Neuropeptide FF (NPFF) interacts with specific receptors to modulate opioid functions in the central nervous system. On dissociated neurons and neuroblastoma cells (SH-SY5Y) transfected with NPFF receptors, NPFF acts as a functional antagonist of μ-opioid (MOP) receptors by attenuating the opioid-induced inhibition of calcium conductance. In the SH-SY5Y model, MOP and NPFF2 receptors have been shown to heteromerize. To understand the molecular mechanism involved in the anti-opioid activity of NPFF, we have investigated the phosphorylation status of the MOP receptor using phospho-specific antibody and mass spectrometry. Similarly to direct opioid receptor stimulation, activation of the NPFF2 receptor by antibody and mass spectrometry. Similarly to direct opioid receptor stimulation, activation of the NPFF2 receptor by antibody and mass spectrometry. Similarly to direct opioid receptor stimulation, activation of the NPFF2 receptor by antibody and mass spectrometry.

**Background:** Neuropeptide FF2 receptors interact with μ-opioid receptors and decrease their activity.

**Results:** The phosphorylation pattern of MOP receptor is similar after homologous (DAMGO) and heterologous (neuropeptide FF) stimulation.

**Conclusion:** GPCR kinase 2 (GRK2) contributes to the NPFF-induced phosphorylation and loss of function of MOP receptor.

**Significance:** GRK2-mediated transphosphorylation within receptor heteromers could play a role in heterologous desensitization of GPCRs.

**μ-Opioid (MOP)** receptors are the main targets of analgesic drugs. However, the use of opiates gives rise to some undesirable effects, including tolerance, pain hypersensitivity, and addiction. Despite more than 40 years of research in the field, the mechanisms of action of these drugs are not completely understood. Tolerance to morphine has been explained by a variety of molecular mechanisms, including functional selective desensitization of receptor signaling and/or endocytosis, as well as cellular counter-adaptations such as hypertrophy of adenylyl cyclase signaling or shift from inhibitory to excitatory G protein coupling (1–5). But tolerance certainly results from complex integrated adaptive processes that contribute to counteract the opioid effects or to change the nociceptive threshold, leading in particular to the so-called paradoxical opioid-induced hyperalgesia (6). Among the peptidergic systems that modulate opiate action, the neuropeptide FF (NPFF) system has recently been shown to contribute to the tolerance to morphine analgesia (7, 8).

Neuropeptide FF (FLFQPQRF-amide, NPFF) belongs to a family of RF-amide peptides involved in the control of pain, cardiovascular functions, appetite, thirst, and body temperature (9). NPFF, together with other related neuropeptides, is produced from a precursor, pro-NPFFA, which is expressed in a few regions of the rodent central nervous system, namely the hypothalamus, the nucleus of the tractus solitarius in the brainstem, and the dorsal horn of the spinal cord (10). It is a high affinity agonist of two G protein-coupled receptors (GPCRs)
called NPFF₁ and NPFF₂ (11). Injection of NPFF analogs in rodents has been shown to modulate both analgesic (12–14) and motivational properties of opioid drugs (15, 16). The NPFF system is therefore considered to be an opioid-modulating system involved in homeostasis that counteracts the action of opioids (17, 18) and thus an interesting therapeutic target for the management of opiate tolerance and dependence (7). In many cases, NPFF pretreatment inhibits opioid effects in rodents. For example, intracerebroventricular injection of NPFF analogs inhibits morphine-induced analgesia in rats (19) and mice (20) and morphine-induced neuronal activity in the mouse nucleus accumbens (21). Similarly to other opioid-modulating peptides (18), an important question is whether this inhibitory effect is indirect (mediated through neuronal circuitry) or direct (mediated by receptor cross-talk within the same neuron). The fact that 1DMe, an NPFF analog, reverses the inhibition of calcium conductance induced by the MOP agonist DAMGO in dissociated rat dorsal root ganglion neurons favors the second hypothesis (22).

To gain information on the molecular mechanisms responsible for the cellular anti-opioid activity of NPFF, we have developed a model by stably transfecting human NPFF₂ receptors in the neuroblastoma-derived cell line SH-SY5Y (23). The neuroblastoma clone SH-SY5Y, derived from a human sympathetic ganglion tumor, endogenously expresses low levels of MOP receptors that are negatively coupled to adenyl cyclase and voltage-gated N-type Ca²⁺ channels through heterotrimeric G₁ₒ proteins. Activation of NPFF₂ receptors in the recombinant (SH₂-D9) cell line inhibits the opioid modulation of Ca²⁺ channels, thus reproducing the cellular anti-opioid activity observed in isolated neurons (23). This anti-opioid effect is not prevented by inhibition of second messenger-dependent kinases PKA and PKC (23), suggesting that it does not involve typical pathways of heterologous desensitization (24).

GPCR function can also be modulated by heteromerization (25). Numerous studies have shown that the MOP receptor is subjected to this type of regulation by interacting with a large number of GPCRs (25, 26). In the case of MOP receptor dimerization with somatostatin ss₂₄ (27), substance P NK1 (28), α₂a-adrenergic (29), and nociceptin ORL1 (30) receptors, cross-desensitization occurs and could result from co-internalization of both partners upon activation of only one receptor or from allosteric changes in binding affinity (31). However, in the case of NPFF₂ and MOP receptors previously shown to physically interact in SH-SY5Y cells (22), the stimulation of NPFF₂ receptors does not induce the internalization of MOP receptors but, conversely, partly blocks its trafficking (32). Moreover, activation of NPFF₂ receptors does not affect the ability of MOP agonists to bind to MOP receptors (23) and to stimulate GTPyS binding on membranes of SH-SY5Y cells (33) nor the distribution of MOP receptors in detergent-resistant membrane microdomains (34). In this study, we have pursued our investigations on the molecular mechanism of the anti-opioid activity of NPFF by testing the possibility that NPFF₂ receptor-induced desensitization of the MOP receptor could result from an atypical mode of cross-phosphorylation.

