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Plasma membrane and lysosomal localization of CB1 cannabinoid receptor are dependent on lipid rafts and regulated by anandamide in human breast cancer cells

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Abstract In this report we show, by confocal analysis of indirect immunofluorescence, that the type-1 cannabinoid receptor (CB1R), which belongs to the family of G-protein-coupled receptors, is expressed on the plasma membrane in human breast cancer MDA-MB-231 cells. However, a substantial proportion of the receptor is present in lysosomes. We found that CB1R is associated with cholesterol- and sphyngolipid-enriched membrane domains (rafts). Cholesterol depletion by methyl-β-cyclodextrin (MCD) treatment strongly reduces the flotation of the protein on the raft-fractions (DRM) of sucrose density gradients suggesting that CB1 raft-association is cholesterol dependent. Interestingly binding of the agonist, anandamide (AEA) also impairs DRM-association of the receptor suggesting that the membrane distribution of the receptor is dependent on rafts and is possibly regulated by the agonist binding. Indeed MCD completely blocked the clustering of CB1R at the plasma membrane. On the contrary the lysosomal localization of CB1R was impaired by this treatment only after AEA binding.

Keywords: Anandamide; CB1 receptor; Endocannabinoids; Intracellular trafficking; Lysosomes; Rafts

1. Introduction

The cannabinoid type I receptor (CB1R), a seven-transmembrane domain protein, is one of the most abundant G-protein-coupled receptors (GPCRs) in the central nervous system and in some peripheral tissues [1–3]. High levels of the CB1 receptor are found in hippocampal neurons which also contain the highest levels of an endogenous ligand, arachidonoyl ethanolamide (anandamide; AEA) [4]. Together with AEA and congeners like 2-arachidonoylglycerol, CB1 and CB2 receptors form the "endocannabinoid system" which regulates several biological events such as vascular relaxation, apoptosis and cell proliferation in human breast cancer cells [5]. Indeed, we have previously observed that AEA inhibits the proliferation of human breast cancer cells (HBCCs) by blocking the G_0/G_1–S phase transition of the cell cycle through interference with CB1 receptor-coupled signal transducing events [6] indicating that endocannabinoids can act as selective inhibitors of human breast cancer cell proliferation through a growth-factor-dependent mechanism [7].

We have also shown that a metabolically stable AEA analog (Met-F-AEA) stops the growth of K-ras-dependent tumors, induced and/or already established, in vivo and it inhibits metastatis in the Lewis lung carcinoma model, two effects that are mediated by CB1 receptors [8,9]. Furthermore we observed that endocannabinoids can induce a non-invasive phenotype in human breast metastatic cells MDA-MB-231 (Bifulco et al., personal communication).

The termination of the endocannabinoid signaling as well as the molecule(s) and the mechanism(s) responsible for the biosynthesis, release and uptake of AEA have not yet been elucidated [3].

CB1Rs are coupled to $G_{i/0}$-proteins which, once activated by the binding of the ligand agonist, initiate various changes in intracellular signalling pathways. It has been reported that the CB1 receptor, like many, but not all G-protein-coupled seven-transmembrane receptors, could undergo agonist-induced [10,11] or constitutive endocytosis [12] cycling between the plasma membrane and endosomes. However, the mechanism of CB1R internalization is not completely understood although recent findings suggest that both clathrin-coated pits and caveolae might be involved in this process [13]. In addition, it has been shown that the cellular accumulation of its ligand AEA is possibly due to caveolae-mediated endocytosis in RBL-2H3 cells [14] and that methyl-β-cyclodextrin (MCD), which extracts cholesterol from the plasma membrane, completely blocks AEA-induced cell death in a variety of cells, including PC12, C6, HEK and HL-60 cells [15]. All these data point towards an involvement of caveolae and of cholesterol-enriched membrane domains in the trafficking

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Abbreviations: AEA, anandamide; Cav1, caveolin 1; DRMs, detergent-resistant microdomains; MCD, methyl-β-cyclodextrin; GPCRs, G-protein-coupled receptors; TNE/TX-100, Tris Na EDTA/Triton X-100 buffer; PBS, phosphate buffer saline

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and function of CB1R. In particular a novel role for lipid rafts in AEA-depending signaling was suggested [15,16].

Lipid rafts are membrane domains biochemically defined by the insolubility of their components in cold non-ionic detergents (like Triton X-100) [17,18]. They are enriched in specific lipids characterized by their saturated long fatty acid chains, like sphingomyelin and sphingolipids, and by cholesterol. Because of their ability of forming more liquid ordered domains within the membranes, lipid rafts also segregate particular proteins and regulate their intracellular trafficking and signal transduction functions within [19].

