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Effect of Lipid Rafts on Cb2 Receptor Signaling and 2-Arachidonoyl-Glycerol Metabolism in Human Immune Cells

Monica Bari,* Paola Spagnuolo,† Filomena Fezza,* Sergio Oddi,‡ Nicoletta Pasquariello,† Alessandro Finazzi-Agrò,* and Mauro Maccarrone2†‡

Recently, we have shown that treatment of rat C6 glioma cells with the raft disruptor methyl-β-cyclodextrin (MCD) doubles the binding of anandamide (AEA) to type-1 cannabinoid receptors (CB1R), followed by CB1R-dependent signaling via adenylate cyclase and p42/p44 MAPK activity. In the present study, we investigated whether type-2 cannabinoid receptors (CB2R), widely expressed in immune cells, also are modulated by MCD. We show that treatment of human DAUDI leukemia cells with MCD does not affect AEA binding to CB2R, and that receptor activation triggers similar [35S]guanosine-5′-O-(3-thiotriphosphate) binding in MCD-treated and control cells, similar adenylate cyclase and MAPK activity, and similar MAPK-dependent protection against apoptosis. The other AEA-binding receptor transient receptor potential channel vanilloid receptor subunit 1, the AEA synthetase N-acyl-phosphatidylethanolamine-phospholipase D, and the AEA hydrolase fatty acid amide hydrolase were not affected by MCD, whereas the AEA membrane transporter was inhibited (−55%) compared with controls. Furthermore, neither diacylglycerol lipase nor monoacylglycerol lipase, which respectively synthesize and degrade 2-arachidonoylglycerol, were affected by MCD in DAUDI or C6 cells, whereas the transport of 2-arachidonoylglycerol was reduced to −50%. Instead, membrane cholesterol enrichment almost doubled the uptake of AEA and 2-arachidonoylglycerol in both cell types. Finally, transfection experiments with human U937 immune cells, and the use of primary cells expressing CB1R or CB2R, ruled out that the cellular environment could account per se for the different modulation of CB receptor subtypes by MCD. In conclusion, the present data demonstrate that lipid rafts control CB1R, but not CB2R, and endocannabinoid transport in immune and neuronal cells. The Journal of Immunology, 2006, 177: 4971–4980.

A

nandamide (or arachidonoyl ethanolamide (AEA))5 and the other endocannabinoid 2-arachidonoylglycerol (2-AG) bind to and activate two inhibitory G protein-coupled receptors (GPCR), namely type-1 (CB1R) and type-2 (CB2R) cannabinoid receptors (1–3). CB1R are localized mainly in the CNS (4), but are also expressed in peripheral tissues like immune cells (5–7). Conversely, CB2R are predominantly expressed peripherally, but they are also present in the brain (8, 9). Therefore, activation of CB1 or CB2 receptors by AEA or 2-AG has many central (10) and peripheral (11) effects. These actions are controlled through not yet fully characterized cellular mechanisms, which regulate the release of endocannabinoids from membrane precursors, their uptake by cells, and finally their intracellular disposal. The key agent in AEA synthesis is the N-acyl-phosphatidylethanolamine-phospholipase D (NAPE-PLD) (12), whereas degradation occurs through a putative AEA membrane transporter (AMT) (13–15) and fatty acid amide hydrolase (FAAH) (16). Besides CB receptors, AEA binds also to type 1 vanilloid receptors (now called transient receptor potential channel vanilloid receptor subunit 1 (TRPV1)), and thus it can be considered a true “endovanilloid” (17). On the other hand, 2-AG is released from membrane lipids by means of an sn-1-specific diacylglycerol lipase (DAGL) (18), and is hydrolyzed by a specific monoacylglycerol lipase (MAGL) (19). The transport of 2-AG through the cellular membrane has been shown to be saturable and energy-independent, and might occur through the same AMT that transports AEA (13, 20, 21). Altogether, AEA and 2-AG, with other congeners like N-arachidonoyl-dopamine, noladin ether, and virodhamine and the proteins that bind, transport, synthesize, and hydrolyze these lipids form the “endocannabinoid system” (22, 23).