Homologous desensitization results from the phosphorylation of activated GPCRs by a family of serine/threonine kinases called G protein-coupled receptor kinases (24). Previous studies have shown that Thr-370 and Ser-375 are the main targets of homologous regulation by G protein-coupled receptor kinases in the C-terminal tail of the rodent MOP receptor (35–38), although a potential role for Thr-180 in the second intracellular loop has also been suggested in Xenopus oocytes (39). Here, we have studied the phosphorylation status of the human MOP receptor after homologous stimulation by its agonist (DAMGO) or after heterologous regulation by NPFF₂ receptors. In our SH-SY5Y model, we have used the only commercially available phospho-specific antibody to specifically monitor the phosphorylation of Ser-377 (corresponding to Ser-375 in the rodent sequence), and we have performed mass spectrometry to identify other phosphorylation sites in the MOP C-tail. Activation of the NPFF₂ receptor by 1DMe induced the phosphorylation of MOP Ser-377. This heterologous phosphorylation was not blocked by inhibition of second messenger-dependent kinases (PKC and PTK) but, in contrast to homologous phosphorylation, was prevented by inactivation of G₁ₒ proteins by pertussis toxin (PTX). We also showed that Ser-377 cross-phosphorylation and MOP receptor cross-desensitization are mediated by GRK2. Moreover, NPFF₂ receptor-mediated MOP receptor phosphorylation was accompanied by the recruitment of β-arrestin2 but not by receptor internalization. Finally, mass spectrometric analysis indicated that the phosphorylation pattern of immunopurified MOP receptor is similar after homologous and heterologous stimulation, but the phosphorylation level is more intense after DAMGO treatment.

**EXPERIMENTAL PROCEDURES**

**Materials**—Oligonucleotides were synthesized by Sigma Genosys. Restriction enzymes were purchased from New England Biolabs (Ozyme, France). Mouse monoclonal anti-GFP antibody was obtained from Invitrogen; rabbit polyclonal anti-GFP antibody and anti-GRK2 antibody were from Santa Cruz Biotechnology, and rabbit polyclonal anti-actin antibody was from from Sigma. The Ser-375 phospho-specific antibody was from Cell Signaling Technology (Ozyme, France), Silencer Select validated siRNA (Ambion Silencer Negative control 1, GRK2 s1127 and GRK2 s1128) were purchased from Ambion (Applied Biosystems, France). 1DMe was synthesized with an automated peptide synthesizer (model 433A, Applied Biosystems). Tyr-d-Ala-Gly-(NMe)-Phe-Gly-ol (DAMGO) was purchased from Bachem (Switzerland); clonidine, H89, and chelerythrine were from Sigma, and PP2 and EGF inhibitor were from Calbiochem. [³H]DAMGO (67 Ci/mmol) and [³H]adenine (26 Ci/mmol) were purchased from GE Healthcare. The NPFF₂ receptor-selective radioligand [³H]EYF (Glu-Tyr-Trp-Ser-Leu-Ala-Ala-Pro-Gln-Arg-Phe-NH₂) was tritiated by Tritec (Switzerland) as described (40).

**Vector Construction**—A truncated version of the enhanced YFP variant Venus encoding amino acids 155–238 of the protein was amplified by PCR using two overlapping forward primers introducing an NcoI restriction site and a DGGSGGGS-8-amino acid linker (5’-CATATACCATGAGCCGGCGAG-CGGCGGCGGCGGCGGCAAG-3’ and 5’-AGCGGGCGGCGGCGGCGGCAAGCGCAAGAGACGCGCATC-3’) and a reverse primer designed to introduce an XbaI restriction site.
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(5′-AATCTCTTAGCTTTGGTACAGCTCGTCCATGCGGAGAGT-3′). The PCR product was ligated in-frame at the 3′ end of the human MOP receptor cDNA (32) in pBluescript II SK− (Stratagene, Agilent Technologies) using NotI and XbaI sites. The complete construct called MOP-VC was finally inserted into the EcoRV-XbaI sites of the mammalian expression vector pEF/B3 bearing the blasticidin selection marker. All constructs were verified by sequencing (Millegen, France).

The cDNA for β-arrestin2 fused to the first 155 amino acids of Venus (β-arrestin2-VN) in a pcDNA3-zeo vector was kindly provided by Dr. N. Holliday (University of Nottingham, United Kingdom) (41).

**Cell Culture and Transfection**—Human neuroblastoma SH-SY5Y cells were grown in Dulbecco’s modified Eagle’s medium (4.5 g/liter glucose, GlutaMAXI) containing 10% fetal calf serum (FCS) and 50 μg/ml gentamicin (Invitrogen), in a 37 °C humidified atmosphere containing 5% CO2. 400 mM NaCl, 5 mM KCl, 1.25 mM MgSO4, 1.5 mM CaCl2, 12738 mM KH2PO4, 25 mM HEPES, 8 mM glucose, 0.5 mg/ml bovine serum albumin, pH 7.4), and then incubated for 15 or 30 min at room temperature with 0.5 ml of buffer (control) or 0.5 ml of buffer plus 1 μM 1DMe or DAMGO. Cells were rinsed three times (with PBS and were fixed with 3.8% formaldehyde (Sigma, France) in PBS containing protease and phosphatase inhibitors (Complete EDTA-free and PhosStop, respectively, Roche Diagnostics) for 20 min at room temperature. After three washes with 0.5 ml of PBS, cells were incubated in PBS containing 10% FCS, 2% BSA, and protease and phosphatase inhibitors for 1 h at room temperature. Coverslips were then incubated with anti-phospho-Ser-377 primary antibody (1:100) in PBS, 2% FCS inhibitor mixtures for 1 h. Cells were rinsed three times for 10 min in PBS, 2% SFV and incubated overnight at 4 °C with Alexa Fluor-633 goat anti-rabbit IgG diluted 1:400 in the same solution for 30 min at room temperature. After three rinses, cells were mounted in Vectashield medium (Vector Laboratories, AbCys, France) and observed on a Zeiss 710 NLO inverted microscope with a ×40 objective (1.3 numerical aperture, oil). Cells were excited at 488 nm (band pass 493–598 nm for emission) and 633 nm (band pass 652–720 nm for emission) under sequential mode. Images were processed with Image J (National Institutes of Health, Bethesda).

**β-Arrestin2 Recruitment**—Bimolecular fluorescence complementation (BiFC) was detected in (SH2-D9)MOP-VC cells 48 h after transient transfection with β-arrestin2-VN in Lab-Tek II chambered coverglasses (Nunc, Thermo Fisher Scientific, France) using FuGENE 6 transfection reagent. Cells were rinsed with KRH buffer, treated with agonists, and observed live at room temperature under an inverted confocal Zeiss 510 microscope (×63 water objective, 488 nm excitation wavelength, LP505). Images were acquired using the AMS software (Zeiss, France).

