MM, Morgan MM, Tortorici V and Fields HL (1994) Disinhibition off-cells and antinociception produced by an opioid action within the rostral ventromedial medulla. *Neuroscience* 63:279-288.
- Horie, K and Paul A. Insel (2000) Retrovirally Mediated Transfer of a G Protein-coupled Receptor Kinase (GRK) Dominant-negative Mutant Enhances Endogenous Calcitonin Receptor Signaling in Chinese Hamster Ovary Cells. GRK Inhibition Enhances Expression of Receptors and Receptor mRNA. *J Biol Chem* 275:29433-29440.
- Koch T, Schulz S, Pfeiffer M, Klutzny M, Schroder H, Kahl E and Hollt V (2001) C-terminal Splice Variants of the mouse  $\mu$ -opioid receptor differ in morphine-induced internalization and receptor resensitization. *J Biol Chem* 276:31408-31414.
- Law P, Erickson LJ, El-Kouhen R, Dicker L, Solberg J, Wang W, Miller E, Burd AL and Loh HH (2000) Receptor Density and Recycling Affect the Rate of Agonist-Induced Desensitization of  $\mu$ -Opioid Receptor. *Mol Pharmacol* 58:388-398.

- Lefkowitz RJ, Pitcher J, Krueger K and Daaka Y (1998) Mechanisms of  $\beta$ -adrenergic receptor desensitization and resensitization. *Adv Pharmacol* 42:416-420.
- Li AH and Wang HL (2001) G protein-coupled receptor kinase 2 mediates mu-opioid receptor desensitization in GABAergic neurons of the nucleus raphe magnus. *J Neurochem* 77:435-444.
- Lou LG, Ma L and Pei G (1997) Nociceptin/Orphanin FQ activates protein kinase C, and this effect is mediated through Phospholipase C/Ca<sup>2+</sup> pathway. *Biochem Biophys Res Com* 240:304-308.
- Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265-275.
- Mandyam CD, Altememi GF and Standifer KM (2000)  $\beta$ -Funaltrexamine inactivates ORL1 receptors in BE(2)-C human neuroblastoma cells. *Eur J Pharmacol* 402:205–207.
- Mandyam CD, Thakker DR, Christensen JL and Standifer KM (2002) Orphanin FQ/Nociceptin-Mediated Desensitization of Opioid Receptor-Like 1 Receptor and  $\mu$  Opioid Receptors Involves Protein kinase C: A Molecular Mechanism for Heterologous Cross-Talk. *J Pharmacol Exp Ther* 302:502-509.
- Mandyam CD, Thakker DR and Standifer KM (2003) Micro-opioid-induced desensitization of opioid receptor-like 1 and micro-opioid receptors: differential intracellular signaling determines receptor sensitivity. *J Pharmacol Exp Ther* 306:965-72.
- Mogil JS, Grisel JE, Zhang G, Belknap JK and Grandy DK (1996) Functional antagonism of  $\mu$ -,  $\delta$ -, and  $\kappa$ -opioid antinociception by orphanin FQ. *Neurosci Lett* 214:131-134.

- Mueller SG, Schraw WP and Richmond A (1995) Activation of Protein kinase C enhances the phosphorylation of the type B Interleukin-8 receptor and stimulates its degradation in Non-hematopoietic cells. *J Biol Chem* 270:10439-10448.
- Ohsawa M, Tanaka S and Kamei J (1999) Possible involvement of spinal protein kinase C in thermal allodynia and hyperalgesia in diabetic mice. *Eur J Pharmacol* 373:181-186.
- Pan ZZ, Hirakawa N and Fields HL (2000) A cellular mechanism for the bi-directional pain modulating actions of orphanin FQ/nociceptin. *Neuron* 26:515-522.
- Pasternak GW, Kolesnikov YA and Babey AM (1995) Perspectives on the N-Methyl-D-Aspartate/Nitric Oxide cascade and opioid tolerance. *Neuropsychopharmacology* 13:309-313.
- Pei G, Ling K, Pu L, Cunningham MD and Ma L (1997) Nociceptin/orphanin FQ stimulates extracellular acidification and desensitization of the response involves protein kinase C. *FEBS Lett* 412:253-256.
- Pu L, Bao GB, Ma L and Pei G (1999) Acute desensitization of nociceptin /orphanin FQ inhibition of voltage-gated calcium channels in freshly dissociated hippocampal neurons. *Eur J Neurosci* 11:3610-3616.
- Schulz S, Mayer D, Pfeiffer M, Stumm R, Koch T and Holtt V (2004) Morphine induces terminal mu-opioid receptor desensitization by sustained phosphorylation of serine-375. *EMBO J* 23:3282-3289.
- Szabo I, Chen XH, Li X, Adler MW, Howard OMZ, Oppenheim JJ and Rogers T (2002) Heterologous desensitization of opioid receptors by chemokines inhibits chemotaxis and enhances the perception of pain. *Proc Natl Acad USA*. 99:10276-10281.