Lipid rafts are subdomains of the plasma membrane that contain high concentrations of cholesterol and glycosphingolipids, and are well-known modulators of the activity of a number of GPCR (24–26). In fact, they modulate signaling and membrane trafficking in many cell types (27), including human immune cells (28–30). Not surprisingly, lipid rafts have been proposed as a potential regulator of CBR activity (3, 31, 32), and indeed, we have shown recently that methyl-β-cyclodextrin (MCD), a membrane cholesterol depleter (33) that is widely used to disrupt the integrity of lipid rafts (24–26), doubles the CB1R binding and signaling in rat C6 glioma cells (34). CB1R activation after MCD treatment could also account for the effect of this raft disruptor to block apoptosis induced in vitro by AEA in the same C6 cells (34, 35). In addition,
two parallel studies have shown that cholesterol depletion by MCD reduces also the activity of AMT (34, 36), possibly by promoting a faster endocytosis of the transporter molecules (37).

This growing evidence suggesting that lipid rafts might modulate the endocannabinoid signaling prompted us to investigate also the possible effect of lipid rafts integrity on CB2 receptors, on AEA metabolism in immune cells, and on the proteins that synthesize, transport, and degrade 2-AG. We have chosen human DAUDI leukemia cells, because they have active AMT and FAAH (38), express functional CB2R (39), and are protected by CB2R activation against AEA-induced apoptosis (38). Overall, DAUDI cells share several aspects of the endocannabinoid system and endocannabinoid-induced apoptosis with C6 cells (38), which we used in our previous study on the effect of lipid rafts on C6R (34). On the other hand, in DAUDI cells lipid rafts regulate important functions like exosome secretion (41), or growth arrest induced by antitumor drugs (41). We perturbed raft integrity also by means of membrane cholesterol enrichment, under the same experimental conditions already used for C6 cells (42). In addition, we checked for the first time the effect of membrane cholesterol depletion or enrichment on 2-AG metabolism in C6 cells. Taken together, this study and the two previous reports (34, 42) monitor the effect of lipid rafts integrity on all the major proteins that bind and metabolize AEA and 2-AG, both in neuronal and immune cells. The results point out that CB1R and endocannabinoid transporters are probably localized within lipid rafts, at variance with CB2R and the other proteins of the endocannabinoid system.

**Materials and Methods**

**Materials**

Chemicals were of the purest analytical grade. AEA, cholesterol, MCD, pertussis toxin (PTX), resinsinatox (RTX), and guanosine-5′-O-(3-thiotriphosphate) (GTPγS) were obtained from Sigma-Aldrich. Methyl-arachidonoyl fluorophosphonate (MAFP) was purchased from Cayman Chemical. Capsazepine (N-(2-(4-chlorophenyl) ethyl)-1,3,4-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide, CPZ) was from Calbiochem, VDM11 was from Tocris-Cookson, and 2-amino-3-methoxy-flavone (PD098059) was from Alexis Corporation. [3H]AEA (223 Ci/ mmol), [3H]C955.940 (126 Ci/mmol), [35S]GTPγS (125 Ci/mmol), [3H]RTX (43 Ci/mmol), adenosine 5′-[γ-32P]triphosphate (3000 Ci/mmol), and sn-1-stearyl-2-[14C]arachidonoyl-glycerol (56 Ci/mmol) were purchased from PerkinElmer Life Sciences. N-[1H]Arachidonoyl-phosphatidyl-ethanolamine ([1H]NAPE, 200 Ci/mmol) and 2-oleyl-[1H]glycerol (20 Ci/mmol) were from ARC; 2-[(3H)JAG was synthesized from 1,3-dibenzoxy-2-propanol and [3H]arachidonic acid (200 Ci/mmol; ARC), as reported previously (43). N-(1S,3S)-endo,1,3-trimethyl-bicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylenephenoxy)-(4-methyl-benzyl)-pyrazole-3-carboxamide (SR144528) and 2-Piperidino-5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-3-pyrazole carboxamide (SR141716) were gifts from Sanofi-Aventis Research (Montpellier, France). 6-Dodecanoyl-2-dimethylamino-naphthalene (laurdan) was obtained from Molecular Probes. The cDNAs encoding for the human cannabinoid receptors 1 and 2 were purchased from the University Missouri cDNA Resource Center. The pcDNA3.1 and pcDNA3.1/CT-GFP expression vectors and LipofectAMINE 2000 were from Invitrogen Life Technologies.