**Binding and Biological Assays**—Membrane preparation, binding of [3H]EYF (NPFF2 receptors) or [3H]DAMGO (MOP receptors), and cAMP measurements were performed as described previously (23). The MOP-VC cell line established in this study expressed 1.3 pmol of receptor/mg of membrane proteins. The receptor was fully functional in cAMP assays. The receptor was fully functional in cAMP assays.

**Western Blotting**—Cells seeded in 6- or 24-well plates (1 million and 300,000, respectively) were incubated in culture medium containing 0.5% FCS for 2 h. Ligands were then applied in HEPES-buffered medium (150 mM NaCl, 2.5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM glucose, 0.1% BSA, 10 mM HEPES; pH adjusted to 7.3) at room temperature. Cells were rinsed with ice-cold PBS and scraped directly in Laemmli sample buffer containing 5% β-mercaptoethanol on ice. Samples were homogenized using a 1-ml syringe, boiled for 5 min, and subjected to 10% SDS-PAGE. Proteins in the gel were transferred to a polyvinylidene difluoride membrane for immunoblotting under standard conditions in Tris-buffered saline (20 mM Tris, pH 7.6, 137 mM NaCl) containing 0.1% Tween and 1% bovine serum albumin. Immunoreactivity was revealed with peroxidase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch) using the ECL Plus Western blotting detection kit (GE Healthcare). X-ray films were scanned using a GS-800 calibrated densitometer (Bio-Rad). When required, blots were quantified using Quantity One software (Bio-Rad) relative to actin used as internal standard.

**Intracellular calcium concentration ([Ca2+]i)** was monitored in living, perfused SH2-D9 cells by quantitative photometry using indicator Fluo-4 (23). The (SH2-D9)MOP-VC cell line was maintained in 400 mM NaCl, 5 mM KCl, 1.25 mM MgSO4, 1.5 mM CaCl2, 12738 mM KH2PO4, 25 mM HEPES, 8 mM glucose, 0.5 mg/ml bovine serum albumin, pH 7.4), and then incubated for 15 or 30 min at room temperature with 0.5 ml of buffer (control) or 0.5 ml of buffer plus 1 μM 1DMe or DAMGO. Cells were rinsed three times (with PBS and were fixed with 3.8% formaldehyde (Sigma, France) in PBS containing protease and phosphatase inhibitors (Complete EDTA-free and PhosStop, respectively, Roche Diagnostics) for 20 min at room temperature. After three washes with 0.5 ml of PBS, cells were incubated in PBS containing 10% FCS, 2% BSA, and protease and phosphatase inhibitors for 1 h at room temperature. Coverslips were then incubated with anti-phospho-Ser-377 primary antibody (1:100) in PBS, 2% FCS inhibitor mixtures for 1 h. Cells were rinsed three times for 10 min in PBS, 2% SFV and incubated overnight at 4 °C with Alexa Fluor-633 goat anti-rabbit IgG diluted 1:400 in the same solution for 30 min at room temperature. After three rinses, cells were mounted in Vectashield medium (Vector Laboratories, AbCys, France) and observed on a Zeiss 710 NLO inverted microscope with a ×40 objective (1.3 numerical aperture, oil). Cells were excited at 488 nm (band pass 493–598 nm for emission) and 633 nm (band pass 652–720 nm for emission) under sequential mode. Images were processed with Image J (National Institutes of Health, Bethesda).

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**Receptor Purification**—MOP-YFP receptors were isolated by immunoprecipitation using anti-GFP monoclonal antibodies. Each sample was prepared from two confluent 140-mm culture dishes. Cells were incubated in culture medium containing 0.5% FCS for 2 h. Ligands were then applied in KRH buffer for 20 min at room temperature, and cells were scraped directly on ice. After a short centrifugation (1000 × g, 5 min, 4 °C), cells were incubated for 1 h at 4 °C with gentle agitation in 1 ml of freshly prepared solubilization buffer consisting of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and protease and phosphatase inhibitor mixtures (Complete EDTA-free and PhosStop respectively, Roche Diagnostics). The homogenate was then centrifuged at 20,000 × g for 2 min at 4 °C. The supernatant was preclarified by incubation with 50 μl of EZView red protein A affinity gel (Sigma) for 3 h at 4 °C and centrifugation at 12,000 × g for 30 s. Immunoprecipitation was then performed with 4 μg of mouse monoclonal anti-GFP antibody for 1 h at 4 °C, followed by overnight incubation at 4 °C with protein A-agarose beads. The washing procedure was
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RESULTS

NPFF₂ Receptor Activation Induces Heterologous Phosphorylation of Ser-377 of the Human MOP Receptor—The ability of the anti-Ser-377 phosphospecific antibody to detect MOP receptor phosphorylation was first tested after a 30-min treatment with 1 mM of the MOP agonist DAMGO. As the antibody was not sensitive enough to detect the endogenous MOP receptor, which is expressed at low levels in SH-SY5Y cells (23), phosphorylation experiments were performed in the SH₂D9 MOP-YFP cell line that stably expresses the human NPFF₂ receptor and the human MOP receptor fused to the yellow fluorescent protein (YFP) at its C terminus (32). Western blots (Fig. 1A) show that the phospho-Ser-377 antibody detects a band (110–120 kDa), previously shown to correspond to the glycosylated MOP-YFP receptor (32), which is about 6-fold more intense after DAMGO treatment. Interestingly, treatment with 1 mM 1DMe alone for 30 min also induced a significant 2-fold increase in MOP receptor Ser-377 phosphorylation (Fig. 1B). This effect seems specific for the NPFF₂ receptor because activation of another G₁α/IRE-coupled receptor endogenously expressed in SH-SY5Y cells, the α₂-adrenergic receptor, with...
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A

control  DAMGO  DAMGO + 1DMe

B

control  1DMe  Clonidine

FIGURE 1. Homologous (A) and heterologous (B) phosphorylation of MOP receptor Ser-377. Representative Western blots show the levels of Ser-377 phosphorylation induced by treatment with 1 µM DAMGO, 1 µM DAMGO + 1 µM 1DMe, 1 µM 1DMe, or 10 µM clonidine for 30 min in (SH2-D9)MOP-YFP cells. Samples were immunoblotted with anti-Ser(P)-377 antibody (P-MOP) followed by anti-actin antibody (actin) for normalization. Upper panels show representative results from at least three independent experiments. The P-MOP band migrated between the 95- and 130-kDa molecular mass markers. The actin band was detected at the level of the 43-kDa molecular mass marker. Lower panels show means ± S.E. of Ser(P)-377 quantification normalized to actin from at least three independent experiments performed in duplicate. c indicates control cells. *, p < 0.05; ***, p < 0.001; one-way ANOVA followed by Bonferroni post-tests.

