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Regulation of CB₁ cannabinoid receptor trafficking by the adaptor protein AP-3

Raphael Rozenfeld and Lakshmi A. Devi¹

Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, New York, New York, USA

Abstract

Cannabinoid receptor 1 (CB₁) is an abundant G protein-coupled receptor, involved in a number of physiological processes. This receptor is localized at the plasma membrane, as well as in intracellular vesicles. The trafficking events leading to this intracellular localization remain controversial. In this study, we examine the differential trafficking of CB₁ receptors and its implication on signaling. We find that the transfected tagged receptors are predominantly at the plasma membrane, whereas endogenous receptors exhibit an intracellular localization. We also find that intracellular endogenous CB₁ receptors do not have an endocytic origin. Instead, these receptors associate with the adaptor protein AP-3 and traffic to the lysosomes. siRNA-mediated AP-3 δ knockdown leads to enhanced cell surface localization of CB₁ receptors. Finally, we show that CB₁ receptors in the late endosomal/lysosomal compartment are associated with heterotrimeric G proteins and mediate signal transduction. These results suggest that intracellular CB₁ receptors are functional and that their spatial segregation is likely to significantly affect receptor function.—Rozenfeld, R., Devi, L. A. Regulation of CB₁ cannabinoid receptor trafficking by the adaptor protein AP-3.

Keywords

endocannabinoid; G protein-coupled receptors; intracellular signaling; lysosomal sorting; addiction; drug of abuse; THC

The Superfamily of G protein-coupled receptors (GPCRs) constitutes one of the largest family of membrane proteins. They respond to an array of stimuli and play a crucial role in a number of biological functions, including neurotransmission, cellular metabolism, secretion, differentiation, growth, inflammation and immune responses. It is a classical notion that GPCRs function at the cell surface (1), freely accessible to their extracellular ligands. Binding of the ligand then leads to receptor activation that “converts” extracellular stimuli into an intracellular response. Requirement of plasma membrane localization is further supported by studies showing that impaired delivery of GPCRs to the plasma membrane significantly impairs their function (2–4). However, the notion that GPCRs have to reach the cell surface to be functional has been challenged by several recent studies. For example, mGluR5 can be found at the nuclear membrane as a functional receptor and is able to mediate calcium signaling (5). GPR30, a GPCR that binds estrogen, is localized mainly in the endoplasmic reticulum (ER), where it is capable of stimulating rapid estrogen-mediated calcium mobilization and phosphoinositide 3-kinase (PI3K) activation (6, 7). Using

lipophilic and nonlipophilic estrogen derivatives, Revankar and coworkers demonstrated that the lipophilic nature of the ligand is crucial for determining its ability to cross biological membranes and activate the intracellular receptors (7). It is likely that GPCRs with lipophilic ligands could bind their ligands and be activated in intracellular compartments. Among the family A GPCRs, cannabinoid receptors bind to and are activated by lipid-derived ligands (anandamide and 2-arachidonoyl glycerol; ref. 8). These endocannabinoid ligands are highly lipophilic (9) and therefore can diffuse through biological membranes (10). In addition, endocannabinoids are actively taken up into the intracellular compartment; the specific transporters responsible for this process has not yet been identified with certainty (11, 12). Cannabinoid ligands are thus readily available in the intracellular milieu and could stimulate intracellular CB₁ receptors in a manner similar to that described for GPR30.

Several studies have reported the presence of CB₁ receptors in intracellular vesicles in a variety of cell types (13–15). In human embryonic kidney (HEK) 293 cells, transfected CB₁ receptors are present in both intracellular vesicles and at the plasma membrane, where it is proposed that it exhibits constitutive activity leading to its internalization and, in turn, to the intense vesicular localization of the receptor. When transfected into neurons, CB₁ receptors are absent from the somatodendritic plasma membrane, where they are localized to vesicles. However, the receptors are associated with the axonal plasma membrane (14, 15). The proposed mechanism underlying the somatodendritic vs. axonal CB₁ receptor polarity has been controversial since the presence/location of the epitope tag significantly affected receptor trafficking (14, 15). In one study, application of the inverse agonist, AM281, was reported to lead to cell surface relocation of C-terminally green fluorescent protein (GFP)-tagged CB₁ receptors, consistent with an activation-dependent internalization of CB₁-GFP (14). In another study, the same treatment had no effect on the localization of an N-terminally GFP-tagged receptor (15). The trafficking and localization of endogenous (untagged) CB₁ receptors have not been studied in great detail.

In this study, we compared the localization and trafficking of endogenous (untagged) to that of transfected (tagged) CB₁ receptors in a variety of cells (HEK293 cells, Neuro2A neuroblastoma cells, and primary hippocampal neurons) and found that the majority of the endogenous CB₁ receptors do not reach the cell surface. In contrast, only tagged CB₁ receptors exhibit significant localization at the plasma membrane (and at the somatodendritic plasma membrane in the case of hippocampal neurons). We also show that native CB₁ receptors associate with the adaptor protein AP-3 and follow an AP-3-dependent trafficking from the biosynthetic compartment to the lysosomal compartment. Finally, we show that intracellular CB₁ receptors interact with Gαi protein subunits in endosomal/lysosomal compartments and mediate signal transduction by stimulating extracellular regulated kinase (ERK) phosphorylation. These results demonstrate for the first time that the intracellular CB₁ receptor is functional. Furthermore, these results suggest a novel role for AP-3 in GPCR trafficking, leading to intracellular receptor localization and signaling.

