Heterologous Regulation of Mu-Opioid (MOP) Receptor Mobility in the Membrane of SH-SY5Y Cells*

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Background: MOP receptor function is presumably linked to a specific dynamic organization in the membrane.

Results: Inhibition of MOP receptor signaling by NPFF2 and α2 receptors is accompanied by diffusion changes, with a particular behavior for heterodimers.

Conclusion: MOP receptor function, diffusion, and confinement are subject to specific heterologous regulation by other GPCRs.

Significance: Specific GPCR regulation is associated with particular dynamic organization in the membrane.

The dynamic organization of G protein-coupled receptors in the plasma membrane is suspected of playing a role in their function. The regulation of the diffusion mode of the mu-opioid (MOP) receptor was previously shown to be agonist-specific. Here we investigate the regulation of MOP receptor diffusion by heterologous activation of other G protein-coupled receptors and characterize the dynamic properties of the MOP receptor within the heterodimer MOP/neuropeptide FF (NPFF2) receptor. The data show that the dynamics and signaling of the MOP receptor in SH-SY5Y cells are modified by the activation of α2-adrenergic and NPFF2 receptors, but not by the activation of receptors not described to interact with the opioid receptor. By combining, for the first time, fluorescence recovery after photobleaching at variable radius experiments with bimolecular fluorescence complementation, we show that the MOP/NPFF2 heterodimer adopts a specific diffusion behavior that corresponds to a mix of the dynamic properties of both MOP and NPFF2 receptors. Altogether, the data suggest that heterologous regulation is accompanied by a specific organization of receptors in the membrane.

The mu-opioid (MOP) receptor belongs to the G protein-coupled receptor (GPCR) family and is involved in the regulation of pain perception and reward pathways (1). The cellular and pharmacological activities of the MOP receptor are modulated by other GPCR systems (2–5). In some case, such heterologous regulation involves receptor heteromerization (6), which is considered to confer new binding and endocytosis properties to the complex and/or to promote novel signaling pathways or, conversely, to impair signal transduction (7, 8). The heteromerization of the MOP receptor with other opioid receptors, DOP, KOP, and NOP (9–12) or with non-opioid receptors such as α2-adrenergic (13–15), somatostatin SST2A (16) or substance P NK1 (17) receptors has been reported. By using FRET and immunoprecipitation methods, we have also provided evidence for a molecular interaction between the MOP receptor and the receptor for the opioid-modulating peptide neuropeptide FF (NPFF) (18). NPFF receptor activation not only promotes MOP/NPFF receptor interaction as revealed by an increased FRET signal but also induces MOP receptor desensitization by a new mechanism involving a G protein-coupled receptor kinase GRK2-dependent transphorylation of the human MOP receptor Ser577 (19). This heterologous desensitization mechanism probably constitutes the molecular basis underlying the cellular anti-opioid activity exerted by the NPFF receptor on the opioid inhibition of voltage-gated Ca2+ channels, previously observed in neurons (20–22) or in neuronal cell models (23, 24), that is suspected to contribute to the In vivo modulation of opioid analgesia (4, 25–27), or opioid reward (28–32), by NPFF analogs.

Much evidence suggests that GPCR function as homo- or heteromers in the context of specific receptor-based multiprotein complexes including G proteins, effectors, arrestins, regulatory, or scaffolding proteins (33). Such signaling platforms are considered to be assembled in specialized membrane microdomains, allowing a better efficacy and specificity of signal transduction (34, 35). In recent years, the development of biological fluorescent probes and of light microscopy-based techniques such as fluorescence recovery after photobleaching (FRAP (36)), fluorescence correlation spectroscopy (FCS (37)), and single particle tracking (SPT (38)) helped to reveal the importance of the lateral diffusion of receptors in the plasma membrane. Various models of GPCR membrane organization and associated trajectories have been elaborated (39). GPCRs are able to freely diffuse over long distances in the membrane, but their movement can also be restricted within membrane...
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microdomains. Such confinement may have different origins: fences generated by cytoskeleton filaments and anchored proteins, preferential distribution into particular areas enriched in cholesterol and saturated lipids called “rafts,” or high affinity protein–protein interactions generating cluster phases (39–42).