10 µM clonidine for 30 min did not modify Ser-377 phosphorylation (Fig. 1B). Co-treatment with 1 µM MOP and NPFF2 agonists did not induce a further increase in phosphorylation (Fig. 1A), indicating that both agonists act on the same population of receptors. An anti-GFP antibody was used to verify that the total amount of MOP-YFP receptor was not changed after a 30-min treatment with DAMGO or 1DMe (supplemental Fig. 1A), indicating that the changes detected with the phosphospecific antibody were indeed due to increased phosphorylation. When testing various doses of 1DMe, we observed that Ser-377 phosphorylation was already detectable at 10 nM (supplemental Fig. 1B). Importantly, 1DMe was unable to induce MOP receptor phosphorylation in cells that did not express NPFF2 receptors (supplemental Fig. 1C).

Ser-377 Heterologous Phosphorylation Time Course Correlates with Kinetics of Desensitization of Opioid Response—The time course of MOP Ser-377 phosphorylation was also assessed, and interestingly, although DAMGO induced a continuous increase in MOP receptor phosphorylation over 60 min (Fig. 2A), 1DMe-induced heterologous phosphorylation was transient, with Ser-377 phosphorylation returning to baseline level after 60 min of treatment (Fig. 2, B and C). In addition, Ser-377 heterologous phosphorylation was not blocked by treatment with 10 µM of the opioid antagonist naltrexone, indicating that it did not involve any direct activation of the MOP receptor (Fig. 2C). Interestingly, the time course of cross-phosphorylation could be correlated to the kinetics of the effect of 1DMe on MOP receptor activity. We have previously reported that, in SH-SY5Y cells, increasing extracellular KCl concentration induced a transient rise in intracellular calcium (resulting from the opening of N-type voltage-gated calcium channels) that was sensitive to opioids (23). As shown in Fig. 2D, stimulation of endogenous MOP receptors by 0.1 µM DAMGO in SH2-D9 cells reduced by 46.5% the calcium influx. This effect of DAMGO was inhibited by 80% in cells that had been pretreated with 1 µM 1DMe for 30 min, revealing the loss of opioid receptor function at a time point when it is phosphorylated by the activation of NPFF receptors. However, when 1DMe pretreatment time was extended to 60 min, the effect of DAMGO returned to control level (Fig. 2D), indicating that MOP receptors recover their activity (resensitization) when Ser-377 is no longer phosphorylated (Fig. 2, B and C). The correlation of kinetic data thus supports a role of the transient MOP receptor Ser-377 phosphorylation in the opioid receptor desensitization and resensitization during 1DMe treatment.

GRK2 Contributes to Ser-377 Heterologous Phosphorylation and Inhibition of MOP Receptor Signaling—Because Ser-375 of the rodent MOP receptor (equivalent to Ser-377 in the human receptor) has been shown to be a GRK2 target (35–37), we tested the possible involvement of this kinase in Ser-377 heterologous phosphorylation by 1DMe. (SH2-D9)MOP-YFP cells were transfected with two validated siRNAs (1127 and 1128) directed against human GRK2. Levels of GRK2 were determined by Western blot 48 h after transfection. Fig. 3A shows that GRK2 was strongly down-regulated after transfection of siRNA 1128 compared with untransfected cells or cells transfected with a negative control siRNA. siRNA 1127 induced a much lower down-regulation of GRK2. When GRK2 was knocked down by siRNA 1128, 1DMe lost its ability to induce the heterologous phosphorylation of Ser-377 (Fig. 3A). This result indicates that in SH-SY5Y cells, GRK2 is the only kinase responsible for Ser-377 cross-phosphorylation induced by the
activation of NPFF₂ receptor. Binding of GRK2 to activated NPFF₂ receptors was confirmed by co-immunoprecipitation experiments (supplemental Fig. 2). In contrast, the homologous phosphorylation induced by DAMGO was not significantly affected by GRK2 knockdown (Fig. 3B). This suggests that, in the case of homologous phosphorylation of Ser-377, either GRK2 is not involved or other G protein-coupled receptor kinases present in the cell can compensate for the lack of GRK2, or finally, the low level of GRK2 still present after siRNA transfection is sufficient for DAMGO-induced phosphorylation.

Having found a way to specifically block 1DMe-induced Ser-377 phosphorylation, we could directly test the role of this phosphorylation in the anti-opioid effect of 1DMe on MOP receptor function. We compared the ability of a 30-min pretreatment with 1 μM 1DMe to affect the inhibition of calcium influx by 0.1 μM DAMGO in (SH₂-D9)MOP-YFP cells (supplemental Fig. 3). Uncoupling receptors from inhibitory G proteins had opposite effects on homologous and heterologous phosphorylation of Ser-377. It potentiated DAMGO-induced phosphorylation but completely blocked the effect of 1DMe (Fig. 4A). Therefore, G protein activation is not necessary for homologous phosphorylation of the MOP receptor on Ser-377, although it is mandatory for MOP receptor heterologous phosphorylation.

Recruitment of GRK2 to the MOP receptor after NPFF₂ receptor activation could result from an indirect activation of GRK2 via its phosphorylation by second messenger activated kinase. GRK2 activity can be enhanced by protein kinase A (PKA), protein kinase C (PKC), or c-Src (44). 1DMe was still able to induce a strong increase in Ser-377 phosphorylation in the presence of 10 μM H89 (PKA inhibitor), chelerythrine (PKC inhibitor), or PP2 (Src inhibitor). Activation of EGFR is also able to phosphorylate and activate GRK2, resulting in cross-phosphorylation of opioid receptors (45). Because GPCRs can transactivate EGFR (46) and SH-SY5Y cells express EGFR (47), we explored the possibility that Ser-377 phosphorylation could

Ser-377 Heterologous Phosphorylation Depends on Inhibitory Heterotrimeric G Proteins but Not on Second Messenger-activated Protein Kinases—We next addressed the mechanism by which NPFF₂ receptor activation could recruit GRK2 to the MOP receptor. We first inhibited G₁₅, heterotrimeric G protein with PTX (100 ng/ml for 18 h). The PTX treatment was efficient because it fully prevented the inhibition of adenylyl cyclase by both DAMGO and 1DMe in (SH₂-D9)MOP-YFP cells (supplemental Fig. 3). Uncoupling receptors from inhibitory G proteins had opposite effects on homologous and heterologous phosphorylation of Ser-377. It potentiated DAMGO-induced phosphorylation but completely blocked the effect of 1DMe (Fig. 4A). Therefore, G protein activation is not necessary for homologous phosphorylation of the MOP receptor on Ser-377, although it is mandatory for MOP receptor heterologous phosphorylation.

