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