In this context, the functional and dynamic organization of the MOP receptor in the membrane and the influence of the lipid environment on its specific membrane location and its interaction with signaling effectors have been well documented (1, 43, 44). In particular, SPT and FRAP experiments performed at variable radius (vrFRAP) have revealed a confined mode of diffusion for the MOP receptor in microdomains with a radius size of ~1 μm (18, 38, 45, 46). The domain size and/or diffusion parameters of the MOP receptor are modulated upon agonist or antagonist administration (45–47), suggesting that a selective pharmacological response could be associated with a specific dynamic organization of the receptors in the membrane.

These parameters are also influenced by changes in membrane composition such as cholesterol depletion or caveolae enrichment (47, 48) or by co-expression of another receptor such as the DOP receptor (49). Interestingly, we have previously reported that activation of the NPFF₂ receptor suppresses the confinement of the MOP receptor and increases its lateral mobility (18). This loss of constraint was suspected to impair interaction of the MOP receptor with signaling partners, contributing to the reduced opioid response in the presence of the anti-opioid peptide.

Altogether, these data indicate that the diffusion of the MOP receptor is influenced by factors modulating its function, which strongly suggests that the diffusion behavior and the functional state of GPCR are linked. Therefore, the purpose of the present study was to investigate whether the regulation of MOP receptor activity by the activation of different GPCRs (NPFF, α₁-adrenergic, and NPY) in SH-SY5Y cells could be associated with a change in its lateral mobility in the plasma membrane. In addition, by using bimolecular fluorescence complementation (BiFC) coupled to vrFRAP, we analyzed whether a particular dynamic behavior could be specific to the heteromeric MOP/NPFF₂ receptor complex.

EXPERIMENTAL PROCEDURES

Materials—The NPFF analog 1DMe ([D-Tyr¹, (NMe)Phe³]NPFF) was synthesized using an automated peptide synthesizer (model 433A; Applied Biosystems). DAMGO (Tyr-D-Ala-Gly-(NMe)-Phe-Gly-ol) was purchased from Bachem (Switzerland), NPY was from Polypeptide laboratories (France), clonidine was from Sigma (France), and naloxone was from Francopia (France). The fluorescent lipid didodecylphosphatidylethanolamine-7-nitrobenz-2-oxa-1,3-diazole-4-yl (dC₁₂-PE-NBD) was home synthesized (50).

Construction of Expression Vectors for MOP/NPFF₂ Receptor BiFC—PCR was used to amplify cDNAs coding Venus protein fragments 1–154 (Vn) and 155–238 (Vc) from a vector containing a full size Venus protein cDNA. PCR primers were designed to introduce a Ncol restriction site and a short sequence coding for a 8-amino acid linker (DGGSNGGS) at the 5’ end, and a stop codon and an XbaI site at the 3’ end of the amplified fragments.

The Vn and Vc fragments were then cloned in frame with human MOP or NPFF₂ receptors, respectively, in pBluescript II SK⁺ vectors (Stratagene, Agilent Technologies). The MOP-Vn construct was inserted into the EcoRV-XbaI sites of the mammalian expression vector pEFIB3 bearing the blasticidin selection marker. The NPFF₂-Vc construct was inserted into the EcoRV-XbaI sites of the mammalian expression vector pEFIN3 bearing the neomycin selection marker. All constructs were verified by sequencing (Mileneg, France). CD8 fused to Vn (generous gift from J. Javitch, Columbia University, New York) was used as a nonbinding protein control in BiFC experiments, by transfection of HEK293 cells with FuGENE 6 (Roche Diagnostics).

Cell Culture and Transfection—SH-SY5Y cells were grown in Dulbecco’s modified Eagle’s medium (4.5 g/liter glucose, GlutaMAX) containing 10% fetal calf serum and 50 μg/ml gentamicin (Invitrogen), in a 37 °C humidified atmosphere containing 5% CO₂. For cells transfected with the human NPFF₂ receptor (SH2-D9 cells), the medium was supplemented with 400 μg/ml G418 (Invitrogen); for cells expressing the human NPFF₂ receptor in addition to the YFP tagged MOP receptor (SH2-D9MOP-YFP cells), 2 μg/ml blasticidin (Cayla, France) was further added to maintain selection (18). Cells expressing the NPFF₂-YFP receptor were cultured in the presence of 5 μg/ml blasticidin. The stable SH-SY5Y cell line expressing the MOP/NPFF₂ BiFC fusion protein was obtained by a two-step transfection procedure using FuGENE 6 (Roche Diagnostics). A stable cell line expressing the NPFF₂-Vc receptor was first isolated with blasticidin (2.5 μg/ml) selection, characterized, and then transfected again with the MOP-Vn construct. After selection with 400 μg/ml G418 and 2.5 μg/ml blasticidin, the clone C2 was chosen based on pharmacological assays and expression at the plasma membrane.