**Cell culture and treatment and determination of apoptosis**

Human DAUDI leukemia cells were cultured in RPMI 1640 medium (Invitrogen Life Technologies), supplemented with 25 mM HEPES, 2.5 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS as described previously (38). Cholesterol depletion was performed by preincubating DAUDI cells for 30 min at 37°C with the indicated amounts of MCD, which removes cholesterol from the plasma membranes (24, 44). After MCD pretreatment, cells were washed in PBS and then were treated with AEA (or vehicle in the controls) as detailed below. Cell viability was assessed by trypan blue dye-exclusion. Rat C6 glioma cells were cultured and treated with MCD as described previously (34). Also primary Sertoli cells, isolated from 16-day-old mice and cultured as reported (45), and primary HUVEC (BioWhittaker), cultured as previously reported (46), were treated with 2.5 mM MCD for 30 min at 37°C. Then, Sertoli cells and HUVEC were subjected to CB2 binding assays as detailed below.

Cholesterol enrichment was performed by preincubating DAUDI cells or C6 cells for 30 min at 37°C with a cholesterol-polyvinylpyrrolidone-BSA dispersion (vehicle in the controls), as described previously (47). Briefly, 20 μl of a stock solution of cholesterol in methanol (250 mg/ml) was added to 5 ml of sterile PBS containing 3.5% polyvinylpyrrolidone and 2% BSA. The dispersion was sonicated four times for 1 min, at 30-s intervals, using a Vibracell sonifier (Sonics & Materials), and was added to the cells. After incubation for 30 min at 37°C, DAUDI cells or C6 cells were washed twice in sterile PBS and were finally resuspended in culture medium. Cell viability was assessed by trypan blue dye-exclusion and was found to be >95%, in keeping with previous studies (47).

Apoptosis was estimated 48 h after treatment with AEA (or vehicle in the controls) (38), by using the cell-death detection ELISA kit (Boehringer Mannheim), based on the evaluation of DNA fragmentation by an immunoassay for histone-associated DNA fragments in the cell cytoplasm (38). This method has been validated recently for DAUDI cells by comparison with cytofluorimetric analysis performed in a FACSCalibur Flow Cytometer (BD Biosciences) (38). This latter technique quantifies apoptotic body formation in dead cells by staining with propidium iodide (50 μg/ml), and DAUDI cells contained <4.0 ± 1.0 apoptotic bodies for every 100 cells analyzed (38).

**Cholesterol quantitation and analysis of cell membrane fluidity**

Membranes were extracted from DAUDI cells (5 × 10^6/test) and cholesterol content was measured by means of cholesterol oxidase (kit from Biovision) (47). Membrane fluidity of the cells was determined by means of the fluorescent probe laurdan, as already described previously (47). Membrane fluidity is inversely proportional to the ratio of laurdan fluorescence at 440 nm vs that at 490 nm (F<sub>440</sub>/F<sub>490</sub>): the higher the ratio, the lower the fluidity (48).

**Receptor binding assays and AEA-stimulated [35S]GTPγS binding**

Binding of [3H]PP5.940 to DAUDI cells (200 × 10^6/test) was performed on membrane fractions by rapid filteration assays, as reported (38). Binding data were elaborated through nonlinear regression analysis, using the Prism 4 program (GraphPad), to calculate maximum binding (B<sub>max</sub>) and dissociation constant (K<sub>d</sub>) of [3H]PP5.940. Saturation binding of [3H]PP5.940 was further analyzed through Scatchard plots, generated by Prism 4. Also the binding of 200 pM [3H]RTX was evaluated by rapid filtration assays, performed as reported previously (49). In all binding experiments, nonspecific binding was determined in the presence of 1 μM "cold" agonist (38, 49).

AEA-stimulated [35S]GTPγS binding was determined essentially as described previously (50, 51). Cells were homogenized in ice-cold assay buffer (50 mM Tris-HCl (pH 7.4), 3 mM MgCl<sub>2</sub>, and 1 mM EGTA) and centrifuged twice at 48,000 × g for 10 min at 4°C. Pellets were resuspended in and homogenized in membrane buffer (50 mM Tris-HCl, 3 mM MgCl<sub>2</sub>, 100 mM NaCl, 50 μM phenylmethylsulfonyl fluoride, and 0.2 mM EGTA), and protein concentration was determined. Then, membrane preparations were preincubated in 4 μM adenosine deaminase (183 U/mg protein; Sigma-Aldrich) for 10 min at 30°C. The binding of [35S]GTPγS stimulated by 1 μM AEA was assayed in the presence of 30 μM GDP, 0.1 mM [35S]GTPγS, 50 μg of protein, and assay buffer in a final volume of 1 ml. Nonspecific binding was determined in the absence of agonist and in the presence of 10 μM unlabeled GTPγS (50, 51).