Materials and Methods

Cell lines, reagents, and plasmids

HEK293 and Neuro2A cells, from the American Type Culture Collection (ATCC; Manassas, VA, USA) were maintained in Dulbecco modified Eagle medium (DMEM) + 10% FBS at 37°C in a humidified 5% CO₂ incubator. Anti-phospho-ERK monoclonal, anti-ERK polyclonal, and anti-myc monoclonal antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Sulfo-NHS-Biotin and avidin-coupled agarose were from Pierce (Rockford, IL, USA). goat-anti-N-ter CB₁ polyclonal, rabbit- and guinea pig-anti C-ter CB₁ polyclonal antibodies were gifts from Dr. Ken Mackie (Indiana University,

Bloomington, IN, USA). Lysotracker was from Molecular Probes (Eugene, OR, USA). CB₁-YFP plasmid was described elsewhere (16). The anti AP-3 δ (anti-delta SA4) monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA, USA). Polyclonal anti-calnexin antibody was from Sigma (St. Louis, MO, USA). The monoclonal anti-AP-2 α antibody was from BD Biosciences (San Jose, CA, USA). The secondary antibodies IRDye 680-labeled anti-rabbit antibody, IRDye 800-labeled anti-mouse and anti-goat antibodies were from Rockland Immunochemicals (Gilbertsville, PA, USA). Lysotracker was from Invitrogen (Carlsbad, CA, USA). Dynasore (3-hydroxy-*N'*-[(Z)-(3-hydroxy-4-oxo-1-cyclohexa-2,5-dienylidene) methyl]napht halene-2-carbohydrazide) was purchased from Chembridge Corporation (San Diego, CA, USA). siRNA to AP-3 δ and CB₁ receptors were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Plasmid transfections

Transfections were performed when cells were 80–90% confluent using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions, as described previously (17).

siRNA transfections

Chemically synthesized, double-stranded siRNAs targeting mouse AP-3 δ were purchased from Santa Cruz. A nonsilencing RNA duplex was used as a control (17). Neuro2A cells and primary hippocampal neurons (1 wk old) were transfected with 100 nM of siRNA using Lipofectamine 2000 (Invitrogen). Cells were processed for Western blot analysis, immunofluorescence, or ELISA experiments 48 h later, as described below.

Coimmunoprecipitation and Western blot analysis

Cells were lysed for 1 h in lysis buffer (1% Triton, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 50 mM Tris-Cl, pH 7.4) containing protease inhibitor cocktail (Sigma). For immunoprecipitation, 100–200 μ g of protein was incubated without (beads control) or with the goat polyclonal anti-CB₁ antibody (1:250 v/v) with 10% v/v protein A/G-agarose (Pierce) overnight at 4°C. The beads were washed 3 $\times$ with lysis buffer and 1 $\times$ with the same buffer without detergent. Proteins were eluted in 60 μ l of 2 $\times$ Laemmli buffer containing 1% 2-mercaptoethanol. Proteins were resolved by 10% SDS-PAGE and were subjected to Western blot analysis using indicated antibodies. Both blotting and imaging with the Odyssey imaging system (LI-COR, Lincoln, NE, USA) were performed by following the manufacturer's protocols. The secondary antibodies that were used included IRDye 680-labeled anti-rabbit antibody, IRDye 800-labeled anti-mouse and anti-goat antibodies (1:10,000).

Immunofluorescence and confocal microscopy

HEK293, Neuro2A, or primary hippocampal neurons, plated on 14-mm coverslips, were processed for immunofluorescence as follows: for extracellular staining, the cells were washed 1 $\times$ with ice-cold PBS and then incubated with the anti-N-terminal extracellular domain of CB₁ goat polyclonal antibody (1:300 in PBS, 2% BSA) for 90 min at 4°C. Then the cells were washed 2 $\times$ with ice-cold PBS before fixation (described below). For staining of intracellular receptors, cells were washed 2 $\times$ with ice-cold PBS, then fixed in 4% paraformaldehyde in PBS for 25 min at 4°C. The cells were washed 2 $\times$ with PBS, then permeabilized in PBS, 1% Triton X-100 for 10 min at 4°C. After one wash in PBS, the cells were incubated for 1 h with PBS, 5% BSA, then incubated with the indicated antibodies overnight: rabbit polyclonal anti-C-ter CB₁ (1:500), rabbit polyclonal anti-calnexin antibody (1:1000), monoclonal anti-AP-3 δ antibody (1:100), and monoclonal anti-pERK (1:150). The next day, cells were washed 2 $\times$ in PBS, 2% BSA then incubated with the indicated

secondary antibodies and DAPI for 1 h in PBS, 2% BSA. Cells were washed 3× with PBS and mounted with Mowiol for confocal microscopy analysis. Slides were visualized with a Leica TCS SP1 confocal microscope equipped with four external lasers (350, 488, 568, and 633 nm, Leica Microsystems, Bannockburn, IL, USA). Images were acquired with an ×20 or ×100/1.32 PL APO objective lens and analyzed in sequential scanning mode. For lysosome staining, cells were incubated before fixation for 2 h with 100-nM LysoTracker (Invitrogen) in complete media.

Primary cell culture

Primary hippocampal cultures were prepared as described by Osten *et al.* (18). Briefly, E16 Sprague-Dawley rat pups were removed from the uterus of the mother and decapitated in a PBS/10 mM HEPES, pH 7.3/0.6% glucose (PHG) solution. Hippocampi were dissected out with the help of a dissection microscope. The hippocampi were cut into 15 to 20 pieces and incubated for 15 min at 37°C with PHG buffer containing 0.25% trypsin. They were then washed 3× with PHG buffer and resuspended in buffer A, consisting of 0.8 ml of plating media (minimal essential medium, 10% fetal bovine serum, 0.45% glucose, 1 mM sodium pyruvate, 25 μM glutamate, and 1× penicillin/streptomycin) along with 0.1 ml of BSA (4% in plating medium) and 0.1 ml of DNase (1 mg/ml). Cell suspension was prepared with the help of a 1-ml pipette and layered onto a 1-ml, 4% BSA/plating media cushion, and centrifuged for 10 min at 300 g. The pellet was resuspended in plating medium, and cells were plated for 3 to 4 h on wells coated with polyornithine/laminin. After 3 to 4 h, medium was changed to Neurobasal A supplemented with B-27 and nerve growth factor (50 ng/ml) (Invitrogen). Experiments were performed on cells grown for 5 days to 1 wk in culture.