Binding Experiments and cAMP Assays—Membranes were prepared as previously described (24). [³H]DAMGO (50 Ci/mmol; Perkin Elmer) and [³H]EYF (72 Ci/mmol, custom-made by RC TRITEC AG, Teufen, Switzerland) were used to specifically label MOP and NPFF₂ receptors, respectively, using binding protocols reported for [³H]DAMGO (24) and [³H]EYF (51). cAMP assays were performed as previously described (24).

Intracellular Calcium Measurement—The intracellular calcium content was monitored in living cells, by calcium imaging using the fluorescent Ca²⁺ indicator Fluo-4 AM (Invitrogen), as previously described (24). Briefly, after loading cells for 30 min at 37°C in the dark with 3.6 μM Fluo-4 AM, they were perfused with HEPES-buffered medium, pH 7.3, containing 10 mM HEPES, 150 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 0.1% BSA, and 3 mM nifedipine to block L-type Ca²⁺ channels. Depolarization was induced by perfusing cells for 10 s with HEPES-buffered medium modified to contain 140 mM KCl and 12.5 mM NaCl. The increase in fluorescence caused by Ca²⁺ entry was monitored at 488-nm excitation wavelength through a 40×/NA0.65 objective, by using a cooled charge-coupled device camera (MicroMax 782 Y; Princeton Instruments) driven by MetaView software (Universal Imaging Corporation). For acute stimulation with agonists, 5 min after the first depolarization, a second depolarization was applied at the end of a 30-s perfusion in the presence of the
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1. For a given observation radius, the apparent diffusion coefficient \( D_{\text{app}} = R^2/4\pi D \) and the mobile fraction \( M \) characterizing the fluorescent molecules that can diffuse through the bleached area (54, 55) were deduced from the recovery curves of 15–20 cells/cover slip and averaged. The results are expressed as means ± S.E. of \( M \) and \( D \) values/cover slip from several independent experiments. The data from FRAP experiments at variable observation radius (vrFRAP) were processed according to the conceptual analysis previously detailed (36, 50), which predicts the existence of domains with restricted diffusion if the \( M \) values linearly decrease as a function of the inverse of the radius \( R \) of the bleached area, together with the quadratic variation of \( D \). In this case, the radius of the diffusion domain \( r \) is deduced from the relationship between \( M \) and \( R \) given by the following equation,

\[
M = 0.634 (r/R) + M_p \quad \text{(Eq. 1)}
\]

where \( M_p \) is independent of \( R \) and represents the permanent mobile fraction able to freely diffuse over long distances through the domains. The diffusion coefficient \( D_{\text{conf}} \) in the confined domain can be calculated from the following equation.

\[
D_{\text{conf}} = 1/2D_{\text{app}}(r/R)^2 \quad \text{(Eq. 2)}
\]

Analysis of the Data—Nonlinear regression and statistical analysis of the data were performed using Prism 5.03 (GraphPad Software Inc, USA).

RESULTS

Modulation of \( Ca^{2+} \) Channels by Endogenously Expressed MOP, \( \alpha_2 \)-Adrenergic, and NPY Receptors, as Well as Transfected NPFF2 Receptors, in SH-SY5Y Cells—SH-SY5Y cells naturally express the Gi/o-coupled MOP, \( \alpha_2 \)-adrenergic, and neuropetide Y (NPY) receptors (56–58). These cells were transfected with the human NPFF2 receptor to give the SH2-D9 cell line with receptor expression (~300 fmol/mg of protein) in the same range as the endogenous opioid and adrenergic receptors (24).