- Thakker DR and Standifer KM (2002) Induction of G protein-coupled receptor kinases 2 and 3 contributes to the cross-talk between opioid receptor-like 1 and  $\mu$  receptors following prolong agonist exposure. *Neuropharmacology* 43:979-990.
- Tian JH and Han JS. (2000) Functional studies using antibodies against orphanin FQ/nociceptin. *Peptides* 21:1047-1050.
- Ueda H, Inoue M, Takeshima H and Iwasawa Y (2000) Enhanced spinal nociceptin receptor expression develops morphine tolerance and dependence. *J Neurosci* 20:7640-7647.
- Winstel R, Freund S, Krasel C, Hoppe E and Lohse MJ (1996) Protein kinase Cross-talk: Membrane targeting of the  $\beta$ -adrenergic receptor kinase by protein kinase C. *Proc. Natl. Acad. Sci. USA* 93:2105-2109.
- Yuan L, Han Z, Chang JK and Han JS (1999) Accelerated release and production of orphanin FQ in brain of chronic morphine tolerant rats. *Brain Res* 826:330-334.
- Zeidman R, Pettersson L, Sailaja PR, Truedsson E, Fagerstrom S, Pahlman S and Larsson C (1999) Novel and classical protein kinase C isoforms have different functions in proliferation, survival and differentiation of neuroblastoma cells. *Int J Cancer* 81:494-501.
- Zhang L, Yu Y, Mackin S, Weight FF, Uhl GR and Wang JB (1996) Differential  $\mu$ -opioid receptor phosphorylation and desensitization induced by agonists and Phorbol Esters. *J Biol Chem* 271:11449-11454.
- Zhang J, Ferguson SS, Barak LS, Bodduluri SR, Laporte SA, Law PY and Caron MG (1998) Role for G protein-coupled receptor kinase in agonist-specific regulation of mu opioid receptor responsiveness. *Proc Nat Acad Sci* 95:7157-7162.

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b) Part of this work was presented previously in abstract form at the International Narcotic Research Conference, 2004, Kyoto, Japan (H.Z. Ozsoy and K.M. Standifer Acute Orphanin FQ/Nociceptin (OFQ/N) Pretreatment Induces Mu Opioid Receptor Phosphorylation and Internalization; O VII-2) and as a dissertation submitted in partial fulfillment of the requirements for the doctoral degree at the University of Houston.

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## Figure Legends

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

**Fig. 2. Characterization of flag-tagged  $\mu$  receptor protein from Flag $\mu$ -BE cells by immunoprecipitation.** Flag $\mu$ -BE cells were lysed and immunoprecipitated using anti-FLAG M2 antibody. The immunoprecipitates were resolved through SDS-PAGE gels and immunoblotted using anti-FLAG M2 HRP-conjugated antibody (A, lanes 1-3). The membrane then was stripped

and reprobed with the rabbit  $\mu$  receptor antibody (A, lanes 4-6). Lanes 2 and 4 were loaded with 10  $\mu$ l immunoprecipate samples and lanes 3 and 6 were loaded with 20  $\mu$ l. U, untransfected BE(2)-C cells; T, Flag $\mu$ -BE cells. The  $\mu$  receptor protein appears as the band at 65-70 kDa, untransfected BE(2)-C cells lack this band (A, lanes 1, 4), and DAMGO-induced phosphorylation significantly occurred at this band only (B). Con, control; DAM, DAMGO treatment (10 min).