Cell fractionation

Cell fractionation was performed using a protocol adapted from Stasyk *et al.* (19). Briefly, Neuro2A (10⁷) cells were washed 3× with ice-cold PBS, scraped, and pelleted at 200 g for 5 min. The cell pellet was rebuffered in homogenization buffer (HB), consisting of 250 mM sucrose in 3 mM imidazole, pH 7.4, 1 mM EDTA, protease and phosphatase inhibitor cocktails (Sigma), and again pelleted at 1,300 g for 10 min. The cells were resuspended in HB buffer and homogenized by 3 passes through a G22 needle. Postnuclear supernatant (PNS) was obtained by centrifugation at 2000 g for 10 min at 4°C. The PNS was adjusted to 40.6% by adding 62% sucrose (1:1.2 v/v) and loaded on the bottom of a SW28 ultracentrifuge tube (Beckman, Fullerton, CA, USA), overlaid with 35% sucrose in the same HB buffer, and HB was added up to the top of the tube and spun down at 100,000 g for 3 h at 4°C. Crude endosomal fractions (500 μl) were collected from the interphase between 35% sucrose and HB buffer. The fractions were analyzed for the presence of various proteins by Western blot analysis as described above.

Membrane biotinylation

Neuro2A cells transfected with control siRNA or siRNA to AP-3δ, at 70% confluency in 6-well plates, were washed 2× with ice-cold PBS/Ca²⁺/Mg²⁺ and incubated with sulfo-NHS-Biotin (Pierce) for 1 h at 4°C. Biotin was quenched by incubating the cells with 0.1 M glycine for 30 min at 4°C, and the cells were solubilized in immunoprecipitation lysis buffer for 30 min. The cell lysate was spun down to eliminate insoluble material, and the supernatant was incubated with avidin-coupled agarose (10% v/v) (Pierce) overnight at 4°C. The beads were washed 4× in lysis buffer, and eluted in 50 μl of 2× SDS sample buffer. Proteins were resolved by 10% SDS-PAGE for immunoblotting using anti-N terminus CB₁ antibody as described above.

ELISA

Cells in 96-well plates were grown to confluency. Cells were treated without or with dimethyl sulfoxide (DMSO) vehicle or Dynasore (1:500) for the indicated time. To measure cell surface receptors, cells were washed in ice-cold PBS, then incubated on ice with primary anti-N terminus CB₁ antibody (1:300) for 90 min. The cells were then washed 2× in PBS and incubated on ice with IRDye 800-labeled anti-goat secondary antibody (1:10,000). Cells were fixed with 4% PFA in PBS. For the effect of Dynasore on cell surface CB₁ receptor expression, cells were directly analyzed for fluorescence intensity as described below. In the case of cells transfected with CB₁-YFP and myc-CB₁, after fixation, cells were permeabilized with 0.1% Triton X-100 and stained with a primary anti C-terminus CB₁ and an IRDye 680-labeled anti-rabbit antibody (1:10,000) to measure total CB₁ receptors. The plates were imaged by scanning simultaneously at 700 and 800 nm with an Odyssey imaging system at 169-μm resolution, medium quality, focus offset of 3.0 mm, and intensity setting of 5 for both 700- and 800-nm channels. Absence of overlap between the channels was assessed since one infrared-tagged secondary antibody did not lead to nonspecific signal in the other channel. Specific fluorescence was obtained by subtracting the fluorescence obtained by incubation with secondary antibodies alone.

Phospho-ERK Assay

Neuro2A cells were transfected with the siRNA to AP-3δ or control siRNA. Cells were seeded on 24-well plates 24-h post-transfection. The next day, the cells were starved for 2 h in serum-free medium prior to stimulation. Cells were preincubated with vehicle, 1 μM SR 141716A or 10 μM hemopressin for 15 min then stimulated with 100 nM WIN-55212 ± inhibitor for indicated times. Cells were solubilized by directly adding 1× SDS buffer prewarmed to 65°C, followed by sonication with a microtip for 5 s. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting with mouse monoclonal anti-phospho-p44/42 MAPK (anti-pERK, 1:1000), and rabbit polyclonal anti-p44/42 MAPK (anti-ERK, 1:1000) antibodies.

Results

C-terminal YFP tag enhances plasma membrane localization of CB₁ receptors

In an effort to characterize the intracellular trafficking of endogenous CB₁ receptors in Neuro2A cells, we examined the subcellular localization of CB₁ receptors by immunofluorescence and confocal microscopy using a polyclonal antibody directed against a 15 amino acid sequence in the C-terminal tail of CB₁ receptors (20). The endogenous CB₁ receptors were found exclusively in intracellular vesicles with no staining at the plasma membrane (Supplemental Fig. 1A, B; Fig. 1). We verified the specificity of this antibody by blocking binding with the antigenic peptide; under these conditions, there was no detectable signal (Supplemental Fig. 2). The intracellular localization of CB₁ receptors is in contrast to previous studies showing plasma membrane localization of transfected CB₁ receptors. To see whether we could reproduce results from previous reported studies, we examined the localization of a C-terminally YFP-tagged CB₁ (CB₁-YFP) or N-terminally myc-tagged CB₁ (myc-CB₁) expressed in HEK293 cells. We found that in HEK293 cells, CB₁-YFP was present mainly at the plasma membrane and lesser amounts in intracellular vesicles (Supplemental Fig. 1A, B), in agreement with previous reports (13). Although some myc-CB₁ receptors are present at the cell surface, a large proportion is in intracellular vesicles (Supplemental Fig. 1A, B). Next, we assessed the plasma membrane localization of CB₁ receptors by immunofluorescence; for that, the cells were stained with an antibody directed against the extracellular N-terminal domain of CB₁, prior to fixation and cell permeabilization, to allow staining of only CB₁ receptors present at the plasma membrane. CB₁-YFP expressed in HEK293 or in Neuro2A cells was labeled by the N-terminal directed