As expected for \( G_{\text{i/o}} \)-coupled receptors, stimulation with opioid, NPY, and NPFF agonists inhibits voltage-dependent \( Ca^{2+} \) channels, characterized as N-type, in SH-SY5Y or SH2-D9 cells (24, 57). A 30-s application of the opioid agonist DAMGO (0.1 \( \mu \)M), the NPFF analog 1DMe (1 \( \mu \)M), the adrenergic agonist clonidine (10 \( \mu \)M), and NPY (1 \( \mu \)M) reduced the depolarization-evoked calcium entry into cells by 46.5 ± 4.1, 54.1 ± 2.2, 35.2 ± 5.6, and 43.7 ± 4.3%, respectively (Fig. 2A), indicating that these receptors were present and functional in our cell model. When the effect of 0.1 \( \mu \)M DAMGO was tested after a preincubation of the cells with NPFF, \( \alpha_2 \)-adrenergic, or NPY agonists, the opioid response was inhibited by 80% with 1DMe and by 47.5% with clonidine but was not modified by NPY (Fig. 2B). This indicates that NPFF and \( \alpha_2 \)-adrenergic, but not NPY, receptors are able to functionally antagonize MOP receptor activity.

Regulation of MOP-YFP Receptor Mobility in the Membrane of (SH2-D9)MOP-YFP Cells by Activation of NPFF, \( \alpha_2 \)-Adrenergic, and NPY Receptors—To investigate whether the lateral mobility of the MOP receptor could be affected by the activa-
tion of other GPCRs, FRAP experiments were conducted in SH2-D9 cells transfected with YFP-tagged MOP receptors. The fluorescence of an area (3.17 μm radius) at the surface of cells was photobleached, and the recovery of fluorescence upon time into this area was monitored (example given in Fig. 1). The mobile fraction \( M \) and the apparent diffusion coefficient \( D_{\text{app}} \) characterizing the surrounding receptors able to diffuse into the bleached area were deduced from the analysis of the recovery curves. In the absence of ligand (control), the \( M \) value measured for MOP-YFP receptors was equal to 61.2 ± 0.7% (Fig. 3A). This value was significantly enhanced by 9 and 10% in the presence of 1 μM 1DMe (\( M = 66.5 \pm 0.8\% \)) or 10 μM clonidine (\( M = 68 \pm 1\% \)), respectively, whereas it was unchanged in the presence of 1 μM NPY (62.2 ± 0.9%). The apparent diffusion coefficient of MOP-YFP receptors (\( D_{\text{app}} = 0.62 \pm 0.04 \mu m^2/s \)) was significantly reduced only in the presence of 10 μM clonidine (\( D_{\text{app}} = 0.43 \pm 0.03 \mu m^2/s \)) (Fig. 3B). The modulatory effects observed with 1DMe and clonidine were not likely due to a nonspecific perturbation of membrane fluidity, because the diffusion parameters of the fluorescent lipid dC12-PE-NBD (\( M = 70.3 \pm 1.8\% \), \( D_{\text{app}} = 1.14 \pm 0.21 \mu m^2/s \)) in the membrane of SH2-D9 cells were not modified by these agonists (Fig. 3, C...
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As shown in Fig. 4, these effects were also not due to MOP-YFP receptor endocytosis because 1DMe and clonidine, in contrast to DAMGO, did not induce the internalization of MOP-YFP receptors. Altogether, these data indicate that the activation of NPFF and adrenergic, but not NPY, receptors is able to specifically modify the mobility of MOP receptors.

To further test whether the modifications of MOP receptor diffusion induced by 1DMe and clonidine required the activation of G proteins, we performed the same FRAP experiments after overnight treatment with 100 ng/ml pertussis toxin (PTX). In cells treated with the toxin (Fig. 5A), the NPFF and adrenergic agonists increased MOP receptor mobility, but this change remained significant only for clonidine treatment (63.8 ± 2.7% in the presence of clonidine versus 56.8 ± 1.1% in the absence of agonist), suggesting that the effect of the NPFF agonist 1DMe on the modulation of MOP receptor mobility is more sensitive to PTX than that of the adrenergic agonist. Because the toxin did not completely abolish the effect of agonists, we evaluated whether conformational changes resulting from direct receptor/receptor interaction could also be involved in this process by conducting FRAP experiments in the presence of the opioid antagonist naloxone. As shown in Fig. 5B, naloxone (1 μM) did not affect MOP receptor mobility (M = 62.7 ± 1.1% in control versus 62.3 ± 1.4% in naloxone treated cells). In the presence of the antagonist, M values were not enhanced by the application of 1DMe (M = 61.9 ± 1.9%) or clonidine (M = 59.1 ± 1.5%), indicating that naloxone prevented 1DMe- and clonidine-induced increase in MOP receptor mobility. Because the opioid antagonist exhibits no affinity for NPFF2 and α2-adrenergic receptors (0 and 15% inhibition of the binding of [3H]EYF and [3H]RX821002 in the presence of 1 μM naloxone, respectively), this indicates that the antagonist-occupied MOP receptor is more resistant to heterologous regulation than the unoccupied MOP receptor.