Caveolin 1 (Cav1), one of the first protein found to be enriched in rafts, oligomerizes to form the proteinaceous coat of caveolae [20], flask-shaped invaginations of the plasma membrane, which represents a subset of organized raft domains [21]. Besides their recognized role in endocytosis, caveolae have been implicated in serving many functions, including the organization of key signalling proteins, cholesterol transport, and potocytosis [20,22].

Both lipid rafts and caveolae are dependent on cholesterol and are disrupted by drugs extracting cholesterol from the plasma membrane, like MCD. Although cholesterol depletion experiments indicated a possible role of both these domains in the trafficking and signalling of CB1R, the molecular mechanism of AEA uptake, the relationship with CB1 receptor and the cellular compartments involved in the signal transduction events deriving from their interaction are not yet defined and even less is known about the cellular mechanisms controlling CB1R intracellular trafficking and signaling.

Therefore, we studied the subcellular distribution of the CB1R in basal conditions, as well as its trafficking in response to agonist stimulation in human breast cancer MDA-MB-231 cell line because of a role of lipid rafts in the regulation of breast tumor cell invasion [23].

In the present study we have investigated the localization of CB1R in human breast cancer MDA-MB-231 cells, and examined whether CB1R was associated with lipid rafts by utilizing both immunocytochemistry studies and biochemical analysis. We found that CB1R is associated with lipid rafts and is localized both on the cells surface and in the lysosomal compartment of MDA-MB-231 cells. We also show that the surface clustering of CB1R is dependent on rafts integrity, while its lysosomal localization is impaired by cholesterol depletion only when the receptor is bound to its agonist AEA.

2. Materials and methods

2.1. Reagents and antibodies

Cell culture reagents were purchased from Gibco Laboratories (Grand Island, NY). The anti-CB1R antibody was from Santa Cruz Biotechnology and anti-Cav1 antibody was purchased from BD Biosciences. The antibodies against BiP, Giantin and LysoTracker from StressGen Biotechnologies Corp. (120-4243 Glanford Ave. Victoria, BC, Canada). Met-F-AEA (2-methyl-2′-F-anandamide) and MCD were purchased from Sigma–Aldrich.

2.2. Reverse transcriptase polymerase chain reaction

Total RNA was extracted from cell lines by guanidinum thiocyanate–isopropanol method. Reverse transcription (RT) was performed using Moloney murine leukaemia virus reverse transcriptase and random oligonucleotide primers. The first strand cDNA was then amplified using two different sets of primers. The sense primer CB1-F (5′-GATGTCTTTGGGAAGATGAACAAGC-3′) and the antisense primer CB1-R (5′-GACGTGTCTGTGGACACAGAC-ATGG-3′) were used to amplify the CB1 receptor; the primers for amplification of alpha actinin were A1F (5′-ATGATCTGGACCAT-CATCCT-3′) and A1R (5′-CTRATGTGGAAGTTRTGCATG-3′). Polymerase chain reactions were performed 30 s at 93 °C, 1 min at 59 °C and 1 min at 69 °C for 25–28 cycles. Amplified DNA was extracted with chloroform and electrophoresed in a 2% agarose gel in 0.5× TBE.

2.3. Western blot analysis

Cells plated in 100 mm dishes in regular medium with serum were washed with ice-cold phosphate buffer saline (PBS) and scraped into lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 0.5% Triton X-100, 0.5% deossicolic acid, 10 mg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin). After removal of cell debris by centrifugation ($4000 \times g$, 5 min), about 50 μg of proteins were loaded on 12% SDS–polyacrylamide gels under reducing conditions. After SDS–PAGE, proteins were transferred to nitrocellulose membranes that were blocked with 5% milk (Bio-Rad Laboratories, Inc., Richmond CA) and incubated with anti-CB1R antibody. After three washes, filters were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat antirabbit secondary antibody. The membranes were then stained using a chemiluminescence system (ECL-Amersham Biosciences) and then exposed to X-ray film (Kodak).

2.4. Immunofluorescent staining

Cells were plated in 24-well plates on coverslips (Becton–Dickinson Labware). When they were 60 ± 80% confluent, they were treated with Met-F-AEA (10 μM 24 h), and/or MCD (10 mM, 15 min). After the incubation with various drugs, the cells were washed twice with PBS and fixed in 3.7% paraformaldehyde in PBS for 20 min and followed by two washes in 50 mM NH_4Cl for 10 min. Permeabilization was achieved by incubating the fixed cells in 0.1% Triton X-100 in PBS for 5 min at room temperature. The cells were then blocked in FDB buffer (1 mM $MgCl_2$, 1 mM $CaCl_2$, 5% foetal calf serum and 2% BSA in PBS) for 30 min at room temperature. All primary and secondary antibody incubations were performed in FDB buffer for 1 h at room temperature. Coverslips were mounted on 50% glycerol in PBS and examined by using a Zeiss Laser Scanning Confocal Microscope (LSCM 410 or 510).