antibody confirming the plasma membrane localization of CB₁ receptors (Fig. 1A, D, F). Myc-CB₁ also exhibited plasma membrane localization in HEK293 and Neuro2A cells, albeit only when the receptors were expressed at high levels (Fig. 1B, E, F). Quantification of cell surface CB₁ as compared to total CB₁ receptors by ELISA revealed that the levels of CB₁-YFP at the plasma membrane were 2- to 3-fold higher as compared to the levels of myc-CB₁ (Fig. 1F). Interestingly, the levels of endogenous CB₁ receptors at the plasma membrane were very low as examined by immunofluorescence and ELISA (Fig. 1C, F), suggesting that the majority of the endogenous CB₁ receptors are present in intracellular vesicles. Taken together, our results indicate that the presence of the YFP tag at the C-terminus of the receptor influences its localization, and the plasma membrane localization of myc-CB₁ is the consequence of receptor overexpression.

Poor trafficking of endogenous CB₁ receptor to the cell surface

The low expression of endogenous CB₁ receptors at the cell surface could be due to rapid trafficking of the receptors from the cell surface to an endocytic compartment. To examine this, cells were transfected with Rab5-mCherry, which allows the visualization of early endosomes. We found that the endogenous CB₁ receptor was not localized with Rab5, *i.e.*, to the early endosomes (Fig. 2A, left panel), whereas CB₁-YFP was localized to early endosomes (Fig. 2A, right panel). To further examine this and test whether endogenous CB₁ receptors might briefly reside in the early endosomes (which would preclude visualization of the receptor in that compartment), we transfected cells with a dominant positive Rab5-mCherry construct (DP Rab5) that induces accumulation of internalized receptors that transit from the plasma membrane to Rab5-positive vesicles (21). Under these conditions, there was no significant accumulation of endogenous CB₁ receptors in the Rab5-containing endocytic vesicles (Fig. 2B, left panel). However, there was a significant accumulation of CB₁-YFP in these vesicles (Fig. 2B, right panel), indicating that CB₁-YFP but not native CB₁ receptors undergo constitutive internalization. This constitutive internalization of CB₁-YFP is in agreement with previous reports (13). To confirm the lack of internalization of native CB₁ receptors, we treated cells with Dynasore, an inhibitor of dynamin GTPase activity, which has been extensively characterized and shown to block dynamin-mediated endocytosis (22). Both immunofluorescence and ELISA studies with an antibody directed against the N-terminal extracellular domain of CB₁ receptors failed to show significant changes in plasma membrane levels of endogenous CB₁ after 3 h treatment with Dynasore. A modest increase in receptor levels could be detected only after 24 h of treatment (not shown). Taken together, these results suggest that only a small fraction of endogenous CB₁ receptors traffics to the cell surface and that the majority of the CB₁ receptors are intracellular and have a biosynthetic origin.

Poor trafficking of hippocampal endogenous CB₁ receptors to the cell surface

To examine whether our observations in Neuro2A cells could be generalized to primary neurons, we first examined CB₁ receptor localization in primary hippocampal neurons with the CB₁ N-terminal domain antibody under nonpermeabilization conditions. There was no significant staining of CB₁ receptors in the somatic plasma membrane. A low level of discrete staining could be observed in the processes (Fig. 3A). This is in agreement with previous studies that showed the presence of CB₁ receptors at the axonal membrane (14, 15). As expected, the transfected CB₁-YFP could be readily detected at the somatodendritic plasma membrane of hippocampal neurons (Fig. 3A), in agreement with our observations in Neuro2A cells.

We then tested the effect of the DP Rab5 expression on receptor localization. In the absence of DP Rab5, endogenous CB₁ and CB₁-YFP were both found in intracellular vesicles. Expression of DP Rab5 did not affect the localization of endogenous CB₁ receptors (Fig.

3B, top panel), which do not colocalize with this early endosomal marker. In contrast, CB₁-YFP was predominantly present in the DP Rab5 containing early endosomes (Fig. 3B, bottom panel). This indicates that, as in Neuro2A cells, endogenous CB₁ receptors do not traffic significantly to the plasma membrane in primary hippocampal neurons. This is in contrast to transfected CB₁-YFP that traffics to the plasma membrane from where it gets endocytosed into Rab5-containing early endosomes.

Endogenous CB₁ receptors are located in late endosomes/lysosomes

Since endogenous CB₁ receptors are poorly associated with the plasma membrane, we examined the compartment of their localization. For this, we carried out colocalization studies with markers for various subcellular compartments: calnexin for ER; galactosyl-transferase tagged with EGFP (GalT-GFP) for Golgi apparatus; Trans-Golgi network 38 tagged with EGFP (TGN38-EGFP) for trans-Golgi network; Lysotracker for lysosome; and transfected Rab7-mCherry and Rab9-YFP for late endosome. We did not observe colocalization of CB₁ with ER or Golgi markers. In contrast, CB₁ receptors exhibited a marked colocalization with the lysosomal and late endosomal markers (Fig. 4A). These results are consistent with the notion that endogenous CB₁ receptors do not traffic to the cell surface but are delivered to the late endosomal/lysosomal compartment.

