Biochemical and Biophysical Research Communications 373 (2008) 80–84

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Neuropeptide FF-sensitive confinement of mu opioid receptor does not involve lipid rafts in SH-SY5Y cells

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ARTICLE INFO

Article history:
Received 27 May 2008
Available online 9 June 2008

Keywords:
Opioid
Neuropeptide FF
Lipid rafts
Membrane fractionation
Cholesterol

ABSTRACT

Mu opioid (MOP) receptor activation can be functionally modulated by stimulation of Neuropeptide FF 2 ($NPFF_2$) G protein-coupled receptors. Fluorescence recovery after photobleaching experiments have shown that activation of the $NPFF_2$ receptor dramatically reduces the fraction of MOP receptors confined in microdomains of the plasma membrane of SH-SY5Y neuroblastoma cells. The aim of the present work was to assess if the direct observation of receptor compartmentation by fluorescence techniques in living cells could be related to indirect estimation of receptor partitioning in lipid rafts after biochemical fractionation of the cell. Our results show that MOP receptor distribution in lipid rafts is highly dependent upon the method of purification, questioning the interpretation of previous data regarding MOP receptor compartmentation. Moreover, the NPFF analogue 1DMe does not modify the distribution profile of MOP receptors, clearly demonstrating that membrane fractionation data do not correlate with direct measurement of receptor compartmentation in living cells.

Neuropeptide FF (NPFF), FLFQPQRFamide, belongs to a family of neuropeptides who, among other roles, modulate the opioid system [1–3]. Peptides issued from two precursors, pro-$NPFF_A$ and pro-$NPFF_B$, bind specifically to two G protein-coupled receptors, $NPFF_1$ and $NPFF_2$ [4]. Studies on isolated neurons have shown that the anti-opioid activity of NPFF could result from a cross-talk between NPFF and opioid systems within the same cell (for review see [5]). In order to study the mechanism of NPFF anti-opioid activity SH-SY5Y neuroblastoma cells that endogenously express opioid receptors were transfected with the human $NPFF_2$ receptor. In this cellular model, NPFF analogs functionally antagonize the inhibition of N-type voltage gated calcium channels by μ and δ opioid ligands [6]. Moreover, fluorescence energy transfer studies revealed that $NPFF_2$ and μ opioid (MOP) receptors could form heteromers [7]. Finally, fluorescence recovery after photobleaching at variable observation radius (vrFRAP) experiments were used to follow the lateral diffusion of YFP-tagged MOP receptors in the plasma membrane. These analyses showed that about 54% of MOP receptors were compartmentalized under control conditions and that treatment with 1DMe ([D-Tyr^1, (NMe)Phe^3]neuropeptide FF), a $NPFF_2$ receptor agonist, increased the percentage of freely diffusing MOP receptors to about 80% [7], indicating that activation of NPFF receptors could change the lateral mobility behavior of MOP receptors.

MOP receptor compartmentation could be due to lipid raft microdomains that are formed by clustering of cholesterol and sphingolipids, resulting in liquid-ordered lipid phases that could serve as signaling platforms [8,9]. Several G protein-coupled receptors [10,11] including μ, δ and κ opioid receptors [12–19] have been proposed to be enriched in such domains, based on membrane fractionation and cholesterol depletion experiments. The fact that MOP receptor lateral diffusion was modified by 1DMe treatment in our cellular model gave us a unique opportunity to test the equivalence between direct measurement of receptor compartmentation by fluorescence techniques in living cells and indirect estimation of receptor distribution by biochemical fractionation of the membrane. Our results show that MOP receptor partitioning is highly dependent upon the method used to purify lipid rafts, questioning the interpretation of previous data obtained from membrane fractionation analyses. Moreover, our data clearly demonstrate that membrane fractionation data do not correlate with direct measurement of receptor compartmentation in living cells.

Materials and methods

Vector constructions. The YFP cDNA (generous gift from R. Tsien, UCSD) was transferred from a pBluescript II SK^- YFP construct [7] to the 3′-end of the human $NPFF_2$ receptor cDNA also in pBluescript II SK^-using NcoI and XbaI restriction sites. The construct was then inserted into the EcoRV–XbaI sites of the mammalian expression vector pEFIB3 bearing the blasticidin selection marker. The construct was verified by sequencing (Genome Express, Meylan, France).

Abbreviations: 1DMe, [D-Tyr^1, (NMe)Phe^3]neuropeptide FF; DRM, detergent resistant membrane; GFP, green fluorescent protein; MβCD, methyl-β-cyclodextrin; MOP, mu opioid; NPFF, neuropeptide FF; vrFRAP, fluorescence recovery after photobleaching at variable observation radius; YFP, yellow fluorescent protein.