2.5. Drugs treatment

MCD treatment was carried out as described elsewhere [24]. Briefly, MDA-MB-231 cells were plated on dishes and MCD (10 mM) was added to the medium containing 20 mM HEPES, pH 7.5, and 0.2% bovine albumin for 15 min at 37 °C. Met-F-AEA (10 μM) was added to the complete culture medium for 24 h. Where indicated, the cells were first cholesterol depleted by MCD (10 mM, 15 min at 37 °C) and then extensively washed and incubated with Met-F-AEA for further 24 h.

2.6. Cholesterol determination

In order to assay cholesterol levels in the cells before and after treatment with MCD we used the following method: MDA-MB-231 cells grown in the presence or absence of MCD were washed twice with PBS, lysed with appropriate lysis buffer and Infinity Cholesterol Reagent (Sigma Chemical Co., St. Louis, MO, code number 401-25 P) was added to the lysates in the ratio 1:10 for 5 min at 37 °C (according to the suggested Sigma protocol number 401). The samples were then measured in a spectrophotometer at 550 nm.

2.7. Assays for DRM-association

OptiPREP™ density gradients: OptiPREP™ gradient analysis of TX-100-insoluble material was performed using previously published protocols [25]. Cells were grown to confluence in 100 mm dishes, washed in PBS C/M and lysed for 20 min in TNE/TX-100 1% buffer (25 mM Tris–HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% TX-100) on ice. Lysates were scraped from dishes, brought to 40% OptiPREP™, and then placed at the bottom of a centrifuge tube. A OptiPREP™ gradient (5–35% TNE) was layered on top of the lysates and the samples were centrifuged at 21 000 rpm, at 4 °C for 4 h in an ultracentrifuge (model SW41 Beckman Inst., Fullerton, CA). One-milliliter fractions were harvested from the top of the gradient. CB1R was revealed by

Western blotting using the anti-CB1R antibody. As control for correct lipid rafts isolation, Cav1 was chosen as raft marker and Bip as non-raft marker.

3. Results

3.1. Characterization of CB1R expression and localization in MDA-MB-231 cells

In order to analyze the expression levels of CB1R in MDA-MB-231 cells we first analyzed its mRNA expression level by reverse transcriptase-PCR (RT-PCR) technique. We found that CB1R was expressed in our cell system and by Western blot analysis, using a specific antibody against CB1R on cell lysates, we corroborated these data showing that MDA-MB-231 cells expressed high significative levels of CB1R (Fig. 1).

We next analyzed the cellular localization of CB1R by indirect immunofluorescence and confocal microscopy on cells grown on coverslips. In non-permeabilized conditions CB1R was localized on the plasma membrane of MDA-MB-231 cells mainly concentrated in large spots (Fig. 2A).

In permeabilized conditions, by using markers of different intracellular compartments, such as giantin for the Golgi complex, Bip for the endoplasmic reticulum, early endosome antigen-1 for early endosomes (data not shown) and LysoTracker to label lysosomes, we found that the receptor was widely distributed in the cytoplasm and was particularly concentrated in lysosomes as shown in the merged signal from the anti-CB1R antibody and LysoTracker (Fig. 2B). In line with these results, it has been demonstrated that many signaling receptors, such as the G-protein-coupled δ opioid receptor, can undergo agonist-induced proteolysis via endocytic trafficking to lysosomes [26].

After activation, most GPCRs are endocytosed from the cell surface and travel to low pH endosomes, leading the ligand to detach before the receptor is recycled back to the cell surface or sent to the lysosomes for degradation [27]. It has been hypothesized that trafficking of GPCRs from early endosomes to lysosomes might be a mechanism for agonist-induced down-regulation. Indeed, several GPCRs, including the thrombin, thyrotropin and cholecystochinin receptors, have been shown to be sorted to lysosomes in an agonist-dependent manner [28]. Contrary to these findings, we found CB1R in lysosomal structures even in absence of its ligand, that is in basal conditions (Fig. 2B).

Lipid rafts represent versatile devices for compartmentalizing cellular membrane processes and form large platforms involved in protein and lipid signaling, processing and transport [19,29,30].