Endogenous CB₁ receptors interact with the AP-3 adaptor protein complex

We next explored the mechanism of intracellular sorting of endogenous CB₁ receptors by examining whether the receptors interact with the heterotetrameric protein adaptor complex 3 (AP-3), a coat component that participates in the sorting of lysosomal enzymes and in the generation of lysosome-related organelles (23–26). First, we examined the colocalization of endogenous CB₁ receptors with the δ subunit of the AP-3 heterotetrameric protein complex (AP-3 δ) by confocal microscopy. There was a substantial colocalization of the receptor with AP-3 δ (Fig. 5A, top panel). In contrast, there was no significant colocalization of CB₁ with AP-2, another adaptor protein involved in endocytic vesicle formation (27) (Fig. 5A, bottom panel). We then tested whether AP-3 associates with CB₁ receptors, an association that could be responsible for the sorting of the receptors to lysosomes. Coimmunoprecipitation experiments showed that AP-3 but not AP-2 could be detected in the CB₁ immunoprecipitates (Fig. 5B), suggesting that these receptors specifically associate with AP-3 and that these proteins are present in an interacting complex. To confirm the specificity of this association, we depleted the cells of CB₁ receptors using a specific siRNA, and examined the presence of AP-3 δ in the receptor immunoprecipitate. Under these conditions (*i.e.*, a significant decrease in CB₁ receptor protein levels; >60%), we could not detect the presence of AP-3 δ in the immunoprecipitate (Fig. 5C). Taken together, these results are consistent with the notion that CB₁ receptors specifically associate with the adaptor protein AP-3 δ , and this could modulate their localization.

A role for AP-3 in modulating CB₁ receptor trafficking

We then tested directly the functional consequence of the association between AP-3 and CB₁ receptors by examining the effect of AP-3 knockdown on receptor localization. For this, Neuro2A cells were transfected with a control siRNA or an siRNA specific to AP-3 δ , and the level of this adaptor protein was examined. Western blot analysis using an antibody specific to AP-3 δ confirmed that expression of the siRNA leads to more than 90% decrease in AP-3 δ levels, without affecting the levels of AP-2 α (Fig. 6A). Next, we examined the effect of AP-3 δ down-regulation on cell surface expression of CB₁ receptors. For this, plasma membrane-bound CB₁ receptors were stained with an antibody directed against the N-terminal domain of CB₁, in cells transfected with a control siRNA or with the AP-3 δ siRNA. In control cells, we observed a very discrete staining for CB₁ receptors at the plasma membrane. In contrast, on AP-3 δ down-regulation, there was a substantial increase in the

CB₁ receptor cell surface immunoreactivity (Fig. 6B). The plasma membrane staining of CB₁ receptor appears to be punctate; this is probably because of constitutive internalization of cell surface receptors. The enhanced cell surface localization of CB₁ receptors was further confirmed by cell surface biotinylation (Fig. 6C) and by ELISA (Fig. 6D), while the total levels of CB₁ receptor was not changed by siRNA transfection (Fig. 6C). We found a significant increase in CB₁ receptor at the plasma membrane on AP-3 down-regulation, indicating that AP-3 governs the trafficking of endogenous CB₁ receptors.

AP-3 controls CB₁ receptor sorting in primary hippocampal neurons

Next, we examined the involvement of AP-3 in localization and trafficking of CB₁ receptors in primary hippocampal neurons. For that, we examined the localization of CB₁ receptors by confocal microscopy. We found that CB₁ colocalizes with the lysosomal marker (lysotracker) and with AP-3 δ (Fig. 7A). Transfection of the siRNA to AP-3 δ leads to a decrease in the expression of this protein in a large number of neurons (Fig. 7B). In these cells, there is a significant increase in cell surface expression of CB₁ receptors (Fig. 7B, C). Taken together, these results indicate that in primary hippocampal neurons, as in Neuro2A cells, AP-3 regulates CB₁ receptor localization and prevents its trafficking to the somatodendritic plasma membrane.

CB₁ receptors are functional in intracellular compartments

Since most of the endogenous CB₁ receptors are found to be located in intracellular vesicles (and do not transit to the cell surface), we considered the possibility that these receptors can function in these intracellular compartments. To assess the functionality of intracellular CB₁ receptors, we took advantage of the fact that CB₁ ligands are lipophilic and therefore can cross the plasma membrane and that a newly identified peptide antagonist of CB₁ receptors, hemopressin (28), does not cross the plasma membrane. We stimulated the cells with a lipophilic CB₁ agonist, WIN-55212 (100 nM) and used hemopressin to block plasma membrane receptors. Hemopressin has only a small effect at inhibiting WIN-55212-mediated ERK phosphorylation (Fig. 8A, left panel), consistent with the notion that the majority of the receptors are intracellular. The lipophilic CB₁ receptor antagonist SR 141716A (able to cross the membranes) completely inhibits WIN-55212-mediated ERK phosphorylation (Fig. 8A, left panel). The fact that the majority of CB₁ receptor-mediated ERK phosphorylation is not blocked by hemopressin indicates that intracellular CB₁ receptors are able to mediate signaling. Because depletion of cells of AP-3 δ , by a specific siRNA leads to an increase in plasma membrane insertion of CB₁ receptors (Figs. 6 and 7), we examined whether hemopressin had an increased effect on blocking receptor-mediated ERK phosphorylation. As expected, in these AP-3 depleted cells, hemopressin caused a larger inhibition (to the same extent as caused by SR 141716A) of CB₁ receptor-mediated ERK phosphorylation (Fig. 8A, right panel). These results, indicating that intracellular CB₁ receptors can mediate ERK phosphorylation after agonist treatment, further suggest that intracellular CB₁ receptors are functional.

CB₁ receptors associate with heterotrimeric G proteins in endosomal compartments

The intracellular activity of endogenous CB₁ receptors suggest that the receptors are coupled to a G protein in intracellular compartments. We examined the presence of G α i protein in CB₁-containing intracellular fractions. Although little is known about the association of G proteins with vesicles, the presence of a complete set of G proteins, including α -, β -, and γ -subunits, have been documented in large dense core vesicles from bovine adrenal medulla (chromaffin granules) and small synaptic vesicles from rodent and bovine brain (29). In addition, G α i protein has been reported to be present on the membranes of synaptic vesicles (30). To demonstrate the presence of G α i protein in intracellular compartments, we performed subcellular fractionation using a sucrose gradient to separate endosomal

compartments (19) and examined fractions by Western blot analysis using antibodies to late endosomes and lysosomes markers. We found that CB₁ receptors were present in the Rab7, and AP-3 containing fractions (Fig. 8B), supporting the presence of the receptor in late endosomal-lysosomal fractions. We also found that Gai protein and pERK were colocalized in this compartment, indicating the presence of these signaling molecules in CB₁ receptor-containing endosomal fractions. As a control, we found that the plasma membrane marker, Na-K-ATPase- α subunit, was absent from the endosomal fractions (Fig. 8B). It should be pointed out that the pERK in this experiment reflects the basal activity of the receptors, since these fractionation studies were performed in the absence of ligand stimulation. This was further confirmed by immunofluorescence and confocal microscopy experiments where we observed colocalization of pERK and CB₁ receptors in intracellular vesicles (Fig. 8C).