Characterization of the MOP/NPFF2 BiFC Receptor—The fact that only the activation of receptors known to heteromerize with MOP receptor produces a change in the diffusion parameters of the opioid receptor prompted us to propose that heteromerization probably plays a part in this regulation. To investigate whether a specific dynamic behavior could be attributed to a particular heterodimer, we have generated a fluorescent MOP/NPFF2 receptor heterodimer by BiFC, which allows to specifically visualize the heterodimer in living cells. As shown in Fig. 6 (A and B), cells expressing MOP receptors fused to the nonfluorescent N-terminal fragment of Venus (MOP-Vn) and NPFF2 receptors fused to the nonfluorescent C-terminal fragment of Venus (NPFF2-Vc) gave rise to Venus fluorescence complementation expressed at the surface of cells, whereas cells transfected with MOPVn or NPFF2Vn and the nonbinding protein control CD8Vc did not exhibit fluorescence complementation (Fig. 6A). This indicates that the fluorescence complementation observed at the plasma membrane was actually...
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due to the specific formation of heterodimeric MOP/NPFF₂ complexes and not induced by nonspecific Vn/Vc interaction.

Incubation of cells with 1 μM 1DMe or DAMGO for 30 min at room temperature induced endocytosis of the fusion protein, as revealed by a decrease of the fluorescence at the plasma membrane and an increase of highly fluorescent intracellular punctae (Fig. 7). This indicates that the receptors within BiFC heterodimers are still responsive to their ligands. This co-internalization could seem in discordance with the data on Fig. 4, but in the case of BiFC, co-internalization occurs because of the high stability of the BiFC complex, in contrast to what is observed in Fig. 4 where the heterodimer is likely to be transient (59–62). Expression levels of receptors were measured by saturation binding experiments. The specific MOP receptor radioligand [³H]DAMGO labeled 2.7 ± 0.2 pmol/mg protein with a $K_D$ of 0.1 ± 0.2 nM ($n = 3$), and the specific NPFF₂ receptor radioligand [³H]EYF labeled 1.5 ± 0.2 pmol/mg protein with a $K_D$ of 2.5 ± 0.5 nM ($n = 3$), thus indicating similar expression levels of both protomers and conserved high affinity. Unfortunately, there is no reliable means enabling the quantification of heteromeric versus monomeric receptor ratio. As shown in Fig. 6C, receptors were also fully functional in the cAMP assay. The EC₅₀ for DAMGO (1.0 ± 0.6 nM) and 1DMe (0.9 ± 0.4 nM) were similar to those measured on MOP-YFP (0.4 ± 0.1 nM) and NPFF₂-YFP (1.9 ± 0.4 nM) receptors expressed alone in SH-SY5Y cells.

vrFRAP Analysis of the Diffusion Mode of the MOP/NPFF₂ BiFC Receptor in Comparison to MOP-YFP and NPFF₂-YFP Receptors—vrFRAP is a method that enables identification of the presence of microdomains and to characterize their size ($r$) and the diffusion coefficient ($D_{conf}$) within these domains (36). We used this resolutive method to compare the mode of diffusion of the MOP/NPFF₂ BiFC receptor with those of MOP-YFP and NPFF₂-YFP individual receptors. As shown in Fig. 8, linear relationships between $M$ and $1/R$ were obtained for all three constructs, indicating a domain organization of receptors in the membrane. However, the diffusion characteristics deduced from the linear regressions were different (Table 1), revealing a specific dynamic confinement for each receptor. As previously observed (18, 46), the MOP-YFP receptor was found to diffuse