**U937 cell culture and transfection**

The human leukaemic monocytic lymphoma U937 cells were cultured in RPMI 1640 medium, containing 10% FBS, 0.11 mg/ml sodium pyruvate, 2 mM t-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS as described previously (38). The pcDNA-CB1R and pcDNA-CB2R protein expression plasmids encoding for type-1 and type-2 cannabinoid receptors, respectively, were produced by inserting the full-length amino acid sequences of human CB1R and CB2R into pcDNA3.1 (Invitrogen Life Technologies). These plasmids were transfected into U937 cells (75 × 10^6/test), using the LipofectAMINE 2000 reagent according to the manufacturer’s instructions. The transfection efficiency was found to be ~25%, by counting GFP-positive cells in at least five fields of vision under the fluorescent microscope (52). The expression of CB receptors was evaluated 24 h after transfection by Western blotting of cell extracts, using specific anti-CB1R or anti-CB2R Abs (Cayman Chemical). Twenty-four hours after transfection, cells were treated with 2.5 mM MCD for 30 min, and were subjected to receptor binding assays as reported above. U937...
cells transfected with the mock plasmid pcDNA3.1/CT-GFP were used as control.

Determination of AEA uptake, synthesis, and hydrolysis

The activity of the AMT in DAUDI cells was measured as described previously (46). Cells (2 × 10⁶/test) were incubated for 15 min, at 37 or 4°C, with 400 nM [³H]AEA, then they were washed three times in 2 ml of PBS containing 1% BSA and were finally resuspended in 200 µl of PBS. Membrane lipids were then extracted (46), resuspended in 0.5 ml methanol, mixed with 3.5 ml of Sigma-fluid liquid scintillation mixture for nonaqueous samples (Sigma-Aldrich), and radioactivity was measured in a LKB1214 Rackbeta scintillation counter (Amersham Biosciences). To discriminate noncarrier-mediated from carrier-mediated transport of AEA through cell membranes, [³H]AEA uptake at 4°C was subtracted from that at 37°C (46). Apparent Michaelis-Menten constant (Km) and maximum velocity (Vmax) of the uptake of [³H]AEA (0–800 nM range) by AMT were determined by nonlinear regression analysis through the Prism 4 software, as reported (46). AMT activity was expressed as pmol AEA taken up per min per mg protein. The synthesis of AEA through the activity of NAPE-PLD (EC 3.1.4.4) was assayed in DAUDI cell homogenates (50 µg/test), using 100 µM [³H]NAPE as reported previously (53). NAPE-PLD activity was expressed as pmol [³H]AEA released per min per mg protein. The hydrolysis of [³H]AEA by the fatty acid amide hydrolase (EC 3.5.1.4; FAAH) activity was assayed in DAUDI cell extracts (20 µg/test) by measuring the release of [³H]arachidonic acid from 10 µM [³H]AEA at pH 9.0, using reversed phase high performance liquid chromatography (38). FAAH activity was expressed as pmol of arachidonate released per minute per milligram of protein.

Determination of 2-AG uptake, synthesis, and hydrolysis.

The uptake of 2-AG by DAUDI cells or C6 cells (2 × 10⁶/test) was assayed as described above for AMT, using 400 nM [³H]2-AG as substrate. Apparent Km and Vmax of the uptake of [³H]2-AG (0–800 nM range) were determined by nonlinear regression analysis through the Prism 4 program (46). The transport activity was expressed as pmol 2-AG taken up per minute per milligram of protein. The activity of DAGL was assayed with 10 µM sn-1-stearoyl-2-[³C]arachidonoyl-glycerol as substrate (18), and that of MAGL was determined using 10 µM 2-oleoyl-[³H]-glycerol as substrate, as reported (19). Both DAGL and MAGL activities were expressed as pmol product per minute per milligram of protein.