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

**Fig. 4. OFQ/N-mediated increase in DAMGO-induced  $\mu$  receptor phosphorylation was blocked with protein kinase C inhibition.** Flag $\mu$ -BE cells were labeled with [ $^{32}$ Pi] and then exposed to Chelerythrine (1  $\mu$ M) for 15 min before addition of OFQ/N (100 nM) as described in Methods. After 1 hr OFQ/N treatment, cells were washed with phosphate-free medium and challenged with DAMGO (1  $\mu$ M) for 10 min. Cell lysates were subjected to immunoprecipitation

with anti-FLAG M2 antibody as described in Methods. Data from immunoblots and autoradiography were quantified by densitometric analysis, and the ratio of the  $^{32}\text{P}$ -Flag $\mu$  receptor band to total Flag $\mu$  receptor immunoreactivity was presented as mean  $\pm$  S. E. M. of four experiments. Top and bottom blots of each panel are representative autoradiography and immunoblots of the  $\mu$  receptor, respectively. \* $p < 0.05$  significantly different from vehicle-treated cells, \*\* $p < 0.05$  significantly different from DAMGO-challenged cells, and # $p < 0.05$  significantly different from OFQ/N-treated+ DAMGO-challenged cells.

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



different from DAMGO-challenged cells, and <sup>#</sup>p < 0.05 significantly different from OFQ/N-treated+ DAMGO-challenged cells.

**Fig. 6. OFQ/N-activated protein kinase C induces GRK2 phosphorylation in Flag $\mu$ -BE cells.** Cells were preloaded with [<sup>32</sup>Pi] for 2 h and then treated with OFQ/N (100 nM) in the absence and presence of Chelerythrine (1 $\mu$ M). 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  $\pm$  S.E.M. of three experiments in the graph. OFQ/N treatment significantly increased GRK2 phosphorylation compared to vehicle-treated cells (\*p<0.05), while inclusion of Che blocked the increase (#p<0.05).

**Fig. 7. The  $\mu$  opioid receptor lacking the Ser<sup>375</sup> residue (HA3A $\mu$ ) does not undergo OFQ/N-induced heterologous  $\mu$  receptor desensitization.** Intact HA $\mu$ -BE (A), HA3A $\mu$ -BE (B), and HA2A $\mu$ -BE (C) cells were incubated with or without OFQ/N (100 nM) for 1 hr. After pretreatment, cells were assayed for the ability of increased concentrations of DAMGO to inhibit forskolin (10  $\mu$ M)-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<sub>50</sub> -7.1  $\pm$  0.2\* vs. vehicle log EC<sub>50</sub> -7.9  $\pm$  0.1) and efficacy in HA $\mu$ -BE cells. The same treatment did not change the agonist potency in HA2A $\mu$ -BE (B; OFQ/N log EC<sub>50</sub> -7.1  $\pm$  0.1 vs. vehicle log EC<sub>50</sub> -

7.3 ± 0.1) cells, but did significantly reduce agonist efficacy. However, OFQ/N reduced neither potency (C; OFQ/N log EC<sub>50</sub> -7.5 ± 0.2 vs. vehicle log EC<sub>50</sub> -7.3 ± 0.2) nor efficacy in HA3Aμ-BE cells. Data are expressed as mean ± S.E.M. of two-three experiments. \*p < 0.05 significantly different from vehicle-treated cells.

**Fig. 8. Ser<sup>375</sup> is responsible for OFQ/N-mediated enhancement in DAMGO-induced μ receptor phosphorylation.** Immunoblots display the Ser<sup>375</sup> phosphorylated form of the μ receptor protein (upper blots) and total μ receptor protein (lower blots) in HAμ-BE (A), HA3Aμ-BE (C) and HA2Aμ-BE cells (D). Mu receptor proteins in cells were partially purified with wheat germ lectin agarose and immunoblotted using phosphoSer<sup>375</sup> antibody as described in Methods. After immunoblotting, the membrane was stripped and reprobed with anti-HA antibody. Panel B shows the 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.

**Table 1. Characterization of the epitope-tagged wild type (Flag $\mu$  and HA $\mu$ ) and mutant (HA2A $\mu$  and HA3A $\mu$ )  $\mu$  opioid receptors stably expressed in BE(2)-C cells.** Radioligand binding studies were performed on membrane preparations as explained in Methods. B<sub>max</sub> values were determined by saturation binding with [<sup>3</sup>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<sub>max</sub>), DAMGO concentration producing 50% of the maximal response (EC<sub>50</sub>) and the maximal response induced by 10  $\mu$ M DAMGO (% Maximal Inhibition). The values shown are means  $\pm$  S.E.M. from at least two independent experiments.