![FIGURE 6. Characterization of the MOP/NPFF₂ BiFC receptor. A, fluorescence complementation 48 h after transient transfection of HEK293 cells with various combinations of transmembrane receptors fused to Venus fragments (MOPVn/NPFF₂Vc, CD8Vn/MOPVc, or CD8Vn/NPFF₂Vc). Cell density was similar in each condition. Fixed cells were observed at λₐc 514 nm with a 40× oil immersion objective on a confocal microscope (Leica TCS SP5). B, confocal image of the stable SH-SY5Y cell line expressing the MOP/NPFF₂ BiFC receptor showing expression at the plasma membrane. C, dose-response curve of the inhibition of forskolin-induced intracellular cAMP accumulation by 1DMe and DAMGO in SH-SY5Y cells expressing the MOP/NPFF₂ BiFC receptor. Points represent means ± S.E. of at least three experiments performed in duplicate.](https://example.com/figure6)

![FIGURE 7. Internalization of the MOP/NPFF₂ BiFC receptor in SH-SY5Y cells, visualized by confocal microscopy. Cells expressing the MOP/NPFF₂ BiFC receptor were incubated for 30 min at room temperature in HEPE-S-buffered medium, in the absence (control) or the presence of 1 μM 1DMe or 1 μM DAMGO. Fixed cells were observed at λₐc 514 nm with a 40× NA1.4 oil immersion objective on a confocal microscope (Olympus FV1000).](https://example.com/figure7)

![FIGURE 8. Comparison of the lateral mobility and domain sizes of MOP-YFP, NPFF₂-YFP, and MOP/NPFF₂ BiFC receptors in the membrane of SH-SY5Y cells by FRAP experiments at variable radius. FRAP experiments were conducted for 30 min at 20–22 °C, by using a set of diaphragms allowing observation of bleached areas of radius 1.88, 2.16, 2.80, 3.17, and 3.92 μm. For each condition, recovery curves from 15–20 cells were recorded per coverslip, and the deduced $M$ values were averaged. Points represent means ± S.E. of the $M$ values from 7–14 coverslips, plotted as a function of the inverse of the illumination radius $R$.](https://example.com/figure8)
TABLE 1
Lateral diffusion parameters of MOP-YFP, NPFF2-YFP, and MOP/NPFF2 BiFC receptors in the membrane of SH-SY5Y cells

|           | M p   | r      | D out       |
|-----------|-------|--------|-------------|
| MOP-YFP   | 33 ± 2 | 1.36   | 5.5 ± 0.8   |
| NPFF2-YFP | 9 ± 3  | 1.88   | 15.4 ± 1.9  |
| MOP/NPFF2 BiFC | 7 ± 3 | 1.3    | 4.6 ± 0.2   |

*p < 0.001; different from the two other values (one-way ANOVA followed by Dunnett’s multiple comparison test).

The present study aimed to explore whether the functional heterologous regulation of GPCR, in the case of heteromeric partners, is related to a specific dynamic behavior of receptors in the membrane, by considering the MOP receptor as an example. NPFF2 and α2-adrenergic receptors are known to modulate opiate analgesia in vivo (63, 64), to heterodimerize with MOP receptors (13, 18, 65), and to affect MOP receptor signaling in cell models (13, 24), as well as in neurons (13, 14, 20, 22). We show here that the activation of endogenous α2-adrenergic receptors, similarly to transfected NPFF2 receptors, inhibits the opioid modulation of voltage-gated Ca2+ channels in SH-SY5Y cells, as observed previously in neurons (14, 20, 22). This effect is accompanied by a change in the diffusion parameters of MOP receptors in the membrane. In contrast, NPY receptors, which are also endogenously expressed and active in these cells but do not modulate opioid response (Fig. 2) and are not known to form heterodimers with MOP receptors (66), have no effect on the diffusion of the MOP receptor. Because NPY receptors activate the same type of G protein as NPFF2 and α2-adrenergic receptors, this means that triggering Gαi/o signaling is not sufficient to modify MOP receptor dynamics. Therefore, knowing that agonists induce heterodimer formation between MOP receptors and α2-adrenergic (13) and NPFF2 receptors (18), our results suggest that heterologous regulation of the MOP receptor through heteromerization impacts its dynamic organization in the membrane.