Other enzymatic assays

DAUDI cells (5 × 10⁶/test) were incubated for 15 min at 37°C with AEA and related compounds, then they were washed, homogenized and subjected to enzymatic assays. Forskolin (1 µM)-stimulated adenylate cyclase (AC) (EC 4.6.1.1) activity was determined according to the amount of cAMP (54), detected in cell extracts with the cAMP Enzyme Immunoassay kit (Amersham Biosciences), as described previously (46). AC activity was expressed as pmol cAMP per min per mg protein. The activity of p2/p4 MAPK (EC 2.7.1.37; MAPK) was assayed in cell extracts by the phosphorylation of MAPK-specific peptide substrate at 30°C with adenosine 5’-γ-[³P]triphosphate (55), using the Biotрак MAPK Enzyme Assay system (Amersham Biosciences) as reported previously (56). MAPK activity was expressed as pmol phosphate per min per mg protein. The effect of pretreatment with PTX on enzymatic activities was determined by preincubating DAUDI cells for 3 h at 37°C with 5 µg/ml PTX before addition of AEA, or vehicle in control experiments (55).

Statistical analysis

Data reported in this article are the means ± SD of at least three independent experiments, each performed in duplicate. Statistical analysis was performed by the nonparametric Mann-Whitney U test, elaborating experimental data by means of the Instat 3 program (GraphPad).

Results

Effect of MCD on CB2 receptors

Human DAUDI leukemia cells were treated with MCD in a concentration range (0.5–5 mM) widely used to disrupt lipid rafts (24–29). In particular, 2.5 mM MCD has been recently shown to double the binding efficiency of CB1 receptors, defined as the Bmax/Kd ratio by analogy with the catalytic efficiency (Vmax/Km) of enzymes, and to enhance ~3-fold CB1R-dependent signaling in nerve cells (34). Therefore, we used the same range of MCD concentration to test the effect of lipid rafts on CB2R binding and signaling in immune cells. Independently of the dose used, MCD did not affect the binding of [³H]CP55.940, a CB1R and CB2R agonist (57). DAUDI cells express type-2 CB receptors (38, 39), and consistently [³H]CP55.940 binding was fully displaced by 0.1 µM SR144528 (Fig. 1A), a selective CB2R antagonist (57). Saturation curves like those shown in Fig. 1B allowed to calculate the constants for [³H]CP55.940 binding, i.e., Bmax values of 301 ± 10 or 296 ± 9 fmol/mg protein, and Kd values of 294 ± 30 or 306 ± 28 pm, for untreated or MCD-treated cells, respectively. These data were further confirmed by Scatchard analysis of the saturation binding of [³H]CP55.940 to control (Fig. 1C) or MCD-treated cells (Fig. 1D), which yielded Bmax values of 306 or 299 fmol/mg protein, and Kd values of 305 or 312 pm, for control or MCD-treated cells respectively. Thus, the binding efficacy of CB2R was always ~1. Incidentally, Bmax and Kd values of CB2R in DAUDI cells are close to those reported for the same receptor from other sources (2, 57). These data show that, unlike CB1R in nerve cells, CB2 receptors in leukemic cells are not affected by lipid rafts disruption. To ascertain whether the cellular environment per se could be responsible for the different effect of MCD on CB1 and CB2 receptors, human lymphoma U937 cells were transfected with plasmids encoding for type-1 or type-2 cannabinoid receptors. These cells are a widely used model of immune cells, and were chosen because they have a functional endocannabinoid system (38). We found that U937 cells are devoid of CB receptors, based on Western blot (data not shown) and binding data (Table I). These observations extend previous findings from our group (38), yet conflicting reports have shown that the same cell line expresses a “brain-type” (i.e., type-1) (58), a type-2 (59), or both type-1 and type-2 (60) cannabinoid receptors. These discrepancies may be due to different subclones of U937 cells, and underline the need to characterize the biochemical background of a cell type before running biological assays (61). Twenty-four hours after transfection, U937 cells expressed CB1 proteins (data not shown), and were treated with 2.5 mM MCD for 30 min to perform receptor binding assays. Table I shows that both CB receptors were functional in transfected cells, and that CB1R or CB2R binding was minimized by the corresponding selective antagonists SR141716 or SR144528 (57), respectively. Yet, MCD enhanced CB1R binding only (Table I), ruling out any effect of the gross cellular environment per se. In addition, we sought to ascertain whether the effect of MCD on CB receptors in immortalized cell lines was representative also of normal cells. To this end, we chose two primary cell types that are known to bind [³H]CP55.940 through CB2R or CB1R only: mouse Sertoli cells (45) and HUVEC (Ref. 46 and references therein), respectively. We could not use human peripheral lymphocytes, because they express both CB receptors (5, 6), thus preventing to dissect the effect of MCD on CB1R vs CB2R. In keeping with the data on DAUDI cells (Fig. 1A) and on C6 cells (34), treatment of Sertoli cells with 2.5 mM MCD did not affect [³H]CP55.940 binding, which instead was increased up to ~160% of the controls in HUVEC (Fig. 2). The selective antagonists SR141716 and SR144528 minimized binding of [³H]CP55.940 to CB1R or CB2R, respectively (Fig. 2).