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doi:10.1016/j.bbrc.2008.05.174

Cell culture and transfection. The $NPFF_2$-YFP cell line was obtained by transfecting SH-SY5Y neuroblastoma cells using FuGENE 6 (Roche Applied Science, Bale, Switzerland) according to manufacturer's instructions. Stable cell lines were obtained by subcloning and selection with 5 μg/ml blasticidin (Cayla, Toulouse, France). Cells expressing the C-terminal yellow fluorescent protein (YFP)-tagged human MOP receptor together with untagged-$NPFF_2$ receptors [7] were grown in high glucose DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum, 50 μg/ml gentamicine (Invitrogen), 400 μg/ml G418 and 2 μg/ml blasticidin in 5% CO_2 at 37 °C. Cells expressing the C-terminal YFP-tagged human $NPFF_2$ receptor were grown under the same conditions except that no G418 but 5 μg/ml of blasticidin were used for selection.

Measurement of intracellular cAMP. Intracellular cAMP was measured after incorporation of [^{3}H]adenine and selective batch elution on acidic alumina columns as previously described [20].

Isolation lipid rafts using detergent. Cells were solubilized in 0.2% Triton X-100 (Sigma, St. Louis, MO) and the detergent resistant membranes were isolated by centrifugation on discontinuous sucrose gradients as previously described [20].

Detergent-free isolation of lipid rafts. The detergent-free procedure was adapted from [21] with minor modifications. Cells (from 2 confluent 150-mm dishes) were harvested in 2 ml of ice-cold 500 mM sodium carbonate, pH 11. The suspension was first homogenized 3×10 s using an Ultra-Turrax T25 (Janke & Kunkel, IKA-Labortechnik, Staufen, Germany) then 3×20 s using a VibraCell sonicator (Bioblock Scientific, Illkirch, France). The homogenate was then mixed at the bottom of 12 ml Polyallomer centrifuge tubes (Beckman, Fullerton, CA) with 2 ml of 90% sucrose prepared in MBS (25 mM MES, 0,15 M NaCl) containing proteinase inhibitors (Complete Mini tablets, Roche). The resulting 45% sucrose layer was overlaid successively with 4 ml of 35% sucrose and 4 ml of 5% sucrose in MBS containing 250 mM sodium carbonate, and centrifuged at 39,000 rpm for 18 h at 4 °C in a SW41 rotor in a Beckman Optima LE-80K Ultracentrifuge. Twelve 1-ml fractions were collected from the top down. Alkaline phosphatase activity was measured in 50 μl of each fraction using Sigma FAST p-nitrophenyl phosphate tablet sets.

Western blot analysis. Samples were solubilized in SDS–PAGE sample buffer containing 5% β-mercaptoethanol by boiling for 5 min at 100 °C. Proteins (an equal volume from each fraction) were subjected to SDS–PAGE on 10% polyacrylamide gels followed by liquid transfer on PVDF membranes (Immobilon-P, Millipore, Bedford, MA). The following antibodies were used for immunoblotting: monoclonal anti-flotillin1 (Transduction Laboratories, Lexington, KY), polyclonal anti-GFP (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-Gαi3 (C-10, reactive with Gαi3 and to a lesser extent with Gαi1 and Gαi2 subunits, Santa Cruz Biotechnology), and anti-Gβ (T-20, reactive with Gβ1, Gβ2, Gβ3 and Gβ4 subunits, Santa Cruz Biotechnology). After ECL + revelation (GE Healthcare Life Sciences, Piscataway, NJ), chemiluminescence was detected using both a Typhoon 9410 imager (GE Healthcare Life Sciences), for quantification, and X-ray films. When required, blots were quantified using the ImageQuant TL software (GE Healthcare Life Sciences).

Data analysis. Experimental data fitting and statistical analysis were performed using Prism (GraphPad Software, San Diego, CA). Statistical significance between groups of data was assessed using Student's *t* test.