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be linked to EGFR signaling. Similarly to what we observed with second messenger activated kinases, 1DMe-induced increase in Ser-377 phosphorylation was unaffected by the presence of 10 μM of the EGFR inhibitor cyclopropanecarboxylic acid-(3-(6-(3-trifluoromethyl-phenylamino)-pyrimidin-4-ylamino)-phenyl)-amide (Fig. 4C). Taken together, these results show that MOP receptor phosphorylation by GRK2 after NPFF2 receptor activation necessitates G protein coupling but is independent from GRK2 regulation downstream of G protein signaling.

NPFF2 Receptor Activation Induces β-Arrestin2-VN Recruitment to MOP Receptors but Not Their Internalization—Ser-375 phosphorylation of rodent MOP receptors has been implicated in receptor internalization in addition to desensitization (36, 37). However, morphine has been shown to induce the phosphorylation of MOP receptor Ser-375 at the plasma membrane without triggering endocytosis of the receptor (37). We thus tested the consequences of DAMGO or 1DMe treatment on trafficking of total (MOP-YFP) and Ser-377 phosphorylated MOP receptors. (SH3-D9)MOP-YFP cells were incubated at room temperature with 1 μM DAMGO or 1DMe for 15 or 30 min, respectively. Incubation with 1DMe was shorter to match the peak of Ser-377 phosphorylation. When total MOP receptors were visualized using the fluorescence of the YFP tag, a strong internalization was observed with DAMGO, whereas receptors had remained at the plasma membrane after 1DMe treatment (Fig. 5). The same pattern was observed when Ser-377 phosphorylated MOP receptors were specifically immuno-labeled by the phospho-specific MOP antibody. Incubation with 1DMe for 30 min did not produce any sign of MOP receptor internalization, although it is able to induce NPFF2 receptor endocytosis (see Ref. 32 and data not shown). This indicates that the Ser-377 phosphorylation of MOP receptor induced by the NPFF2 agonist is not sufficient to trigger MOP receptor internalization.

β-Arrestin2 has been implicated in MOP receptor desensitization, endocytosis, and recycling (3). We used BiFC to test whether NPFF2 receptor activation could recruit β-arrestin2 to MOP receptors (48). SH3-D9 cells stably expressing MOP receptors fused to the C-terminal part of the fluorescent protein Venus (MOP-VC) were transiently transfected with β-arrestin2 fused to the N-terminal part of the Venus protein (β-arrestin2-VN). 48 h later, living cells were incubated with 1 μM DAMGO or 1DMe at room temperature and observed under an inverted confocal microscope for 30–60 min to allow for the refolding of the fluorescent protein. Compared with control, cells incubated with DAMGO exhibited highly fluorescent intracellular punctae (Fig. 6), indicating that activated MOP receptor could recruit β-arrestin2-VN, enabling fluorescence complementation and internalization of the complex. Cells treated with 1DMe showed a different profile.
complementation also occurred, demonstrating β-arrestin2-VN recruitment to MOP receptors, but in this case the fluorescence remained at the plasma membrane (Fig. 6). Thus, 1DMe-induced recruitment of β-arrestin2 to MOP receptor is not sufficient to trigger its intracellular trafficking. A ligand-dependent selective association of β-arrestin1 and β-arrestin2 to the MOP receptor has been recently described (49). Using BiFC, we confirmed that DAMGO induced β-arrestin1 association to the MOP receptor and endocytosis of the complex in our model, but because constitutive association was observed at the membrane of control cells, it was not possible to conclude for NPFF2 receptor activation (data not shown).

**Mass Spectrometric Analysis Reveals a Similar Pattern for Homologous and Heterologous Phosphorylation of MOP Receptor**—The difference in MOP receptor trafficking after DAMGO and 1DMe could be due to quantitative or qualitative differences in the receptor phosphorylation pattern, similarly to what has been suggested for morphine and DAMGO differential effects (38, 50). MOP receptor phosphorylation was thus analyzed by on-line nanoLC-MS/MS LTQ Orbitrap. Receptors were purified from (SH2-D9)MOP-YFP cells treated or not with 1 μM DAMGO or 1 μM 1DMe for 30 min in the presence of 10 μM of protein kinase inhibitors (representative of three independent experiments). Western blot showing the levels of Ser-377 phosphorylation induced by treatment with 1 μM DAMGO or 1 μM 1DMe for 30 min in control cells (C) or cells pretreated with 100 ng/ml of PTX for 18 h. Samples were immunoblotted with anti-Ser(P)-377 antibody followed by anti-actin antibody for normalization. Histogram shows the quantification of Ser-377 phosphorylation, and data are expressed as means ± S.E. of three independent experiments.

**FIGURE 5.** Differential localization of Ser-377-phosphorylated MOP receptors after DAMGO or 1DMe treatment in (SH2-D9)MOP-YFP cells. Cells were treated with buffer alone, 1 μM DAMGO, or 1DMe for 30 or 15 min at room temperature, respectively, before formaldehyde fixation. Total MOP receptors were visualized using the fluorescence of the YFP tag, MOP receptors phosphorylated on Ser-377 were visualized by immuno-fluorescence using the phospho-specific MOP antibody. Confocal images were taken with a Zeiss 710 NLO inverted microscope with ×40 objective as described under “Experimental Procedures.”

**FIGURE 6.** β-Arrestin2 bimolecular fluorescence complementation assay. (SH2-D9)MOP-VC cells were transiently transfected with β-arrestin2-VN. 48 h post-transfection, living cells were incubated at room temperature in KRH buffer alone (Control) or containing 1 μM DAMGO or 1DMe and observed for 30 to 60 min under a Zeiss LSM 510 inverted confocal microscope with ×63 objective at λexc 488 nm. Representative images of the 30-min time point are shown.
sequence with 39.9–41.5% coverage, depending on the sample. No phosphorylation was detected in the parts of the intracellular loops from which tryptic peptides were present, so we focused our analysis on the C-tail. Full coverage was obtained for the 351–408 sequence that starts between the two putatively palmitoylated cysteines (Fig. 7B). The phosphorylated or unphosphorylated peptides identified within this region are presented in Table 1, and the detailed MS/MS spectra of phosphorylated peptides are displayed in supplemental Fig. 4. The effects of agonists were evaluated by calculating the relative abundance ratios for each phosphorylated peptide (see “Experimental Procedures”).