Effect of MCD on CB2-dependent signaling pathways

The main signaling pathways triggered by agonist binding to CB2 receptors include inhibition of AC and stimulation of MAPK, both mediated by Gi/o proteins (for review, see Refs. 1–3, 57). Here, pretreatment of DAUDI cells with MCD did not affect the binding of [³H]GTPγS stimulated by various amounts of AEA, up to a saturating concentration of 1 µM (data not shown). The effect of 1 µM AEA was fully prevented by 0.1 µM SR144528 at all concentrations of MCD (data not shown). In addition, pretreatment...
with 2.5 mM MCD did not affect the basal activity of AC or that of MAPK, nor did it significantly potentiate the effect of AEA up to 1 μM on these enzymes; at the latter saturating concentration AEA inhibited AC down to 50% of the controls, and increased MAPK up to 240% (Fig. 3, A and B, and data not shown). The activity of AC and MAPK in AEA-treated control cells was 55 and 225% of the basal levels, respectively (Fig. 3, A and B). Additionally, the effects of 1 μM AEA on AC and MAPK were abolished by 0.1 μM SR144528, or by 5 μg/ml PTX, an inhibitor of Gi/o proteins (55, 56) (Fig. 3, A and B). These findings suggest that MCD does not affect CB2R binding nor the agonist-induced CB2R signaling through Gi/o proteins.

Effect of MCD on TRPV1 receptors, AEA synthesis, uptake, and degradation

We further investigated the effect of MCD on the other proteins of the endocannabinoid system that bind and metabolize AEA. The binding of [3H]RTX, a selective TRPV1 agonist (49, 62), to DAUDI cell membranes was not affected by MCD, yet it was fully inhibited by 1 μM CPZ, a selective TRPV1 antagonist (49, 62) (data not shown). Independently of the dose, MCD did not affect the activity of NAPE-PLD, responsible for AEA synthesis (12), nor that of the AEA-hydrolase FAAH (data not shown), whereas it reduced the activity of the AEA transporter AMT down to 55% at 2.5 mM (Fig. 4A). As expected, the activities of AMT and of

Effect of MCD on TRPV1 receptors, AEA synthesis, uptake, and degradation

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Table 1. Effect of cellular environment on CBR modulation by MCD

<table>
<thead>
<tr>
<th>Treatment of U937 Cells</th>
<th>CBR Binding (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>ND</td>
</tr>
<tr>
<td>+2.5 mM MCD</td>
<td>ND</td>
</tr>
<tr>
<td>+CB1R-plasmid</td>
<td>50 ± 8 (100%)</td>
</tr>
<tr>
<td>+CB1R-plasmid + 0.1 μM SR141716</td>
<td>10 ± 3 (20%)*</td>
</tr>
<tr>
<td>+CB1R-plasmid + 2.5 mM MCD</td>
<td>78 ± 8 (156%)*</td>
</tr>
<tr>
<td>+CB2R-plasmid</td>
<td>55 ± 7 (100%)</td>
</tr>
<tr>
<td>+CB2R-plasmid + 0.1 μM SR144528</td>
<td>11 ± 3 (20%)*</td>
</tr>
<tr>
<td>+CB2R-plasmid + 2.5 mM MCD</td>
<td>60 ± 7 (109%)*</td>
</tr>
</tbody>
</table>

* Denotes p < 0.01 compared with CB1R- or CB2R-transfected cells, respectively (p > 0.05 in all other cases).

Values in parentheses represent percentage of the control, set to 100.

Substrate was 400 pM [3H]CP55.940.

ND. Not detectable.