Results and discussion

Mu opioid receptors do not behave as canonical lipid raft proteins in SH-SY5Y cells

Isolation of detergent-resistant membranes (DRMs) is the most widely used method for studying the affinity of membrane proteins for lipid rafts [22]. Solubilization of SH-SY5Y cells expressing MOP–YFP receptors in 0.2% Triton X-100 followed by sucrose gradient centrifugation indeed produced detergent-resistant low density fractions containing two classical lipid raft markers: GPI-anchored alkaline phosphatase and palmitoylated flotillin-1 (Fig. 1, left panel, fractions 3 and 4). Both proteins are totally absent from heavy fractions. MOP–YFP behaved in an opposite way, being almost undetectable in DRMs (Fig. 1, left panel). Previous studies that have described the enrichment of MOP receptors in lipid raft fractions have used alternative detergent-free methods of isolation based on homogenization in 500 mM sodium carbonate at pH 11 [15,16,19]. By using a similar protocol, the large majority of MOP–YFP receptors are observed in low density fractions together with the raft markers alkaline phosphatase and flotillin (Fig. 1, right panel, fractions 4 and 5). After detergent-free extraction, some alkaline phosphatase and flotillin were also present in heavy fractions in contrast to MOP–YFP receptors (Fig. 1, right panel, fractions 9 to 12). Thus, even when it is found in lipid raft fractions, the MOP–YFP receptor does not behave as do classical lipid raft markers. In the present study we used YFP-tagged MOP receptors in order to compare with FRAP experiments that had been performed using this fusion protein. However, a possible influence of YFP on the partitioning of MOP receptors can be ruled out as similar results were obtained using a receptor bearing only a small T7 tag at its N-terminus (data not shown).

Another indirect way of assessing the importance of lipid raft localization for a GPCR is to study the consequences of cholesterol depletion by methyl-β-cyclodextrin (MβCD) on its signaling in living cells. One hour treatment with 5 mM of MβCD induced a partial redistribution of MOP–YFP receptors towards the heavy fractions of the sucrose gradient after detergent-free extraction (Fig. 2A). This redistribution was correlated with a statistically significant 5.4-fold increase of the EC_{50} of the MOP receptor agonist DAMGO for the inhibition of adenylyl cyclase ($p < 0.05$, 1.6 vs 8.6 nM). This result indicates that the lipidic environment of the receptor plays a part in its activity [23]. However, the fact that MOP receptors are excluded from DRMs clearly shows that their "raftophilicity" [22] is rather low. This conclusion strengthens previous reports demonstrating that, contrary to GPI-anchored or palmitoylated proteins, integral membrane proteins are often excluded from DRMs despite being found in low density fractions after detergent-free extraction [24–26]. A possible explanation for this discrepancy could be that the physicochemical properties of the MOP receptor do not favor its association with lipid rafts fractions but that it is still loosely associated with this compartment by specific protein/lipid or protein/protein interactions that resist detergent-free extraction. The necessity for these specific interactions could also explain why lipid rafts association and cholesterol dependency of the MOP receptor is region-specific in the rat brain [16].

$NPFF_2$ receptors are predominantly found outside lipid rafts in SH-SY5Y cells

A stable SH-SY5Y cell line was also constructed in order to study the distribution of the human $NPFF_2$ receptor fused to YFP at its C-terminus. The correct expression of the construct was confirmed by confocal microscopy (data not shown) as well as by the fact that 1DMe was able to potently inhibit adenylyl cyclase in these cells (Fig. 3B). As previously described [7], 2 forms of $NPFF_2$ receptors at about 130 and 90 kDa were detected by Western blot against GFP. Concerning the lower form, Triton X-100 and detergent-free extraction methods gave similar results with no detectable $NPFF_2$ receptors in lipid raft fractions (Fig. 3A). However, the distribution of the band corresponding to the heavier form along the sucrose gradient showed some variability between repeated detergent-free extraction experiments (10% to 50% of this heavy form was found