To confirm the association of intracellular CB₁ with Gai protein, we carried out immunoprecipitation experiments using the fraction that is enriched in endosomes (fraction 5 from the subcellular fractionation experiments). We found that Gai could be detected in the CB₁ receptor immunoprecipitate (Fig. 8D), indicating that CB₁ and Gai interact in endosomal compartments. This was further confirmed by immunofluorescence and confocal microscopy experiments in which we found colocalization of CB₁ receptors with Gai-EGFP in the lysosomes (Fig. 8C), in agreement with the notion that CB₁ receptors in this intracellular compartment represent functional receptors.

Discussion

In this study, we show that trafficking and subcellular localization of CB₁ receptors are affected by the receptor tag and overexpression. Immunolabeling of the extracellular domain of CB₁ receptors and quantification of plasma membrane receptors (by ELISA) indicate that C-terminal YFP tag leads to enhanced cell surface expression and subsequent constitutive endocytosis of these receptors. This is further supported by the robust colocalization of CB₁-YFP with DP rab5. In contrast to the YFP-tagged receptors, the cell surface expression of endogenous CB₁ receptors is significantly lower since it can be detected only after prolonged inhibition of endocytosis. These results suggest a mechanism other than that of constitutive endocytosis of membrane-bound receptors for the intracellular localization of endogenous CB₁ receptors. The fact that we find CB₁ receptors in an interacting complex with AP-3 and that down-regulation of AP-3 leads to increased cell surface receptor localization, suggests an alternative mechanism of intracellular localization and lysosomal targeting of CB₁ receptors. The AP-3 complex has previously been shown to be involved in the trafficking of proteins between the trans-Golgi network and lysosomes/melanosomes (31–34). AP-3 consists of four subunits δ , β 3, μ 3 and σ 3. β 3, μ 3 and σ 3 exist as two isoforms; the ubiquitous and the neuronal isoform (35, 36). Deletion of the δ subunit leads to the degradation of all neuronal and ubiquitous AP-3 subunits (37, 38). The cargo proteins that are transported through the AP-3 pathway contain dileucine- or tyrosine-based sorting signals in their cytoplasmic domains (35, 39–42). The CB₁ receptor sequence contains putative dileucine- or tyrosine-based sorting signals in its cytoplasmic domains, and these could directly interact with the AP-3 adaptor protein. Lamp I and Lamp II are lysosomal membrane proteins that contain tyrosine-based signals, and tyrosinase is a melanosomal protein that contains a dileucine-based sorting signal. These proteins use AP-3 for trafficking. It has been proposed that these proteins reach lysosomes/melanosomes through two distinct pathways: a direct pathway, which is mediated by AP-3, and an indirect pathway, where proteins are transported to the plasma membrane and then endocytosed to intracellular compartments *via* the AP-2 complex (43). In AP-3 β null cells, the transport of both Lamp I and Lamp II to the cell surface is increased, consistent with the idea that in the absence of AP-3 complex, these proteins enter the default pathway (44). Our results show that endogenous CB₁ receptors traffic *via* the direct pathway since we find an increased cell

surface localization of CB₁ receptors on AP-3 down-regulation. Taken together, these results suggest that CB₁ receptors could traffic *via* the same pathway as lysosomal proteins such as Lamp I and II. Overexpression of Lamp family members (such as lysosomal membrane glycoprotein lgp120 and of lgp-A and lgp-B) leads to their enhanced cell surface expression (45, 46), implying that lysosomal targeting involves a saturable intracellular sorting site (such as AP-3). Our findings indicate that overexpression of CB₁ (and of myc-CB₁ as we describe) leads to enhanced receptor cell surface expression, consistent with saturation of the AP-3-mediated lysosomal targeting of CB₁ receptors.

It is possible that the mechanism of CB₁ axonal targeting (14, 15) could involve an AP-3-mediated axonal transport of CB₁, implying an important role for AP-3 in neuronal vesicular trafficking to membrane domains. Previous studies have shown that AP-3 δ -deficient mice have neurological defects (37). Furthermore, recent reports have indicated an important role for neuronal AP-3 in the regulation of neurotransmitter-containing vesicle trafficking, and in particular, a role in the regulation of the basal and stimulated exocytosis of synaptic vesicles (47). Our data, for the first time, demonstrate that AP-3 can also participate in the regulation of GPCR trafficking and localization. This is of interest since GPCRs such as the delta opioid receptor have been found to traffic to the axon in neurotransmitter-containing secretory vesicles (48). It is possible that AP-3 could be involved in the axonal trafficking of a variety of GPCR and could contribute to the spatial segregation of receptors leading to distinct biological functions. Further studies are needed to dissect the molecular mechanism of AP-3 mediated GPCR trafficking and regulation and to characterize the contribution of neuronal and ubiquitous AP-3 in this process.