The first peptide, EFCIPTSSNIEQQNSTR, existed as three forms as follows: unphosphorylated; phosphorylated on Ser-365; and phosphorylated on Ser-358. Phosphorylation of Ser-365 (corresponding to Ser-363 in the rodent sequence) was unambiguously identified in control condition (ETD MS/MS spectrum presented in supplemental Fig. 4), whereas the only obtained CID MS/MS spectra did not allow us to discriminate between Ser-365 and Thr-366 for DAMGO and 1DMe treatments. However, the agonists had minimal effect on the relative amount of this phosphopeptide suggesting it is constitutively phosphorylated on Ser-365. The Ser-358 phosphorylated EFCIPTSSNIEQQNSTR peptide was present, albeit in very low abundance, only in the DAMGO sample.

The second peptide, DHPSTANTVDR, containing Ser-377, existed as four forms as follows: unphosphorylated; bearing a single phosphorylation either on Ser-377 or Thr-378, and with a dual phosphorylation on these two residues (Table 1). 1DMe and DAMGO induced an increase in single phosphorylation, in agreement with immunoblotting data focusing on Ser-377. Interestingly, the peptide with dual phosphorylation was only present in agonist-treated samples. It is worth noting that the DAMGO effect was always more intense than the 1DMe effect, especially when considering dual versus single phosphorylation (DAMGO/1DMe abundance ratio = 6.6- and 1.9-fold, respectively).

A third peptide, QNTRDHPSTANTVDR, containing the second peptide sequence extended at its N terminus resulted from a trypsin missed cleavage. Four different forms of this peptide were identified as follows: unphosphorylated; phosphorylated on Thr-372 (corresponding to Thr-370 in the rodent sequence); bearing a dual phosphorylation on Thr-372 and Ser-377 or Thr-378; and a triple phosphorylation on Thr-372, Ser-377, and Thr-378 (Table 1). The nonphosphorylated form was present in very low relative abundance, probably because the missed cleavage was favored only in the presence of a phosphate on Thr-372, adjacent to the digestion site. Similarly to the shorter DHPSTANTVDR peptide, DAMGO and 1DMe treatment favored the appearance of multiphosphorylated species (causing a parallel decrease in monophosphorylated peptides), the triphosphate peptide being present only after agonist treatments (Fig. 7C). Again, although both ligands induced the same qualitative pattern, DAMGO was much more efficacious.
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A two-residue extended IRQNTRDHPSANTVDR peptide containing two missed cleavages was also detected, but its detailed analysis was not presented for the sake of clarity because it behaved exactly as the QNTRDHPSANTVDR peptide described above. Finally, the peptide TNHQLENLEATVPPLPGPMVSK, corresponding to the last 16 amino acids of the C-tail followed by the first 8 residues of the fused YFP, was present in all samples but exclusively in an unphosphorylated form. Unexpectedly, our proteomics analysis thus revealed that stimulation of the NPFF$_2$ receptor induces the same pattern of phosphorylation of MOP receptor as the one induced by direct MOP receptor activation. However, the dual phosphorylation on Ser-377 and Thr-378 shows a strong "selectivity" for DAMGO versus 1DMe with a DAMGO/1DMe abundance ratio of 6.6, and even 20.1, when associated with Thr-372 phosphorylation.

**DISCUSSION**

All GPCRs possess several phosphorylation sites (serine, threonine, and tyrosine) in their intracellular domain (51–53). Differential phosphorylation, tissue-, cell-, or agonist-dependent, has recently been postulated to create a "phosphorylation bar code" that offers a way to regulate signaling and trafficking of GPCRs (52). Such a mechanism could explain, for example, the differences in MOP receptor desensitization and trafficking induced by morphine versus DAMGO (5, 38, 49, 50, 54) or could determine the signaling pathway (PKC$_e$ versus $\beta$-arrestin2) by which MOP agonists activate ERK (55). Cross- or trans-phosphorylation between receptors offers an additional level of complexity to the regulation of GPCR activity and trafficking (56) that could be involved in the functional inhibition of MOP receptors by NPFF receptors. Indeed, activation of NPFF receptors negatively modulates MOP receptor pharmacological effects in vivo, as well as MOP receptor signaling in cellular and neuronal models (17). Because MOP and NPFF$_2$ receptors have been shown to heteromerize in recombinant SH-SY5Y cells (32), we have investigated here the phosphorylation status of MOP receptor in relation to the anti-opioid effect produced by NPFF$_2$ receptor activation in the SH-SY5Y model.

Our results show that, similarly to the MOP agonist DAMGO, the NPFF$_2$ agonist 1DMe induced the phosphorylation of the C-terminal tail of the human MOP receptor, in particular at a residue (Ser-377) known to play a key role in desensitization and internalization of the opioid receptor (35–37). This heterologous phosphorylation was specific to NPFF receptor activation because it did not occur when another $\alpha_2$-adrenergic receptor, was activated. NPFF$_2$-induced MOP receptor phosphorylation on Ser-377 was GRK2-dependent and was associated with a reduction of opioid response on calcium signaling. However, contrary to opioid stimulation that induced recruitment of $\beta$-arrestin2-VN and internalization of MOP receptor, stimulation of the NPFF receptor promoted the association of $\beta$-arrestin2-VN with MOP receptor but not the internalization of the opioid receptor that remained associated with arrestin and phosphorylated on Ser-377 at the plasma membrane. This suggests that the recruitment of $\beta$-arrestin2 is not sufficient for receptor internalization and that NPFF-induced phosphorylation of MOP receptor Ser-377 contributes to the loss of opioid receptor function rather than to the receptor internalization. Finally, mass spectrometric analysis of MOP receptor phosphorylation revealed that 1DMe induced the same pattern of phosphorylation as DAMGO, but with a lower phosphorylation rate, indicating that differences between NPFF and opioid agonist effects do not result from a specific pattern of phosphorylation but rather from a quantitative phosphorylation threshold, or other events not dependent on phosphorylation.