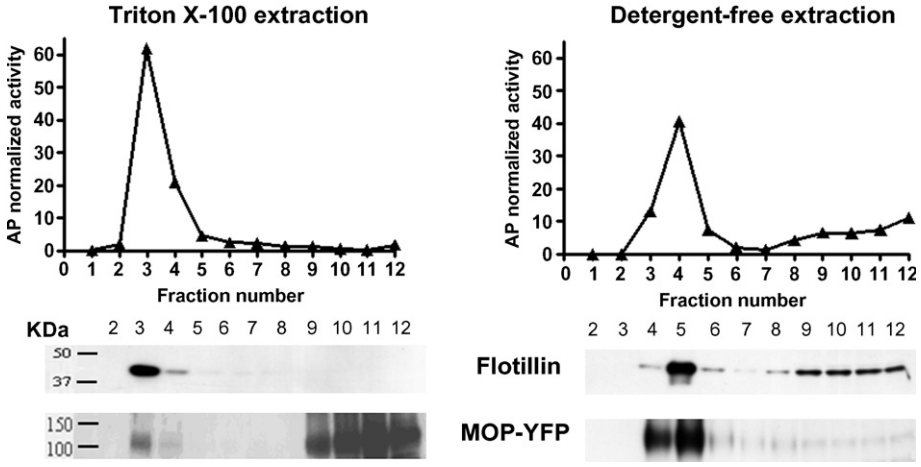

Fig. 1. MOP–YFP receptor distribution is dependent upon the method used to prepare lipid raft fractions. Left panel, cells were solubilized in 0.2% Triton X-100. Right panel, cells were solubilized in 500 mM sodium carbonate pH 11. AP, alkaline phosphatase activity expressed as a percentage of the total activity along the gradient. Samples from each fraction were loaded on 10% SDS–PAGE gels and immunoblotted using antibodies directed against flotillin or GFP to detect YFP-tagged MOP receptors. Data are representative of at least three independent experiments.

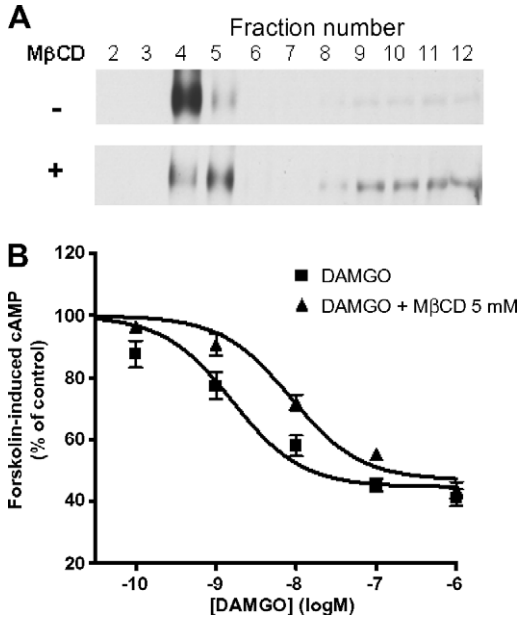

Fig. 2. Methyl-β-cyclodextrin treatment alters MOP–YFP receptor distribution and signaling. (A) Cells were treated with ±MβCD 5 mM for 1 h at 37 °C before detergent-free raft preparation. MOP–YFP receptors were detected using antibodies directed against GFP. (B) DAMGO dose–response curves for the inhibition of forskolin-induced cAMP production. Control refers to forskolin (5 μM) in the absence of DAMGO. Data are means ± SEM of five experiments performed in duplicates.

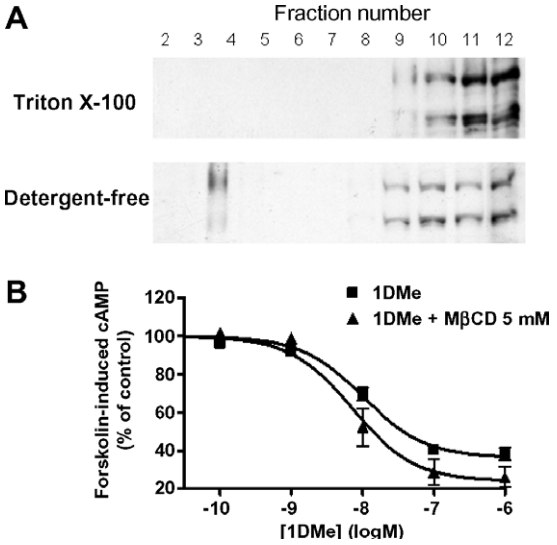

Fig. 3. $NPFF_2$ receptors are predominantly distributed outside lipid rafts and their signaling is unaffected by cholesterol depletion. (A) Cells were solubilized either in 0.2% Triton X-100 or in 500 mM sodium carbonate pH 11. Samples from each fraction were loaded on 10% SDS–PAGE gels and immunoblotted using antibodies directed against GFP to detect YFP-tagged $NPFF_2$ receptors. Two bands at about 130 and 90 kDa correspond to the receptor. Data are representative of at least three independent experiments. (B) 1DMe dose–response curves for the inhibition of forskolin-induced cAMP production. Control refers to forskolin (5 μM) in the absence of 1DMe. Data are means ± SEM of three experiments performed in duplicates.

in buoyant fractions). Nevertheless, unlike MOP–YFP receptors, cholesterol depletion by 5 mM MβCD had no significant effect on $NPFF_2$-YFP receptor signaling (EC_{50} of 1DMe for the inhibition of adenylyl cyclase was 10 nM in control cells and 7.2 nM in depleted cells, Fig. 3B). We can thus conclude that, in SH-SY5Y cells, $NPFF_2$-YFP receptor signaling is not confined in putative cholesterol-rich lipid raft microdomains.