$\mu$ Receptor- expressing cell line	B <sub>max</sub> (fmol/mg protein)	DAMGO EC <sub>50</sub> (Log M)	Maximal inhibition (%)
Native BE(2)-C	33 $\pm$ 2.4	-7.6 $\pm$ 0.2	70 $\pm$ 6.4
Flag $\mu$	560 $\pm$ 31	-8.0 $\pm$ 0.1	76 $\pm$ 4.2
HA $\mu$	577 $\pm$ 19	-7.9 $\pm$ 0.1	86 $\pm$ 2.1
HA2A $\mu$	522 $\pm$ 32	-7.3 $\pm$ 0.3	94 $\pm$ 7.4
HA3A $\mu$	487 $\pm$ 40	-7.3 $\pm$ 0.1	95 $\pm$ 3.3

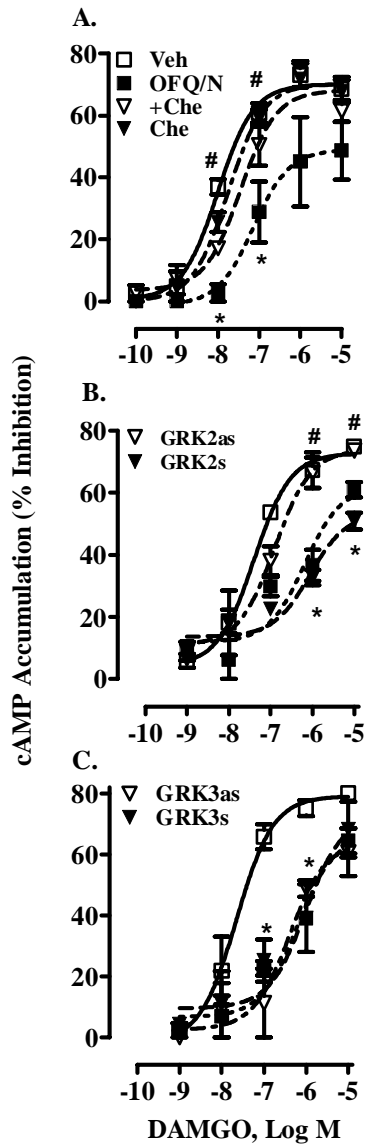


Figure 1

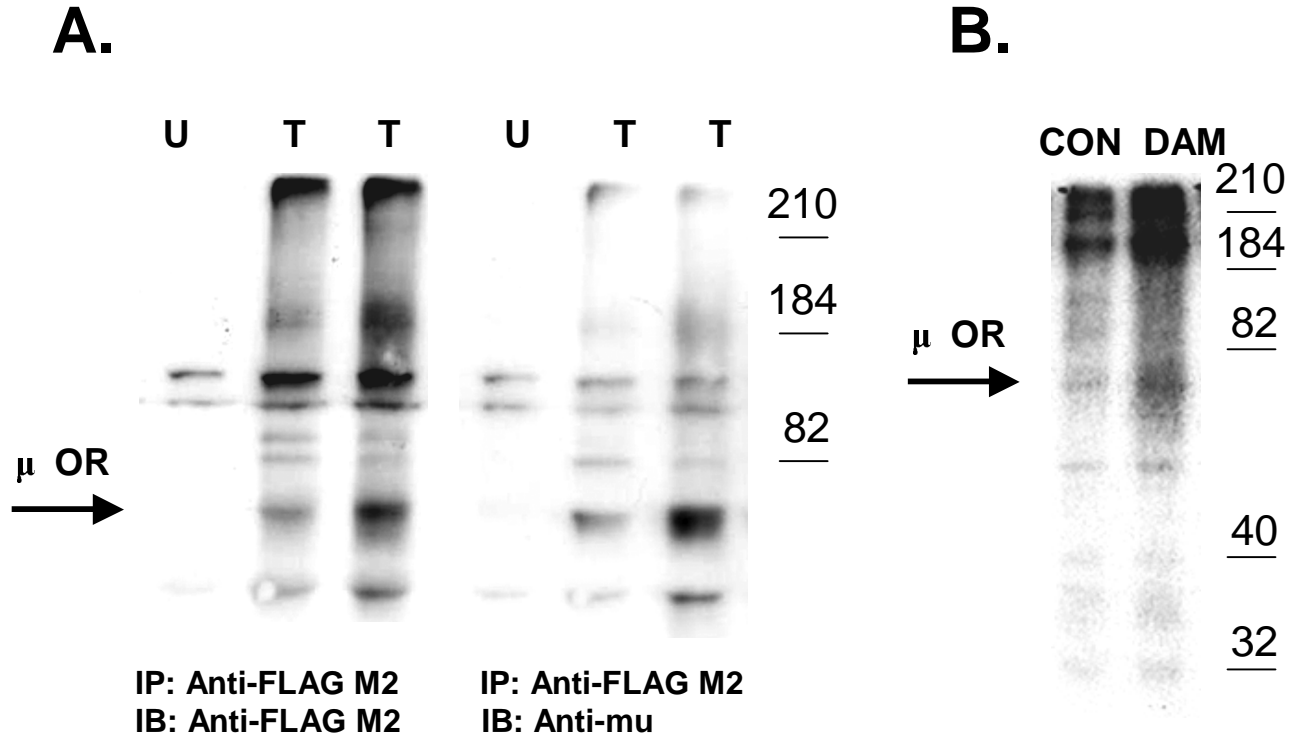


Figure 2