Because raft domains exist both at the plasma membrane and lysosomes [31], and a lysosomal degradative pathway for CB1 receptor has not yet been demonstrated, we sought to analyze whether the intracellular compartmentalization of CB1R was dependent on lipid rafts. To this aim we studied the intracellular distribution of CB1R after lipid rafts disruption by cholesterol depletion. Cholesterol is an important functional and structural component of lipid rafts and its depletion by using different drugs has been demonstrated to alter the raft composition and as a consequence the raft functions [17,32]. After incubation with MCD which extracted ~60% of the total cholesterol (see Section 2), we found that lysosomal localization of CB1R was not affected as shown by colocalization with LysoTracker (Fig. 2B, MCD, see merge in the right panel). On the contrary, cholesterol depletion strongly altered the cell surface CB1R localization inducing a more uniform plasma membrane distribution of the receptor (Fig. 2C). These data suggest that the plasma membrane clustering of CB1 is dependent on lipid rafts, while the receptor does not appear associate to cholesterol-dependent domains in the lysosomes. Thus, these results indicate that lipid rafts do not appear to regulate the route of CB1R to the lysosomes where presumably degradation of CB1R occurs.

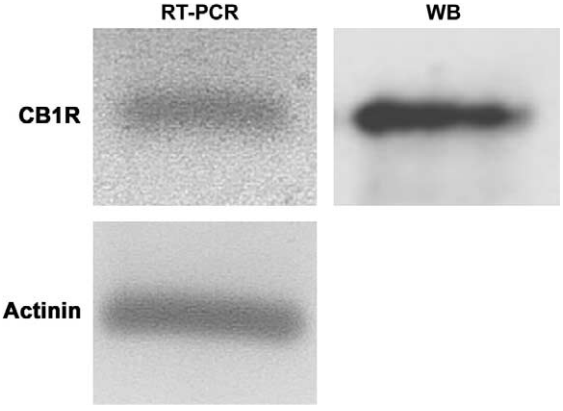

Fig. 1. Expression levels of CB1R in MDA-MB-231 cells. Cells grown in RPMI 1640 medium supplemented with 10% inactivated foetal bovine serum and 2 mM L-glutamine were subjected to total RNA extraction and reverse transcription (RT) was performed using Moloney murine leukaemia virus reverse transcriptase and random oligonucleotide primers for CB1R and actinin were used. Polymerase chain reactions were performed 30 s at 93 °C, 1 min at 59 °C and 1 min at 69 °C for 25–28 cycles to assess saturation of the signal (left panel, RT-PCR). The cells grown in the same conditions described above, were scraped in lysis buffer and 50 μg proteins were subjected to SDS–PAGE. CB1R was revealed by Western blotting on nitrocellulose and hybridization with monoclonal anti-CB1R antibody in 5% milk (right panel, Western blot: WB).

3.2. CB1R localization after anandamide treatment

Several endogenous ligands for cannabinoid receptors have been identified, most notably anandamide (AEA) and noladin ether.

Because the nature of CB1R interaction with AEA, as well as the exact cellular site for their interaction are not yet known, we decided to study the effect of anandamide on CB1R intracellular localization, by using its synthetic analogous methanandamide (Met-F-anandamide) which is more stable to the fatty acid amide hydrolase hydrolysis than the anandamide itself [33].

As shown by immunofluorescence in permeabilized conditions (Fig. 3), the presence of Met-F-AEA (10 μM, for 24 h) added to the extracellular medium did not change the intracellular distribution of CB1R (compare Fig. 3, AEA, with Fig. 2B, control). Interestingly, pre-treatment with MCD (10 mM for 15 min at 37 °C) before agonist incubation, significantly changed the intracellular distribution of the receptor which assumed a more diffuse cytoplasmic localization and lost its lysosomal localization (see Fig. 3, MCD + AEA). Thus, these data indicate that the presence of the agonist modifies the intracellular localization of the receptor when lipid rafts are perturbed by cholesterol depletion, suggesting that the intracellular pathway followed by CB1R to the lysosomes, after the binding of the ligand anandamide, is depending on lipid rafts integrity.