Clonidine and 1DMe were found to increase the mobile fraction of MOP receptors in the membrane, with a slight decrease of the apparent diffusion coefficient in the case of the adrenergic agonist only. This contrasts with what is generally observed when receptors are activated by their own agonists. For a majority of GPCR, the binding of agonists decreases the mobility of receptors by reducing their diffusion coefficient and/or by restricting their movement in smaller membrane domains (39, 46, 67). This probably reflects the early events preceding endocytosis processes. In previous FRAP experiments performed at 14 °C to avoid endocytosis in SH-SY5Y cells, two distinct populations of MOP receptors were observed in the presence of DAMGO, one diffusing freely with a fast diffusion coefficient, and the other confined in isolated closed domains smaller than those without ligand. The effect of DAMGO was not sensitive to PTX but was abolished by high sucrose, suggesting that it was related to the endocytosis process (46). A more recent study on neuropeptide Y (NPY1 and NPY2) receptors, combining FCS associated with photon counting and BiFC, showed that the NPY-induced receptor slowing down and clustering (measured as an increase in particle brightness) was due to the recruitment of β-arrestin, the first step before internalization (67). On the other hand, the binding of antagonists has been generally described to produce no modification of the lateral mobility of receptors (37, 39, 46, 67, 68), as we observed here with naloxone. It has to be noticed that one study investigating MOP receptor diffusion by FCS showed the opposite behavior for agonists and antagonists, but these observations were acquired under endocytosis conditions (47). Our finding that heterologous regulation induces a change in MOP receptor diffusion different from that described upon homologous stimulation is consistent with the fact that 1DMe and clonidine do not induce MOP receptor internalization in SH-SY5Y cells (this study and Ref. 18). A lack of internalization of MOP receptors after α2 receptor activation was also observed in transfected HEK cells (65), whereas co-internalization of endogenous receptors was found to occur in DRG cells (14), pointing to a possible dependence on the cell model.

The increase of MOP receptor mobility observed in the presence of adrenergic or NPF agonists should thus reflect a specific rearrangement of the domain organization of the opioid receptor in the plasma membrane. MOP receptors have been described to partition into lipid microdomains (rafts) in HEK cells (69), but this is not suspected to be the case here, because MOP receptors were not found to be localized in detergent-resistant membrane fractions from SH-SY5Y cells (46, 70). Rather, interprotein interactions are thought to be responsible for the confinement of MOP receptors. As a consequence, the enhanced MOP receptor mobility observed upon heterologous regulation by NPFF and adrenergic agonists reflects a less constrained diffusion that is most probably due to changes in the interactions of the opioid receptor with signaling partners contributing to impair cellular opioid response. This scenario is supported by our previous observation that NPFF receptor activation by 1DMe modifies the content and the nature of the G protein subunits associated with the MOP receptor, as revealed by Western blots after MOP receptor immunoprecipitation (71). Also, the stimulation of NPFF receptors induces the recruitment of β-arrestin to the MOP receptor with a retention at the plasma membrane and no endocytosis, as observed by
confocal imaging of fluorescence complementation between the MOP receptor and β-arrestin (19). Finally, as for glycine and kainate receptors where phosphorylation drives the dynamic exchange between synaptic and extrasynaptic location (72, 73), such post-translational modifications could play a role in MOP receptor mobility changes. As described below, this probably occurs in the case of the MOP/NPFF receptor couple.

Even if both 1DMe and clonidine enhance MOP receptor mobility, the mode of regulation by the two agonists could be different as indicated by PTX experiments showing that 1DMe is more sensitive to the toxin than clonidine. This is consistent with the fact that different mechanisms have been reported to explain the loss of MOP receptor function induced by NPFF and α2-adrenergic receptors. We have previously shown that 1DMe promotes a rapid and transient phosphorylation of the Ser377 residue in the human MOP receptor C-terminal tail, a residue known to be involved in the homologous desensitization of the opioid receptor (19). This heterologous phosphorylation is abolished by PTX treatment or GRK2 knockdown. Because no other kinase, including G protein-dependent kinases, were found to be involved, it is suspected that the G11 protein is principally required to form or to stabilize the MOP/NPFF heterodimer, as is the case for MOP/DOP heterodimers (74). In contrast, clonidine was not found to phosphorylate the MOP receptor on Ser377 in SH-SY5Y cells (19). The cross-talk between α2-adrenergic and MOP receptors involves a mutual cross-desensitization of receptors (13, 75), not sensitive to PTX (15) and shown to implicate β-arrestin and p38 MAP kinase in DRG neurons (14). It has been also shown by intramolecular FRET that a rapid and direct, PTX-insensitive, conformational switch between MOP and α2-adrenergic receptors occurs upon agonist stimulation and could stabilize an inactive conformation of receptors (15). This later hypothesis may explain why in the presence of the opioid antagonist naloxone, clonidine failed to increase the mobility of the MOP receptor, which may be refractory to conformational change when occupied by an antagonist. Although the detailed mechanisms involved in the regulation of MOP receptors by NPFF2 receptors are different, a conformational switch may also occur for this heteromer because the increase in MOP receptor mobility induced by 1DMe was also abolished by the opioid antagonist. Thus, agonist-promoted heterodimerization within the plasma membrane could induce conformational changes of MOP receptors that either directly affect the dynamic properties of the heteromeric complex or indirectly through post-translational modification of MOP receptors.