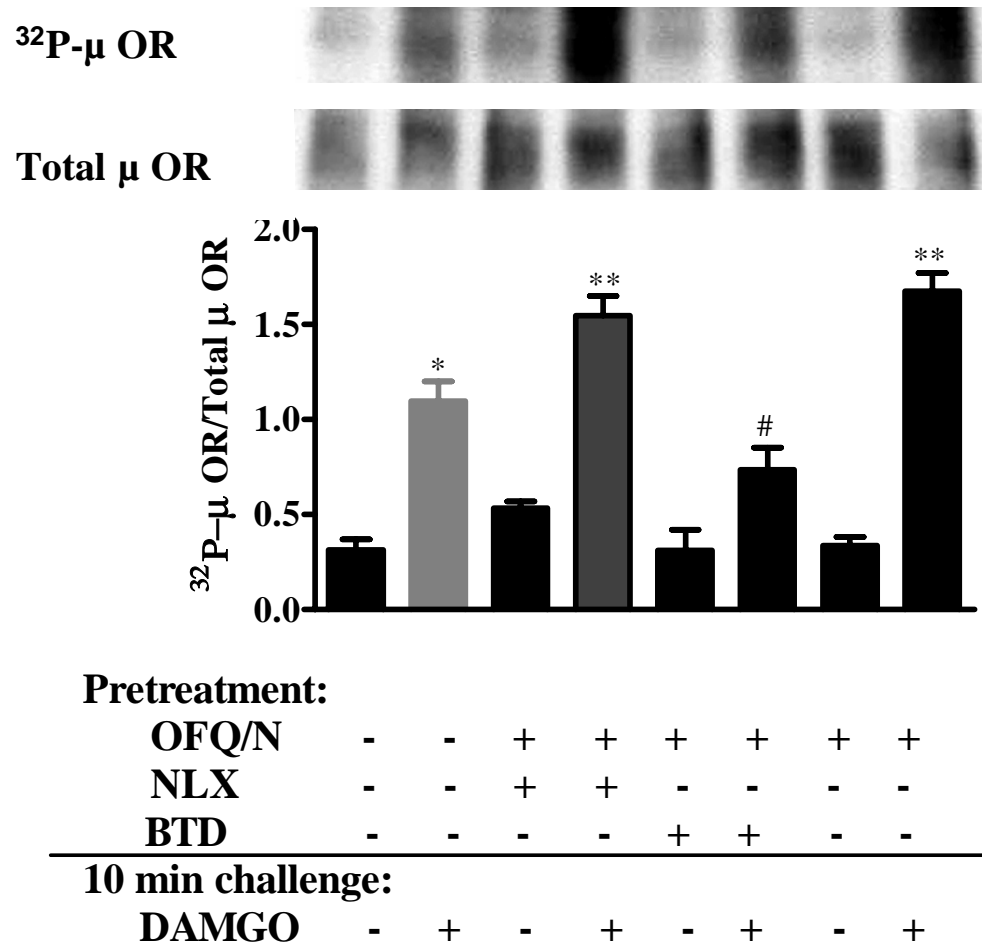


Figure 3

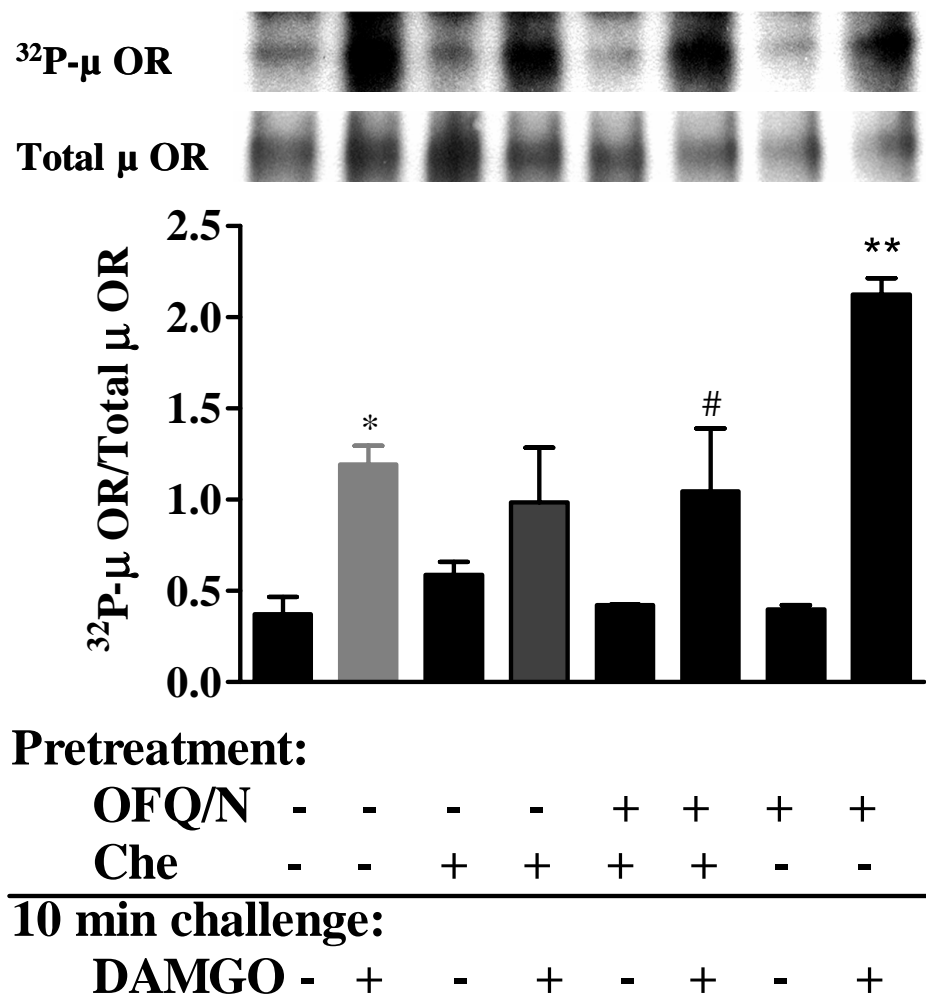
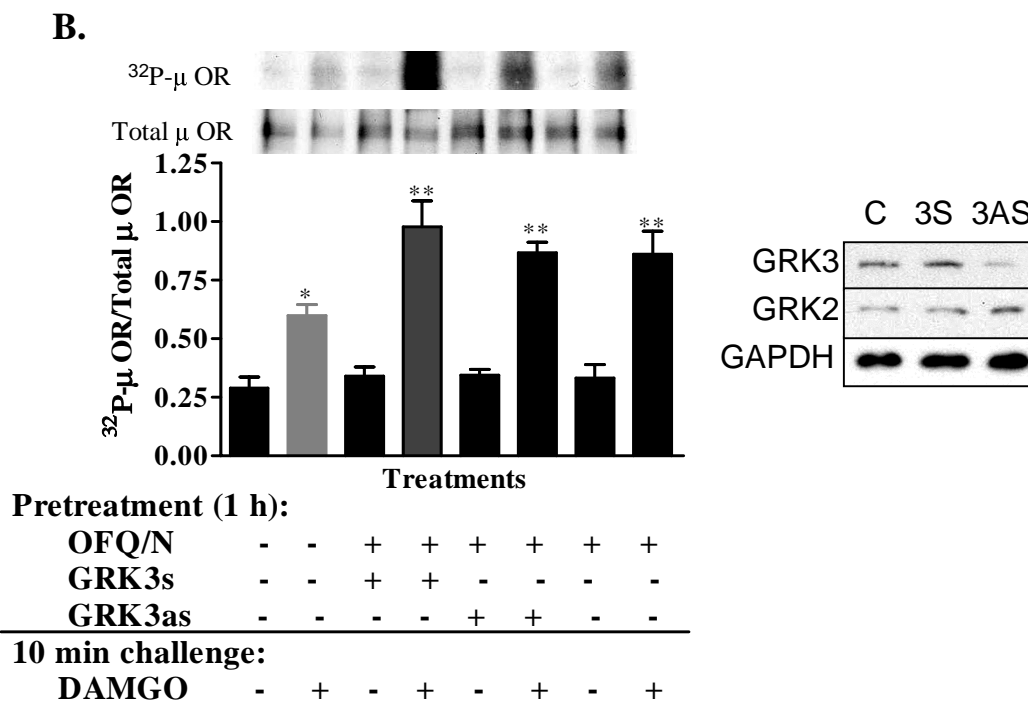
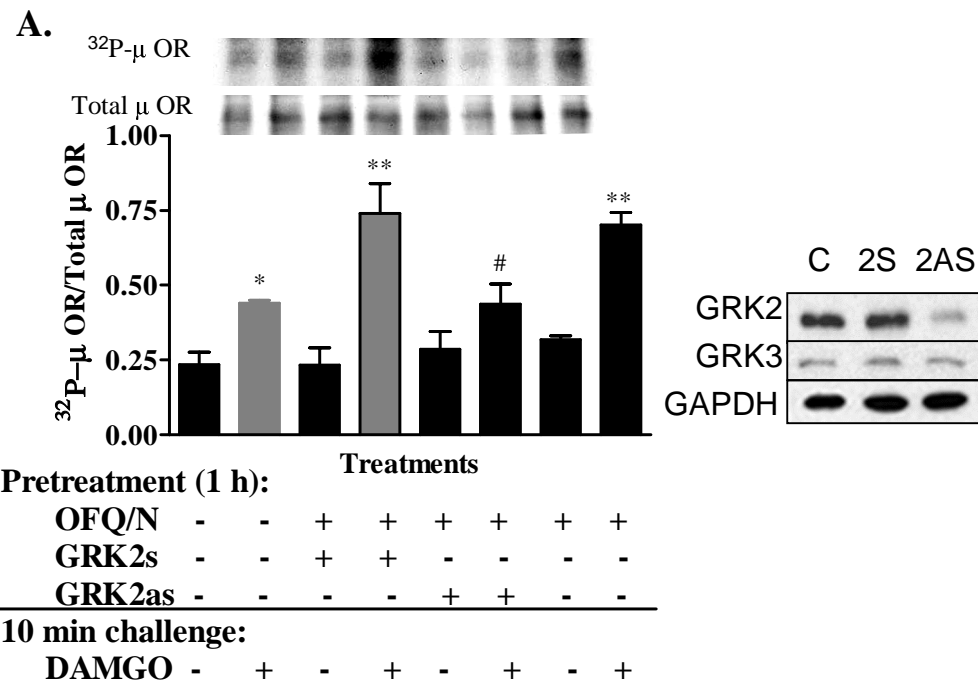