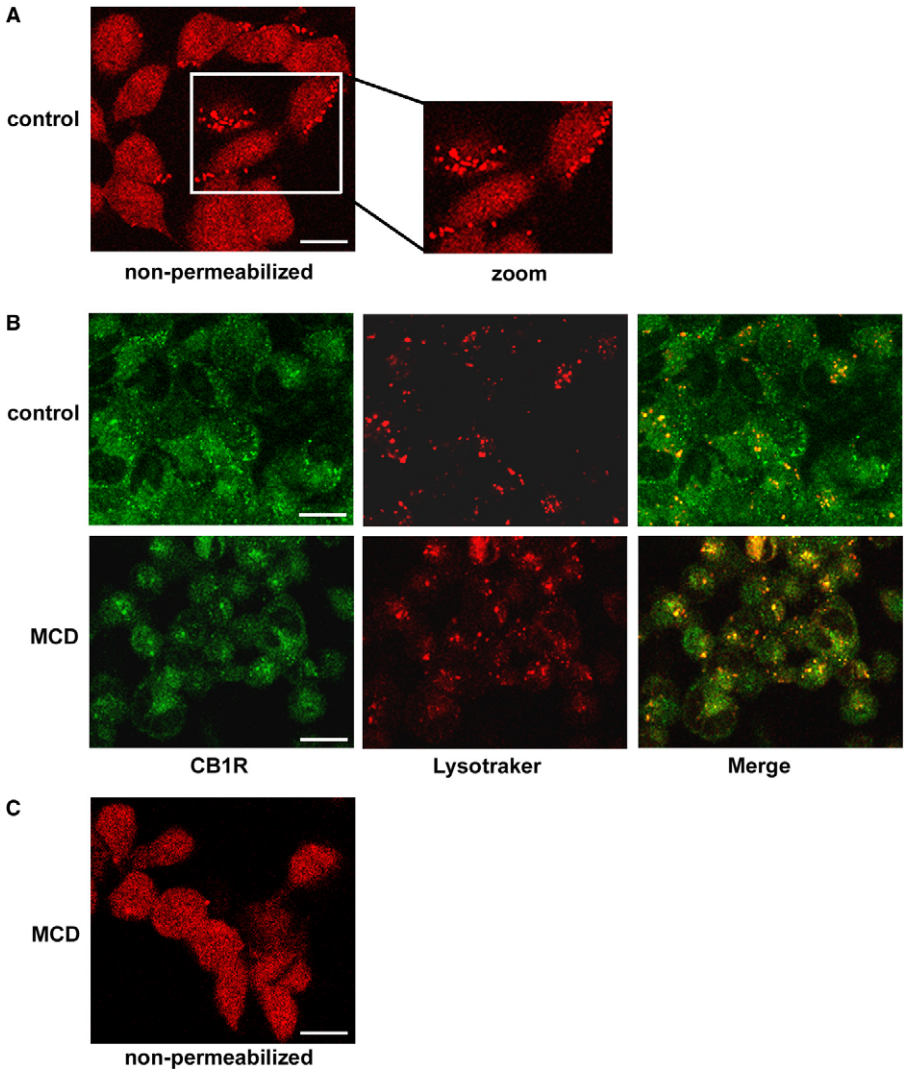

Fig. 2. Surface distribution and intracellular localization of CB1R. (A) The cells were grown on coverslips and fixed with paraformaldehyde. CB1R was revealed by incubating the cells with the first antibody and then with TRITC-conjugated secondary antibody under non-permeabilized conditions. (B) The cells were grown on coverslips without (control) or with MCD for 15 min at 37 °C and were incubated 1 h with LysoTracker 1:10000 in complete culture medium. They were then fixed, permeabilized with TX-100 (see Section 2) and CB1R was revealed with a FITC-conjugated secondary antibody. All the immunofluorescence samples were observed using a Laser Scan Confocal microscope as described in Section 2. The yellow spots indicate that lysosomes and CB1R colocalize. (C) Cells were subjected to immunofluorescence analysis as in A, with the exception that here the cells were analyzed after cholesterol depletion with MCD (15 min at 37 °C). Bar: 10 μm.

3.3. *Characterization of CB1R-association with detergent-resistant microdomains*

Association of CB1R with lipid rafts has not yet been formally demonstrated. Thus, we analyzed whether it was associated with detergent-resistant microdomains (DRMs) by performing TX-100 extraction and flotation-based assays (see Section 2). Indeed, resistance to non-ionic detergent extraction at 4 °C (e.g., TX-100) and association with DRMs is one of the major biochemical characteristics of lipid rafts and raft components [22,34–36]. We found that CB1R was TX-100 insoluble at steady-state (not shown) and specifically ~80% of the receptor floated in the fractions 4–5 of the gradients, which are the typical DRMs containing fractions (Fig. 4A, control), as shown by the flotation of Cav1, an extensively characterized raft marker (Fig. 4A, Cav1) [23]. In particular, Western blot analysis on an aliquot of the individual fractions, confirmed an enrichment of Cav1 in fraction 5 (Fig. 4A), confirming that we had successfully isolated DRM-domains. We also verified that a typical non-raft marker, the endoplasmic reticulum resident protein Bip/GRP78, was excluded from these fractions (Fig. 4A). Of note, the prevalent enrichment of both CB1 receptor and Cav1 in the high density fraction 5 more than in fraction 4 of the gradient (Fig. 4A, control) suggested that the two proteins were distributed in lipid rafts of similar density.