The heterologous phosphorylation of MOP receptors following the activation of other GPCRs (CCCR5 chemokine (57), somatostatin sst2A (27), substance P NK1 (28), and nociceptin (58)) has been described previously but without identification of the phosphorylated residues nor characterization of the kinases involved (except for nociceptin receptor, see below). Our study is the first one that identifies sites of MOP receptor heterologous phosphorylation after activation of another GPCR. It also shows that the NPFF$_2$-induced phosphorylation of MOP receptor on Ser-377 and inactivation of the MOP receptor are mediated by GRK2, a protein kinase described to mediate GPCR homologous desensitization, although it is able to contribute also to heterologous phosphorylation by indirect

**TABLE 1**

List of C-tail MOP receptor phosphorylated and unphosphorylated peptides identified by nanoLC-MS/MS CID and/or ETD analysis from cells treated for 20 min at room temperature with KRH buffer alone (CTRL), 1 nM DAMGO, or 1 nM 1DMe.

| Theo. Mass  | MC | Position  | Sequence | Phospho-residue | Present in sample | Agonist effect |
|------------|----|-----------|----------|----------------|------------------|---------------|
| 2009.9113  | 0  | 351–367   | EFCIPTSSIEQQNSTR Ser-365 | ✓ ✓ | ✓ | No increase 1.1 |
| 2089.8776  | 0  | 351–367   | EFCIPTSSIEQQNSTR Ser-358 | ✓ ✓ | ✓ | Increase 1.9 |
| 2089.8776  | 0  | 351–367   | EFCIPTSSIEQOQNSTR Ser-358 | ✓ ✓ | ✓ | Increase 6.6 |
| 1211.5531  | 0  | 374–384   | DHPISTANTVDR Ser-377 or Thr-378 | ✓ ✓ | ✓ | Increase 1.9 |
| 1291.5194  | 0  | 374–384   | DHPISTANTVDR Ser-377 or Thr-378 | ✓ ✓ | ✓ | Increase 1.9 |
| 1371.4587  | 0  | 374–384   | DHPISTANTVDR Ser-377 or Thr-378 | ✓ ✓ | ✓ | Increase 1.9 |
| 1710.8034  | 1  | 370–384   | QNTRDHPSANTVDR Thr-372 | ✓ ✓ | ✓ | Decrease 0.4 |
| 1790.7697  | 1  | 370–384   | QNTRDHPSANTVDR Thr-372 | ✓ ✓ | ✓ | Decrease 0.4 |
| 1870.7360  | 1  | 370–384   | QNTRDHPSANTVDR Thr-372 or Ser-377 or Thr-378 | ✓ ✓ | ✓ | Increase 2.6 |
| 1950.7023  | 1  | 370–384   | QNTRDHPSANTVDR Thr-372 or Ser-377 or Thr-378 | ✓ ✓ | ✓ | Increase 20.1 |
| 2644.3530  | 0  | 385–408   | TNHQLENLEATVPPLPGPMVSK | ✓ ✓ | ✓ | Increase 20.1 |

* No distinction between Ser-365 and Thr-366 was obtained for the phosphorylation site. All MS/MS spectra of phosphorylated peptides were manually interpreted and are displayed in supplemental Fig. 4. For each peptide, the normalized DAMGO/1DMe relative abundance ratios were calculated from peak area automatically extracted using the label-free module of the in-house developed MFPaQ version 4.0.0 software as described under "Experimental Procedures."
regulation via second messenger-activated kinases (24, 44). Such indirect regulation has been described in the case of MOP and nociceptin receptors in BE(2)-C neuroblastoma cells, where activation of nociceptin receptors potentiates DAMGO-induced phosphorylation of the MOP receptor by GRK2, by a mechanism that necessitates PKC activation and the presence of the MOP agonist (58). In our case, the fact that inhibitors of several protein kinases (PKC, PKA, Src, or EGF receptor tyrosine kinase), all known to stimulate GRK2 activity (44, 45), did not inhibit the 1DMe-induced phosphorylation of the MOP receptor indicates that GRK2 is directly involved in this heterologous regulation. Transphosphorylation within GPCR oligomers has been previously described for rhodopsin (59) and chemokine CCR5 receptor in homo-association or hetero-association with C5a anaphylatoxin receptor (60) and could be a general regulatory mechanism (56). In the case of chemokine receptors, one part of the transphosphorylation was attributed to direct G protein-coupled receptor kinase activation, as observed in our study.

Because MOP and NPFF₂ receptors interact physically in an NPFF-regulated way (32), the heterologous phosphorylation described here could result from the recruitment of GRK2 by activated NPFF₂ receptors within a MOP/NPFF heteromer by two possible mechanisms: (i) GRK2 could be recruited to the activated NPFF₂ receptor (supplemental Fig. 2) and, because of the close proximity of MOP receptor within the complex, could transphosphorylate, with a low efficiency, the C-terminal tail of the opioid receptor; (ii) the dimerization induced by NPFF receptor stimulation could allosterically modify the conformation of MOP receptor toward an activated-like state able to recruit GRK2. In both cases, interaction with a G₁αi protein is a prerequisite because PTX prevents the MOP Ser-377 phosphorylation induced by 1DMe. The G protein could be necessary for the formation and/or the stability of the heteromer as reported for some receptors (61–63). Moreover, although it was originally thought that translocation of GRK2 to the plasma membrane was primarily mediated by interaction with βγ subunits released by activated receptors (44), it has been recently reported that GRK2 could also interact with Ga (64) and could be already present in preassembled receptor-G protein complexes (65). In our case, we have previously observed that activation of NPFF receptors increased the association of Gβγ and some Go subtypes to the MOP receptor (33), and we show here that activated NPFF2 receptors recruit GRK2. However, the precise stoichiometry and dynamic organization of MOP-NPFF₂-G protein-GRK2 complexes, as well as the putative contribution of phosphorylation-independent properties of GRK2 (64–67) to the regulation of MOP signaling, remain to be determined.

We used mass spectrometry to analyze whether DAMGO or 1DMe treatment induced a different pattern of MOP receptor phosphorylation that could explain the differences in MOP receptor trafficking. A recent study used similar techniques to compare the effect of DAMGO and morphine on the phosphorylation pattern of the MOP C-tail (50). As an unexpected result, the phosphorylation pattern of MOP receptor is the same whether it is stimulated by the opioid agonists DAMGO or morphine (50) or by the NPFF agonist 1DMe (this study). However, the phosphorylation level was lower with morphine and 1DMe, suggesting that, in the case of MOP receptor, the specificity of ligand-induced signaling is not encoded by a specific pattern of phosphorylation but rather depends on quantitative variations in phosphorylation, as proposed by Lau et al. (50).