Figure 4

Figure 5





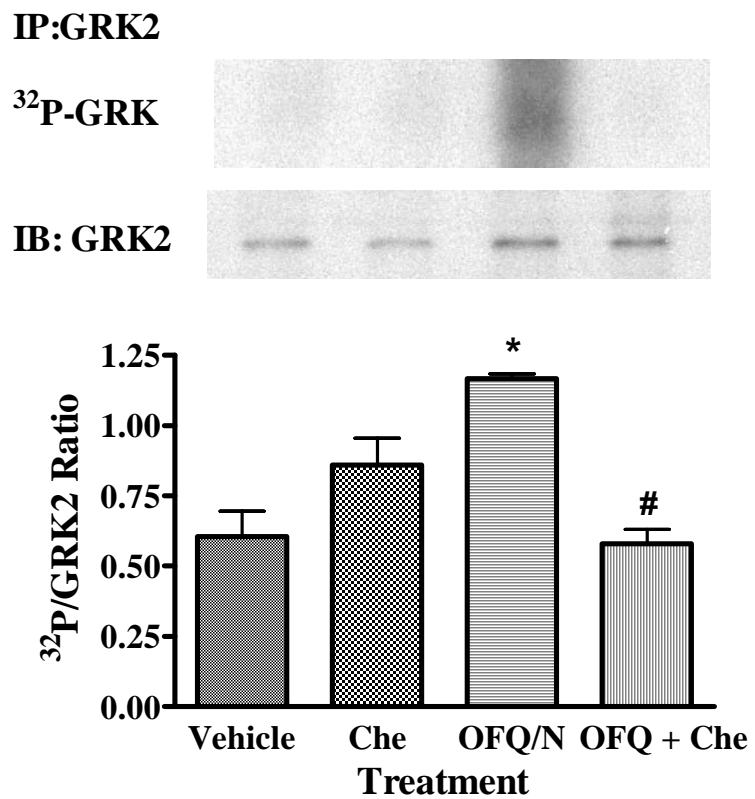


Figure 6

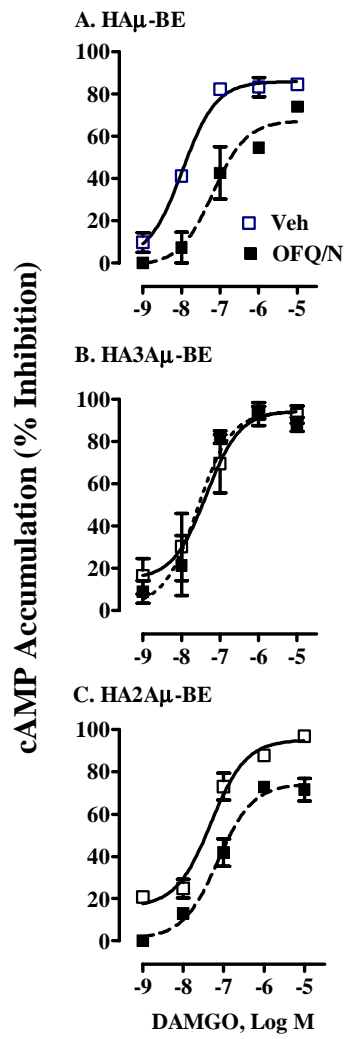


Figure 7

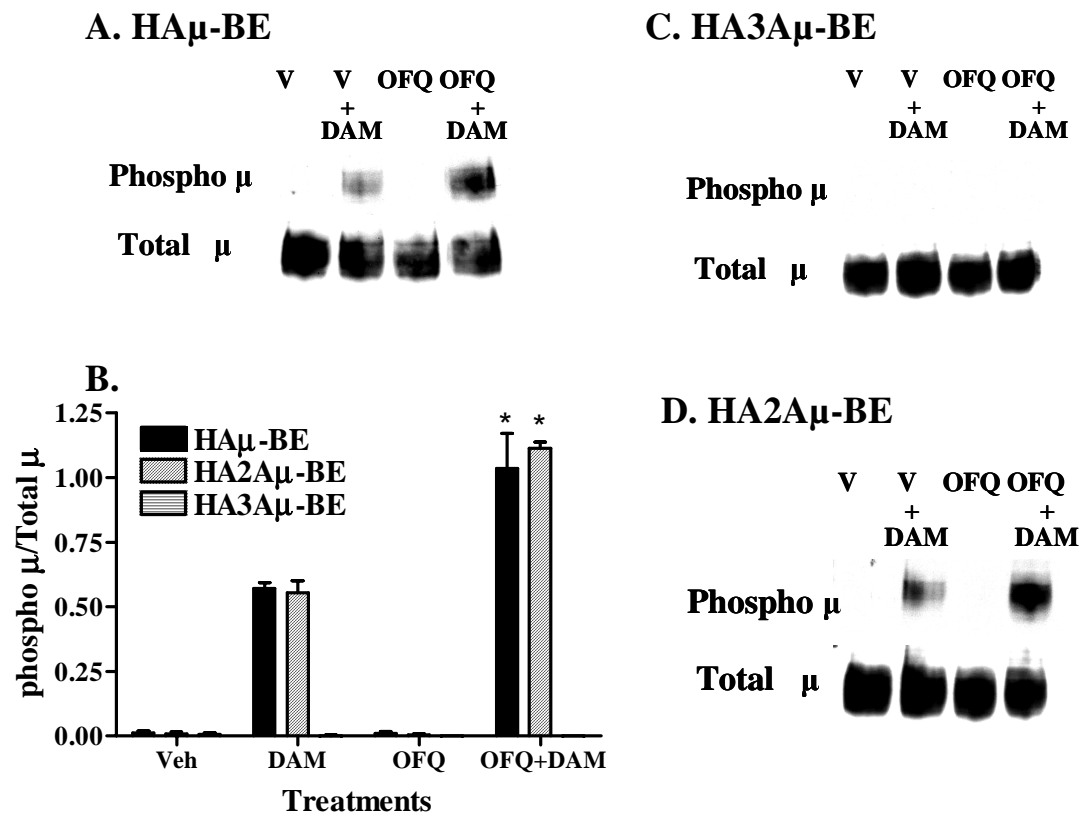


Figure 8