Because cholesterol is an important structural and functional component of rafts [17,29], we determined whether CB1R raft-association was cholesterol-dependent. After treatment with MCD (10 mM, 15 min 37 °C), the amount of CB1R present in the lighter fractions (fractions 4 and 5) of the gradient significantly decreased, and it was estimated to be ~10% of the total amount of protein distributed in the gradient (Fig. 4 and compare fractions 4–5 of the control with fractions 4–5 of the MCD treatment). As expected and previously shown in

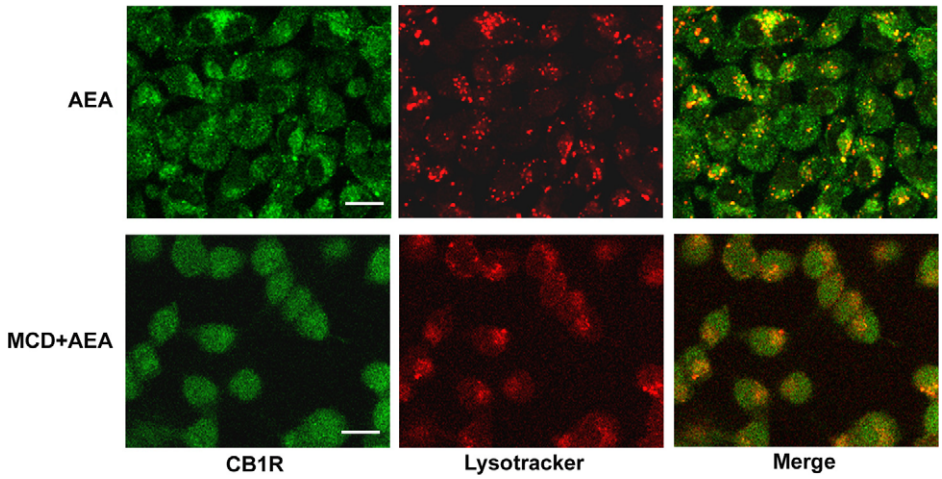

Fig. 3. Localization of CB1R after Met-F-AEA incubation in control or cholesterol depleted cells. MDA-MB-231 cells were grown on coverslips in culture medium containing Met-F-AEA (10 μM, 24 h). Where indicated, the cells were first cholesterol depleted with MCD and then treated with Met-F-AEA (see Section 2). CB1R was revealed by indirect immunofluorescence analysis as in Fig. 2B. Samples were observed using a Laser Scan Confocal microscope. Bar: 10 μm.