A number of studies have reported the presence of functional intracellular GPCRs (6, 49–51). Receptors for lipophilic agonists such as prostaglandins, platelet activating factor, estrogen, and lysophosphatidic acid can be constitutively localized intracellularly, where they may mediate intracrine signaling events (49). On the basis of these reports and our observations that CB₁ receptors are expressed predominantly in the intracellular compartment, we explored whether this intracellular receptor pool might be functionally active, particularly because cannabinoid ligands are freely permeable to membranes. Using a recently identified peptide (nonmembrane permeable) CB₁ antagonist, hemopressin, we showed that intracellular CB₁ receptors are functional. This and the fact that we find Gai colocalized with CB₁ and pERK in endosomal/lysosomal compartment, indicate that intracellular receptors are able to mediate signaling. The possibility that signaling events mediated by CB₁ receptors from distinct locations (cell surface and endosomes) could lead to differential cellular responses and that endosomal/lysosomal vesicles can function as a signaling compartment distinct from the plasma membrane (52) is an exciting possibility. Hence, spatial and temporal compartmentalization of receptor-mediated signal transduction may contribute to the repertoire and diversity of cannabinoid signaling.

In conclusion, the adaptor protein AP-3 directs CB₁ receptors to the late endosomes/lysosomes. There, CB₁ interacts with G proteins and is able to mediate signal transduction. This spatial segregation of CB₁ receptors could play a role in the regulation of cannabinoid signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Cell surface localization of endogenous and transfected CB₁ receptors. A–E) HEK293 cells transfected with CB₁-YFP (A), myc-CB₁ (B), and Neuro2A cells not transfected (C) or transfected with CB₁-YFP (D) or myc-CB₁ (E), were incubated with an anti-N-terminal extracellular domain of CB₁ antibody (red). The cells were then fixed and permeabilized for staining of total CB₁ using an anti-C-terminus CB₁ antibody (blue) and an anti-myc monoclonal antibody (green). YFP fluorescence is shown in green. Nuclei were stained with DAPI (gray). Cells were processed for confocal microscopy as described in Materials and Methods. Micrographs are representative of at least 3 independent experiments. Scale bar = 20 μ m. F) Quantification of plasma membrane vs. total CB₁ by ELISA. Cells were incubated on ice with primary anti-N terminus CB₁ antibody for 90 min then with IRDye 800-labeled anti-goat secondary antibody to obtain the levels of cell surface receptors. After fixation and permeabilization, the cells were incubated with a primary anti-C-terminus CB₁ and an IRDye 680-labeled anti-rabbit antibody to obtain the levels total CB₁. The plates were imaged by scanning simultaneously at 700 and 800 nm with an Odyssey imaging system as described in Materials and Methods. Specific fluorescence was obtained by subtracting the fluorescence obtained by incubation with secondary antibodies alone. Data represent mean $\pm$ SE of the ratio of membrane to total CB₁ receptors ($n=3$).

Figure 2.

Influence of the C-terminal YFP tag on the cell surface localization of CB₁ receptors. *A, B*) Neuro2A cells were transfected with Rab5-mCherry wild-type (Rab5) (*A*) or dominant positive (DP Rab5) with or without CB₁-YFP (*B*). Cells were stained for CB₁ using an anti-C-terminus CB₁ antibody and processed for confocal microscopy analysis as described in Materials and Methods. Nuclei were stained with DAPI. Micrographs are representative of at least 3 independent experiments. *C*) Neuro2A cells were treated with vehicle or with the dynamin inhibitor, Dynasore (1:500 v/v) as described in Materials and Methods. Cell surface expression of CB₁ receptors was assessed by incubating cells with an anti-N terminus CB₁ antibody prior to all fixations. *D*) Cell surface localization of CB₁ receptors was quantified by ELISA using an anti-N terminus CB₁ antibody as described in Materials and Methods. Values obtained with vehicle-treated cells were taken as a control. Data represent mean $\pm$ SE ($n=3$) and are expressed relative to control. Scale bars = 20 μ m.

Figure 3.

Influence of the C-terminal YFP tag on the cell surface localization of CB₁ receptors in primary hippocampal neurons. Five-day-old primary neurons were transfected with or without Rab5-mCherry-dominant positive with or without CB₁-YFP. *A*) Neurons were incubated with an anti-N terminus CB₁ antibody. The cells were then fixed and permeabilized for staining of total CB₁ receptors using an anti-C-terminus CB₁ antibody. *B*) Cells transfected with DP Rab5 with or without CB₁-YFP were stained for CB₁ using an anti-C-terminus CB₁ antibody. Cells were processed for confocal microscopy analysis as described in Materials and Methods. Micrographs are representative of at least 3 independent experiments. Scale bars = 20 μ m.

Figure 4.

Examination of endogenous CB₁ subcellular localization. Neuro2A cells were stained using an anti-C-terminus CB₁ antibody. Localization of CB₁ was compared to that of calnexin for ER localization, transfected galactosyl-transferase tagged with EGFP (GalT-GFP) for Golgi apparatus localization, transfected Trans-Golgi network 38 tagged with EGFP (TGN38-EGFP) for trans-Golgi network localization, and transfected Rab7-mCherry and Rab9-YFP for late endosomal localization and lysotracker for lysosome localization. Cells were processed for confocal microscopy analysis as described in Materials and Methods. Micrographs are representative of at least 3 independent experiments. Scale bars = 20 μ m.

Figure 5.

CB₁ receptors interact with the adaptor protein AP-3δ. **A)** Colocalization of CB₁ receptors with AP-3δ. Neuro2A cells were stained using an anti-C-terminus CB₁ antibody and an anti-AP-3δ or an anti-AP-2α antibody. Cells were processed for confocal microscopy analysis as described in Materials and Methods. Micrographs are representative of at least 3 independent experiments. Scale bar = 20 μm. **B)** Immunoprecipitation experiments: Neuro2A cells were lysed in lysis buffer, and CB₁ receptors were immunoprecipitated using an anti-N terminus CB₁ antibody. Presence of AP-3δ and AP-2α in the immunoprecipitate and in the cell lysate was assessed by Western blot analysis using specific antibodies to AP-3δ and AP-2α. **C)** Neuro2A cells were mock transfected or transfected with an siRNA to the murine CB₁ receptor. After 48 h, cells were lysed as described in Materials and Methods, and cell lysates were subjected to immunoprecipitation using an anti-N terminus CB₁ antibody. Decrease in the level of CB₁ expression in lysate from siRNA-transfected cells was assessed by Western blot analysis using a specific antibody to CB₁. Presence of AP-3δ in the immunoprecipitate and in the cell lysate was assessed by Western blot analysis using a specific antibody to AP-3δ. Micrographs are representative of 4 independent experiments.