* Denotes p < 0.01 compared with CB1R- or CB2R-transfected cells, respectively (p > 0.05 in all other cases).
FAAH were fully inhibited by the corresponding selective inhibitors VDM11 (10 μM) and MAFP (100 nM) (62). The lack of specific inhibitors of NAPE-PLD did not allow to test the inhibition of this enzyme. The kinetic analysis of AMT in control DAUDI cells yielded Km and Vmax values of 100 ± 15 nM and 145 ± 4 pmol/min per mg protein (Table II), in keeping with a previous report (38). In DAUDI cells treated with 2.5 mM MCD the values of Km and Vmax were 92 ± 3 nM and 81 ± 1 pmol/min per mg protein, respectively (Table II). Overall, MCD reduced the catalytic efficiency of AMT, defined as the Vmax/Km ratio, to ~60% of the control. Furthermore, the uptake of 400 nM [3H]AEA was reduced to ~45% by 400 nM 2-AG.

Effect of MCD on 2-AG synthesis, uptake, and degradation
MCD did not affect at any concentration the activity of DAGL, which is responsible for 2-AG synthesis (18), nor that of MAGL, the main responsible for 2-AG hydrolysis (19) (data not shown). Yet, it dose-dependently reduced the uptake of [3H]2-AG, down to ~50% at 5 mM (Fig. 4B). Interestingly, the uptake of [3H]2-AG was fully inhibited by the AMT inhibitor VDM11 (Fig. 4B). Specific inhibitors of DAGL or MAGL are not yet commercially available, thus further inhibition experiments were not feasible. Additionally, kinetic analysis of the uptake of [3H]2-AG in control or MCD-treated DAUDI cells yielded Km and Vmax values of 134 ± 11 nM and 80 ± 2 pmol/min per mg protein, or 111 ± 33 nM and 50 ± 3 pmol/min per mg protein, respectively (Table II). Therefore, MCD reduced the catalytic efficiency of [3H]2-AG uptake to 75% of the control. Finally, the uptake of 400 nM [3H]2-AG was reduced to ~55% by 400 nM AEA.

Effect of MCD on membrane properties
To confirm that MCD treatment affected the membrane properties of DAUDI cells as it does in C6 cells (34), we checked the cholesterol content and membrane fluidity of DAUDI cells. MCD is a cholesterol depletor (33), and indeed it produced a dose-dependent decrease in membrane cholesterol content (Table III). This effect was paralleled by a dose-dependent decrease in the fluorescence ratio of laurdan (Table III), which is an index of membrane fluidity: the higher the ratio, the lower the fluidity (48). At the concentration of 2.5 mM, MCD reduced cholesterol content and fluorescence ratio of DAUDI cell membranes to 38 and 63% of the control values, indicating that membranes of MCD-treated cells were more fluid than those of controls. These changes in DAUDI cells were superimposable on those recently reported for C6 cells (34), suggesting that the different effect of MCD on CB1 and CB2 receptors was not due to a different effect of this substance on cell membranes.
induced apoptosis is blocked by activation of CB2R in DAUDI cells, and of CB1R in C6 cells (38). We have already demonstrated that in C6 cells MCD enhanced CB1R-mediated protection against apoptosis induced by AEA, by reinforcing the CB1R signaling through MAPK (34). These data are in keeping with the concept that activation of CB receptors is antiapoptotic (38, 63, 64).

In the present study, we show that also in DAUDI cells 10 μM CPZ, a selective TRPV1 antagonist (49, 62), quenched the AEA-induced apoptosis down to ~40% of controls, whereas 1 μM SR144528 or 20 μM PD98059, a MAPK inhibitor (55), further increased it up to ~170 or ~150% of controls, respectively (Fig. 5). However, treatment with MCD (2.5 or 5 mM) did not protect at all DAUDI cells against AEA-induced apoptosis, in much the same way as it did not enhance CB2R binding and signaling in these cells (compare Fig. 5 with Figs. 1 and 3).