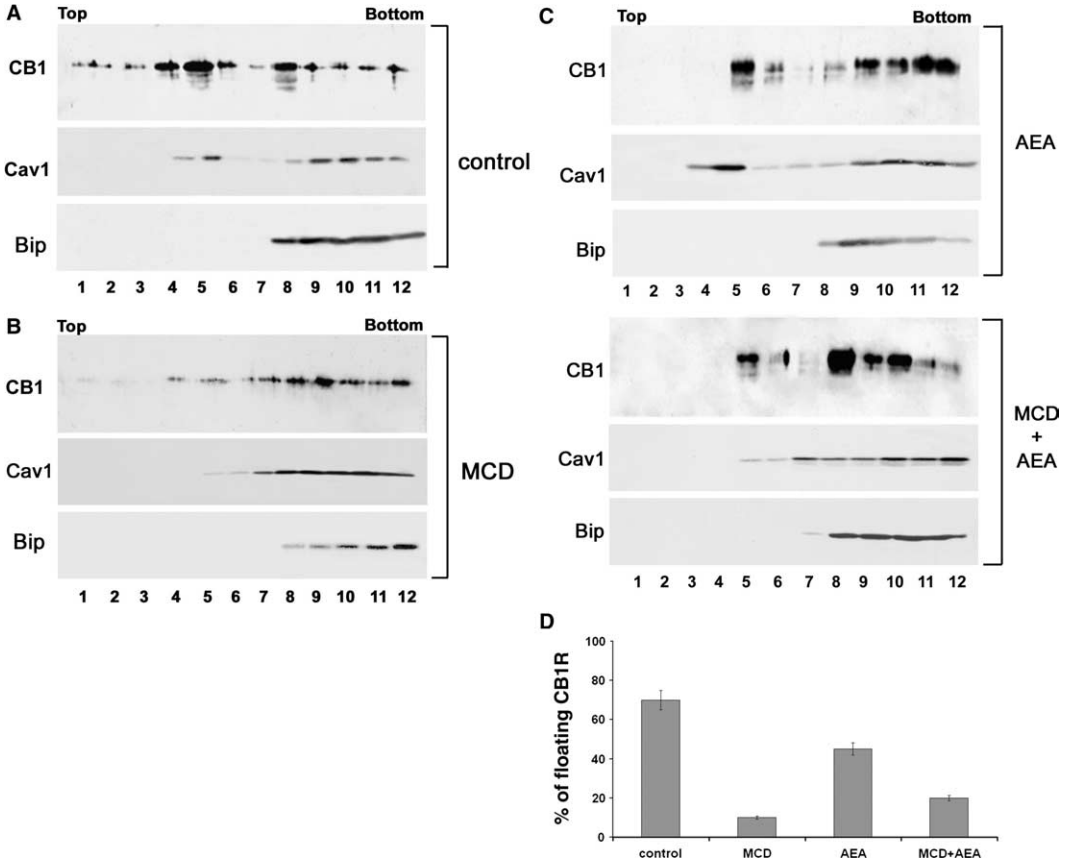

Fig. 4. Purification of CB1R on OptiPREP™ density gradients and effect of MCD and anandamide on CB1R raft-association. (A) The cells were grown in 100 mm dishes in control conditions. They were then lysed for 20 min in cold TNE/TX-100 buffer and then run through a "Two Step" OptiPREP™ gradient (5–40% gradient). One-milliliter fractions were collected from the top of the gradient after centrifugation to equilibrium. An aliquot of each fraction was loaded on 12% gel and revealed by Western blotting and ECL. The distribution of Cav1 and Bip was analyzed by Western blot, respectively, with the anti-Cav1 and anti-Bip antibody and ECL. (B) CB1R purification from the OptiPREP™ gradients was performed after cholesterol depletion with MCD. CB1R, Cav1 and Bip were revealed following the procedure described above. (C) The cells were grown in 100 mm dishes and treated with Met-F-AEA in control condition or after cholesterol extraction by MCD (MCD + AEA) and they were then subjected to purification of CB1R, Cav1 and Bip from OptiPREP™ density gradient fractions. An aliquot of each fraction was loaded on 12% gel, separated with SDS–PAGE and revealed by Western blot and ECL. (D) Quantification of the amount of CB1R in the raft fractions 4 and 5 of the gradients, in different indicated conditions, is reported as a percentage of the total amount of the protein in the gradient and was performed by a densitometric analysis of the bands by NIH image program for McIntosh. Error bars are indicated in the graph and represent the average of three independent experiments.

MDA-MB-231 cells [23], following cholesterol depletion there was a significant reduction of the raft marker Cav1 in the lipid raft fraction (fractions 4–5) but not of the non-raft protein Bip (Fig. 4B). These findings suggest that both CB1R and Cav1 raft-association are dependent on cellular cholesterol levels.

3.4. Effect of anandamide on CB1R raft-association

In order to directly test the influence of the CB1R agonist on its raft-association, we included the metabolically stable anandamide analogue Met-F-AEA in the culture medium of MDA-MB-231 cells and then performed the flotation assay. We found that ~50% of total CB1R floated in the DRM-fractions (Fig. 4C and D, see fractions 4–5 AEA) in the presence of the ligand. Thus, differently from basal conditions in which ~80% of the receptor was found in the light fractions 4–5 (Fig. 4A, control), the anandamide binding lead to a redistribution of the receptor in the soluble fractions of the gradient (fractions 8–12). It is noted that after AEA treatment, Cav1 and Bip distribution in the OptiPREP™ preparation was the same of the control condition suggesting that, AEA affects specifically CB1R raft-association inducing a decrement of CB1R flotation which was exclusively related to fraction 5. Furthermore, contrarily to CB1R, we found that the flotation profile of Cav1 was not affected by AEA but exclusively dependent on cholesterol. After MCD treatment, in the presence of its agonist only ~20% of CB1R floated in the OptiPREP™ gradient and interestingly the majority of the receptor accumulated in the fraction 8 (non-raft membrane) (Fig. 4C, MCD + AEA and 4D and compare with Fig. 4A, control). These results clearly suggest that the profile of CB1R distribution in the gradient fractions is affected by its ligand. Cholesterol depletion further reduces the flotation of the receptor indicating that part of the AEA binding could occur in cholesterol sensitive domains.

4. Discussion

We have investigated the intracellular localization of CB1R and its association with lipid rafts in order to elucidate how the presence of its ligand anandamide and the intracellular cholesterol levels affect CB1R trafficking in a human breast cancer cell line.

It has been previously shown that in HEK-293 cells, as well as in LLC-PK1 epithelial cells or SHSY-5Y neuroblastoma cells, CB1R is predominantly localized in endosomes at steady-state [37], as a result of a constitutive recycling between the plasma membrane and endosomes, mediated by the small GTPases Rab5 and Rab4 [12,38]. By confocal analysis of indirect immunofluorescence we found that CB1R is expressed on the plasma membrane of MDA-MB-231 cells. However, a substantial proportion of the receptor is present in lysosomes (Fig. 2B). Thus, differently from the previous reports [12,38], at our knowledge we provide the first evidence for a lysosomal localization of this receptor in the cells.