Analysis by vrFRAP of the domain organization of MOP, NPFF2, and MOP/NPFF2 BiFC receptors in the membrane revealed differences likely to indicate that receptors are localized in different domains or that they are prone to different interprotein interactions. Compared with NPFF2 receptors, MOP receptors were found to exhibit a 3-fold lower diffusion coefficient and to reside in smaller domains. This could reflect either tighter interactions with partners contributing to crowding, or a localization of MOP receptors in a different membrane environment with higher viscosity. Also, oligomerization of MOP receptors (49, 76) could account for the slower diffusion. Knowing that a large variation of mass is necessary to induce only a moderate change of the diffusion coefficient (77), it would implicate that other proteins such as G proteins are associated with these oligomers and contribute to increase friction. The higher mobility of NPFF2 receptors in larger domains indicates a less crowded environment than that of MOP receptors, delimited by physical barriers or the tethering to a partner because the low $M_p$ value indicates a markedly reduced probability to escape from them.

Access to the dynamic properties of receptor dimers is not easy because they have been described to transiently associate and dissociate (59–62). Although the BiFC approach might not reflect the complexity of receptor interactions, because it quasi-irreversibly stabilizes the heterodimer, it nevertheless allows to characterize the diffusion parameters of the dimeric complex. Here, the combination of vrFRAP experiments with BiFC offers the great opportunity to specifically investigate the dynamic properties of the MOP/NPFF2 heterodimer. By using for the first time this approach, we show that the fluorescent MOP/NPFF2 heterodimer adopts a specific diffusion behavior that corresponds to a mix of the dynamic properties of both MOP and NPFF2 receptors. The diffusion coefficient and domain size of the MOP/NPFF2 BiFC receptor were found to be similar to those of the MOP receptor, suggesting that the heterodimer is located in the same membrane environment as the MOP receptor or is part of opioid receptor oligomers as observed for the MOP/DOP heteromer in Neuro2A cells (49). However, the low $M_p$ value indicates that the presence of the NPFF2 protomer prevents the BiFC receptor from moving out of the domains, in contrast to MOP receptors. The exact description of the domain organization of the MOP/NPFF2 dimer is difficult to provide without complementary analyses such as for example SPT to determine the exact trajectories of each receptors or dual color FRAP to monitor two receptors at the same time (78). However, it is interesting to note that another study combining BiFC with FCS analysis to compare the diffusion behavior of homo- and hetero-dimers between A1 or A2 adenosine receptors (79) also showed that the heterodimeric A1/A2 BiFC receptor exhibited a different diffusion behavior from homodimeric receptors, which was explained by a different oligomeric clustering or a change in membrane environment. Altogether, these data therefore support the idea that the diffusion of heteromeric receptors exhibits characteristic features specific to the GPCR pair.

The dynamics of MOP receptors in the membrane has previously been shown to be subject to regulation by homologous stimulation in a manner specific to the nature of the agonist, suggesting that functional selectivity could be linked to particular diffusion characteristics of receptors in the membrane. Here we show that heterologous activation of receptors able to heterodimerize with MOP receptors, namely NPFF2 and α2-adrenergic receptors, leading to functional inhibition of MOP receptors, is also accompanied by specific changes in the diffusion behavior of MOP receptors. Our data also suggest that a particular dynamic behavior may be associated with a given heteromeric pair of receptors. We can therefore conclude that homologous and heterologous regulation of receptors are asso-
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correlation with specific changes in their diffusion properties in the membrane.

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