Figure 6.

Effect of AP-3δ down-regulation on CB₁ receptor localization. *A*) Neuro2A cells were mock transfected, transfected with control siRNA, or siRNA to AP-3δ, and the levels of AP-3δ were measured by Western blot analysis as described in Materials and Methods. AP-2α was used as a control. *B*) Examination of cell surface localization of CB₁ receptors on siRNA-mediated AP-3δ down-regulation. Presence of CB₁ receptors at the cell surface was assessed by incubating cells with an anti-N terminus CB₁ antibody before fixation. Cells were processed for confocal microscopy analysis as described in Materials and Methods. Micrographs are representative of at least 3 independent experiments. Scale bar = 20 μm. *C*) Cell surface biotinylation. Neuro2A cells were transfected with a control siRNA or an siRNA to AP-3δ. After 48 h, cell surface proteins were biotinylated and isolated by incubation with agarose-coupled avidin. CB₁ receptors, in the biotinylated fraction, were detected by Western blot analysis using an anti-N terminus CB₁ antibody as described in Materials and Methods. Results are representative of 3 independent experiments. *D*) Quantification of surface CB₁ receptors by ELISA. Neuro2A cells were transfected with a control siRNA, or an siRNA to AP-3δ. After 48 h, cells were treated without or with the dynamin inhibitor, Dynasore (1:500 v/v) for 3 h, and cell surface expression of CB₁ receptors was measured by ELISA using an anti-N terminus CB₁ antibody as described in Materials and Methods. Values obtained with cells transfected with the control siRNA are taken as control (and normalized to 1.0). Values represent mean ± SE (*n*=3). **P* < 0.05 vs. control; #*P* < 0.05. vs. untreated cells transfected with the siRNA to AP-3δ.

Figure 7.

Effect of AP-3δ down-regulation on CB₁ receptor localization in primary hippocampal neurons. *A*) Localization of CB₁ receptors in primary neurons. One-week-old neurons were fixed and stained using an anti-C-terminus CB₁ antibody, lysotracker, or anti-AP-3δ antibody. Colocalization of CB₁ receptors with lysosomes or with AP-3δ was performed by confocal microscopy analysis, as described in Materials and Methods. Micrographs are representative of at least 3 independent experiments. *B*) Effect of AP-3δ down-regulation on CB₁ receptor levels at the plasma membrane. Primary neurons (5 days old) were transfected with an siRNA to AP-3δ or a control siRNA. After 48 h, cells were stained using an anti-N terminus CB₁ antibody before fixation and an anti-AP-3δ antibody following fixation with 4% PFA and permeabilization. Cells were processed for confocal microscopy analysis as described in Materials and Methods. *C*) Neurons were incubated with an anti-N terminus CB₁ antibody. The cells were then fixed and permeabilized for staining of total CB₁ receptors using an anti-C-terminal CB₁ antibody and staining for AP-3δ. On the same micrograph, a cell that lacks AP-3δ expression (asterisk) adjacent to a cell expressing AP-3δ is shown. Both cells express CB₁ receptors, but the receptor is present only at the cell surface of the cell that does not express AP-3δ. Micrographs are representative of 3 independent siRNA transfections. Scale bars = 20 μm.

Figure 8.

Intracellular CB₁ receptor is functional. **A**) Neuro2A cells were mock transfected or transfected with the siRNA to AP-3δ. Cells were preincubated with vehicle, 10 μM hemopressin (Hp, peptidic antagonist), or 1 μM SR 141716A (SR, lipophilic antagonist) for 15 min, followed by stimulation with 100 nM WIN-55212 (WIN, lipophilic agonist) ± inhibitor for indicated times. Cell extracts were subjected to Western blot analysis using pERK and ERK antibodies, as described in Materials and Methods. The amount of ERK phosphorylation in each lane was quantified using the Odyssey imaging system and normalized by expressing the data as a ratio of pERK over total ERK levels. Results are expressed as a percentage of the maximum response at 5 min of mock-transfected, WIN-55212-stimulated Neuro2A cells. Data represent mean ± SE (*n*=3–5). **P* < 0.05 vs. control. **B**) Association of CB₁ with G proteins in endosomes. Purification of endosomes of nonstimulated Neuro2A cells by differential centrifugation in discontinuous sucrose gradients. Total endosomal fraction containing late and early endosomes was purified by flotation in three-step sucrose gradient consisting of 40.6%, 35%, and 8.6% sucrose and collected from the interphase between 35% and 8.6% cushions of sucrose solutions. Fractions were probed with antibodies to early endosome antigen-1 (EEA-1), Rab7, AP-3δ, CB₁ receptors, Na-K-ATPase α subunit (a plasma membrane marker), Gαi, and pERK1/2. Representative of 3 fractionations. **C**) Immunofluorescence experiments showing

colocalization of CB₁ receptors (stained with the anti-C-terminus CB₁ antibody) and G α i EGFP in lysotracker-containing vesicles, and of CB₁ receptors (stained with the anti-C-terminus CB₁ antibody) and pERK in intracellular vesicles of nonstimulated Neuro2A cells. Micrographs are representative of at least 3 independent experiments. Scale bars = 20 μ m. D) Fraction 5 from the subcellular fractionation of nonstimulated Neuro2A cells was used for immunoprecipitation experiments. CB₁ receptors were immunoprecipitated from this fraction using an anti-N terminus CB₁ antibody as described in Materials and Methods. Presence of G α i was assessed by Western blot analysis using an antibody to G α i as described in Materials and Methods. Results are representative of 2 independent experiments.