Lysosomes are structures with different kind of critical functions. Among these, the main lysosomes functions could be summarized as follow: (1) the lysosomes can operate enzymatic digestion of endocytosed materials; (2) can mediate events in the programmed cell death called apoptosis; (3) can play an important role in receptor-mediated endocytosis and mediate events of receptor recycling and the shutting down of events of cell communication [39]. This sequence of events involves a receptor binding to its ligand followed by their uptake into coated vesicles. Hence, our finding that CB1R shows a lysosomal localization could represent fundamental basis to further studies about receptor internalization, downregulation and eventual resensitization which constitute essential steps to analyze receptors function and signalling within the cells. For several receptors, the cellular responses to effectors have been shown to be regulated in part by the compartmentalization of the receptors and their effectors within the cells [40], therefore elucidating the mechanism underlying the trafficking of CB1R is critical for understanding the physiological response to a variety of ligands.

Interestingly, we found a significant change in CB1R cell surface distribution after cholesterol depletion by MCD. Indeed, the clustered distribution of the receptor at the cell surface was lost and it assumed a more diffuse staining thus indicating that the plasma membrane clustering of CB1R was dependent on cholesterol-enriched microdomains (lipid rafts or DRMs). It remains to be established whether the CB1 cholesterol-dependent plasma membrane localization is of any functional significance in regulating CB1-mediated signalling.

It has been reported that cholesterol depletion by MCD can affect the transport of cholera toxin from endosomes to Golgi but not from Golgi to lysosomes [41]. The reason that cholesterol can regulate some transport steps but not others is unknown, but presumably depends on cholesterol levels in the compartments/vesicles that mediate transport. In addition cholesterol depletion can alter the internalization pathways of different surface receptors [42].

In the case of CB1R, at least in our cell system, cholesterol depletion alters its plasma membrane localization but not the lysosomal one, thus we propose that the receptor might exhibit a different mode of interaction with the membrane lipid bilayer of the plasma membrane respect to that of lysosomal membranes. This hypothesis is supported by the finding that different kinetics and sorting of lipid analogs out of endosomes depend exclusively on their hydrophobic chains [43]. Furthermore, while on one hand cholesterol depletion did not alter the receptor lysosomal localization in control conditions, on the other hand induced a remarkable redistribution of CB1R in the cytoplasm and an impairment of the receptor lysosomal localization after extracellular addition of the agonist anandamide (see Fig. 3). These results indicate that the intracellular pathway followed by CB1R to the lysosomes after binding of the agonist might depend on lipid rafts. Further detailed analysis will be necessary to understand whether this results come from an impairment of the CB1R endocytic pathway that normally directs the receptor to the lysosomal route for its processing, or whether under cholesterol depletion there is also a mis-sorting of the newly synthesized protein.

Based on cholesterol depletion studies it has been proposed that lipid rafts might have a crucial role in both AEA uptake/recycling [14] and AEA-induced apoptosis [15,16,44]. However, these experiments did not address whether and how lipid depletion affected CB1R localization, its raft-association and the regulation of CB1R-dependent signaling by the agonist anandamide.

Thus, in order to test the hypothesis that lipid rafts might be involved in CB1R trafficking in high invasive human breast cancer cell line MDA-MB-231, in which we have previously observed that endocannabinoids regulates cell proliferation

and can induce a non-invasive phenotype, we analyzed the raft-association of the CB1 receptor in these cells. We found that CB1R was associated with DRMs and that cholesterol depletion by MCD decreases its raft-association, which was also differently controlled by the presence of its agonist anandamide. We found that Met-F-AEA reduces significantly CB1R DRM-association both in the absence or presence of MCD (Fig. 4C and D).

In this contest, our observation that cholesterol depletion, before AEA incubation displaces CB1R from rafts and causes an intracellular redistribution, is particularly intriguing because recent evidences suggest that the cellular uptake of anandamide could be mediated by lipid rafts [14] and that MCD treated CB1R-expressing C6 cells are protected from AEA-induced apoptosis [15,44]. Complementarily with these data, we show for the first time that CB1R is associated with DRMs and suggest that they might represent a cellular device for its intracellular trafficking as well as favorable platform to regulate CB1R signalling.

We are currently checking the implication of lipid rafts/caveolae for the signalling of CB1R and whether the data described in this report could give significant clues to study the endocannabinoid system. However because endocannabinoids through CB1R-dependent mechanism have been shown to have a role in cancer [45,46] and lipid rafts play a pivotal role in breast tumor cell invasion [23], the current data support the view that perturbation of lipid rafts may represent a useful tool to control CB1R signalling and could lead to the development of a novel therapy for endocannabinoids-related diseases, such as cancer.

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