MOP-containing lipid domains isolated by detergent-free extraction are not equivalent to confinement areas observed in living cells

We and others have demonstrated using FRAP technique that MOP receptors lateral diffusion at the surface of SH-SY5Y cells is confined in microdomains with radii ranging from 0.7 to 1 μm [7,27]. We have also shown that MOP receptor confinement was disrupted by 1DMe treatment [7]. If the MOP-enriched membrane domains obtained after detergent-free extraction (Fig. 1, right panel) were equivalent to the domains responsible for receptor compartmentation observed by fluorescence techniques in living cells, then 1DMe treatment should also disrupt receptor distribution along the sucrose gradient. Pre-treatment of the cells with 1 μM 1DMe for 20 min at 20 °C did not induce any change in the distribution of MOP receptors along the sucrose gradient (Fig. 4A). Moreover, the distribution of inhibitory G protein subunits, which are coupled to the MOP receptor and are also enriched in lipid raft fractions, was unaffected by 1DMe (Fig. 4B). It is thus clear that the

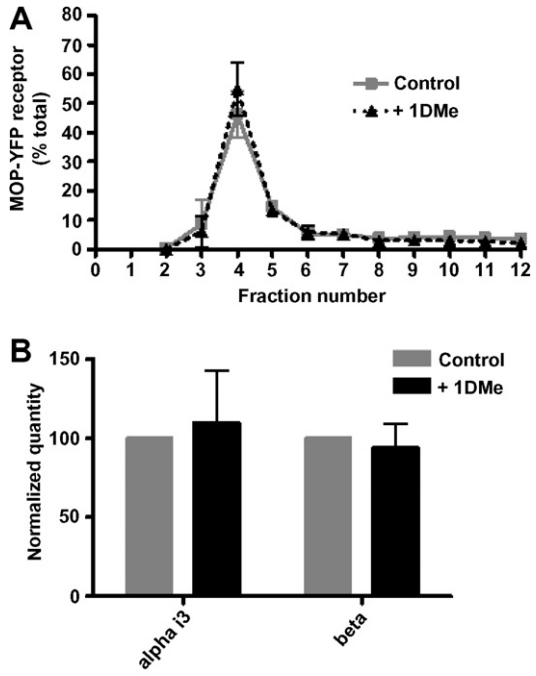

Fig. 4. 1DMe pre-treatment does not alter the distribution of MOP receptors and inhibitory G proteins. Cells were pre-treated with 1 μM of 1DMe for 20 min at 20 °C. Lipid rafts were prepared using the detergent-free method. (A) The quantity of MOP receptors in each fraction is expressed as a percentage of the total amount of receptors along the gradient (as assessed by image analysis of immunoblots). (B) The quantity of $G\alpha_{i3}$ and Gβ subunits detected on immunoblots in lipid raft fractions (4–5) is expressed as a percentage of control untreated cells. Flotillin was used for normalization. Data are expressed as means ± SEM of three independent experiments.

membrane compartmentation of MOP receptor that is affected by 1DMe in living cell is unrelated to the receptor confinement that is extrapolated from detergent-free isolation of buoyant membrane fractions. This implies that the basis for 1DMe-sensitive MOP receptor confinement in living cells might not be the lipidic microcompartmentation of the membrane. Beside lipid rafts, MOP receptor confinement could be due to the cytoskeleton acting as a fence [28] or to other types of protein/protein interactions [29]. These interactions could be disrupted by 1DMe-induced MOP–$NPFF_2$ oligomerization [7] leading to a change in lateral diffusion.

We have shown here a total lack of correlation, not only between MOP receptor confinement observed in living cells and in membrane fractions, but even between results obtained with two membrane fractionation methods. Our data emphasize that, in the case of the MOP receptor, care should be taken when extrapolating results obtained from membrane fractionation and cholesterol depletion to explain the organization of receptor signaling in living cells. This is another example of the methodological issues that arise when trying to probe the reality of lateral heterogeneity in biological membranes [30,31]. There is an evident need for complementing biochemical isolation data with more direct and dynamic approaches in living cells such as FRAP, fluorescence correlation spectroscopy or single particle tracking [32,33].

Acknowledgments

This research was supported by the French Agence Nationale de la Recherche, ‘‘Neurosciences, Neurologie et Psychiatrie” Program, Grant No. ANR-06-NEURO-041-04.

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