Our study confirms the data from Lau et al. (50), with more precision on the identification of two residues. Like them, we found no phosphorylated residue in the last 16 residues of the MOP C-tail, thus confirming that Thr-396 (Thr-394 in rodent) is not phosphorylated under basal conditions and after DAMGO (or 1DMe) treatment, contrary to what was suggested by indirect studies (68). In agreement with previous studies (38, 50), we found that Ser-365 (Ser-363 in rodent), the main site of MOP receptor phosphorylation by PKC when the kinase is directly activated by phorbol ester (69), was phosphorylated under basal conditions and not affected by DAMGO treatment nor by 1DMe treatment. This is in accordance with the fact that the effects of 1DMe are not sensitive to PKC inhibitors (this study and see Ref. 23). Lau et al. (50) found one phosphorylated residue in the TSST (354–357) human sequence that was subjected to agonist regulation (50). We identified Ser-358 in the equivalent TSS (356–358) rodent sequence, as a phosphorylated residue, only after DAMGO treatment. However, as this phosphopeptide was present at the detection limit, it was not possible to say if this phosphorylation was specific to DAMGO stimulation. In any case, there is some discrepancy in the literature about the TSST motif because MOP receptor phosphorylation, internalization, and β-arrestin2 association are not impaired when this motif is mutated by alanine substitution (36, 50). Finally, similarly to what was shown in HEK cells (50) but with faster kinetics (20 min instead of 3 h), we also found that DAMGO treatment induced multiple phosphorylation on Thr-372 + Ser-377 + Thr-378. Thr-372 (Thr-370 in rodents) and Ser-377 (Ser-375 in rodents) were known from the literature (36, 38, 50) but Thr-378 is identified for the first time by a direct method. This triple phosphorylation was also present, however, in much lower relative abundance compared with DAMGO in morphine- (50) and 1DMe (this study)-treated samples (15- and 20-fold, respectively). Taken together, all these data show that the same phosphorylated residues are identified in both homologous and heterologous conditions. This is consistent with the fact that both treatments are able to induce the recruitment of β-arrestin2 to the MOP receptor. However, dual phosphorylation on Ser-377 and Thr-378 and triple phosphorylation on Thr-372, Ser-377, and Thr-378 show a strong selectivity (up to 20-fold) for DAMGO versus 1DMe that could explain the difference in receptor trafficking.

Of particular interest is the finding that the MOP receptor appears to be similarly regulated by the NPFF agonist 1DMe (this study) and the partial opioid agonist morphine (37, 50). This could have some mechanistic importance because NPFF receptors have been involved in the tolerance to morphine analgesia (7, 8). Both ligands are able to increase the phosphorylation of the same residues in the C-terminal tail of the MOP receptor but to a lower (up to 15–20-fold) extent compared with DAMGO (Ref. 50 and this study). They recruit β-arrestin2 (Ref. 49 and this study), but, in contrast to DAMGO, they promote no or low internalization of MOP receptor, leaving the
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under a phosphorylated state at the plasma membrane (Ref. 37 and this study). Alanine substitution of Ser-375 in the rodent MOP receptor shows that Ser-375 phosphorylation is responsible for morphine-induced desensitization of MOP receptor signaling (adenylyl cyclase inhibition and ERK activation) (37). Here, we show that depletion of GRK2 abolishes both 1DMe-induced phosphorylation of Ser-377 and inhibition of MOP receptor function. These data therefore support the hypothesis that an initial step of Ser-377 (Ser-375) phosphorylation leads to acute MOP receptor desensitization not only in the case of homologous stimulation but also in the case of heterologous stimulation, but a higher level of phosphorylation implicating multiple residues is necessary for internalization. However, in contrast to morphine-induced modification of Ser-375 and desensitization, which were shown to be persistent (37), the Ser-377 phosphorylation and MOP receptor desensitization induced by 1DMe were transient (return to basal after 1 h, Fig. 2, B and D), confirming the recent observation that MOP receptor endocytosis and recycling are not necessary for dephosphorylation and resensitization (3, 38). This also suggests that, while sharing a common initial step, 1DMe effect on opioid signaling is of short duration, whereas morphine can induce prolonged alterations in receptor signaling and trafficking likely to contribute to tolerance (3).

Because NPFF2 and MOP receptors are able to heteromerize, one could have thought that NPFF2 receptor internalization induced by 1DMe could result in the co-internalization of the associated MOP receptor, as suggested for other receptor pairs (27, 28). The absence of co-internalization suggests that the interaction between the two receptors is transient or, in contrast, that the dimer is resistant to internalization, as recently observed for angiotensin AT1–AT2 receptor complexes, specifically visualized by BiFC (70). β-Arrestin is a major partner for desensitization and internalization (3, 44). Its interaction with GPCRs is regulated by receptor phosphorylation, but it also highly depends on receptor-active conformation (71). In the case of MOP receptor regulation, a recent study has shown that for many opioid agonists, including morphine and DAMGO, there is a good correlation between the rate of phosphorylation of Ser-375 and the ability to recruit β-arrestin2 and to induce internalization (72). Accordingly, MOP receptor heterologously phosphorylated by activation of NPFF2 receptor can recruit β-arrestin2, but because its phosphorylation level is too low, and probably also because it does not adopt a proper agonist-active conformation, the receptor–β-arrestin complex could either be not stable enough or in an inappropriate conformation to recruit the endocytic machinery. However, β-arrestin2 can also play a role in desensitization independently from internalization (5, 71) and could therefore contribute to NPFF2-induced MOP receptor loss of function by disrupting G protein activation or by means of its scaffolding properties.

In conclusion, our data show that NPFF2 receptor activation is able to induce the heterologous phosphorylation of MOP receptor on three residues (Thr-372, Ser-377, and Thr-378). In the case of Ser-377, the phosphorylation is mediated by GRK2, is transient, and is associated with the NPFF2-induced decrease in MOP receptor ability to inhibit N-type voltage-gated calcium channels. Phosphorylated Ser-377 is thus the first post-translational modification characterized at the molecular level related to the anti-opioid activity of NPFF agonists. Moreover, our data support the concept that GRK2 plays a role in some type of heterologous desensitization, in addition to its granted contribution to homologous desensitization. The rapid and reversible heterologous regulation described here could be a way for NPFF agonists to switch off MOP receptor signaling in neurons that co-express the two types of receptors. The detailed molecular organization of these events, in particular the possible role of direct protein/protein interactions, remains to be characterized. Considering that opioid tolerance is linked in part to MOP phosphorylation/desensitization, arrestin association, and endocytosis (3), the possibility that the mechanism of heterologous phosphorylation presented here contributes to the regulation of the pharmacological effects of opioids by NPFF analogs in vivo deserves further investigation.

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