**Effect of cholesterol enrichment on the endocannabinoid system of DAUDI cells**

We sought to extend the study of the role of lipid rafts perturbation on the endocannabinoid system also in the opposite way, i.e., by increasing membrane cholesterol (65, 66). Table III shows that cholesterol treatment of DAUDI cells led to a ~300% increase in membrane cholesterol content and a ~50% increase in fluorescence ratio (i.e., decrease in membrane fluidity). These changes in cell membranes were superimposable on those reported in C6 cells under the same experimental conditions (42). In parallel, in cholesterol-treated DAUDI cells AMT activity was enhanced up to ~180% of untreated controls, whereas TRPV1 binding, NAPE-PLD activity, and FAAH activity were not affected (Table IV). Kinetic analysis of AMT in cholesterol-treated DAUDI cells showed apparent Km and Vmax values of 96 ± 10 nM and 255 ± 5 pmol/min per mg protein, respectively, indicating an improved

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**Table II. Effect of MCD or cholesterol treatment on the kinetic properties of AMT or 2-AG uptake in DAUDI cells**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cholesterol Content</th>
<th>Fluorescence Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.00 ± 0.35 (100%)</td>
<td>1.90 ± 0.20 (100%)</td>
</tr>
<tr>
<td>+ 0.5 mM MCD</td>
<td>2.49 ± 0.25 (83%)</td>
<td>1.71 ± 0.22 (90%)</td>
</tr>
<tr>
<td>+ 1 mM MCD</td>
<td>2.22 ± 0.25 (74%)</td>
<td>1.61 ± 0.20 (85%)</td>
</tr>
<tr>
<td>+ 2.5 mM MCD</td>
<td>1.14 ± 0.15 (38%)**</td>
<td>1.19 ± 0.15 (63%)**</td>
</tr>
<tr>
<td>+ 5 mM MCD</td>
<td>0.90 ± 0.11 (30%)**</td>
<td>1.05 ± 0.12 (55%)**</td>
</tr>
<tr>
<td>+ Cholesterol</td>
<td>8.70 ± 0.80 (290%)**</td>
<td>2.95 ± 0.24 (155%)**</td>
</tr>
</tbody>
</table>

*p Values in brackets represent percentage of the control, set to 100.

*a* Denotes *p* < 0.05 compared with control; ** denotes *p* < 0.01 compared with control (*p* > 0.05 in all other cases).
catalytic efficiency (~180% of untreated controls) (Table II). Altogether, these data recall those obtained about the cholesterol-dependent modulation of the endocannabinoid system in C6 glioma cells (42). However, a major difference between these two cell types is that cholesterol enrichment did not affect CB2R binding of AEA in DAUDI cells (Table IV), whereas it halved the CB1R binding in C6 cells (42). Taken together with the effect of MCD, these data demonstrate that CB2R, unlike CB1R, is not modulated by perturbation of lipid rafts integrity. In addition, cholesterol enrichment did not significantly affect DAGL and MAGL activity in DAUDI cells, whereas it increased [3H]2-AG uptake up to 175% of untreated controls (Table IV). Kinetic analysis demonstrated that in cholesterol-treated DAUDI cells apparent Km and Vmax values for [3H]2-AG uptake were 121 ± 7 nM and 137 ± 2 pmol/ min per mg protein, respectively, yielding a catalytic efficiency of ~180% of that controls (Table II).

**Effect of MCD or cholesterol treatment on 2-AG metabolism in rat C6 glioma cells**

To extend the analysis of the effect of cholesterol depletion or enrichment also to 2-AG metabolism in neuronal cells, we investigated the effect of MCD or cholesterol addition on the activity of DAGL and MAGL, and on [3H]2-AG uptake in C6 glioma cells. Table V shows that 2.5 mM MCD or cholesterol treatment had no effect on DAGL and MAGL activity, whereas they reduced to ~60% or enhanced to ~190% [3H]2-AG uptake, respectively. These effects of MCD and cholesterol on C6 cells mirrored those on DAUDI cells (Table V), suggesting that 2-AG synthesis or hydrolysis is not modulated by membrane cholesterol content in either neuronal or immune cells.

**Discussion**

We report here unprecedented evidence that the cholesterol depleter MCD does not affect the binding of endocannabinoids to CB2 receptors, and subsequent G protein-dependent signaling through AC and MAPK. This is at variance with CB1 receptors, whose binding and signaling are almost doubled by MCD treatment under similar experimental conditions (34). In addition, we demonstrate that the different effect of MCD on CB1 or CB2 receptors does not depend on the gross cellular environment, and is observed also in normal cells. Furthermore, we show that MCD treatment reduces the uptake of 2-AG without affecting its metabolism via DAGL and MAGL activity, in much the same way as it reduces AMT activity without affecting the other proteins that bind (TRPV1) or metabolize (NAPE-PLD or FAAH) AEA. Conversely, 2-AG uptake and AMT activity of DAUDI cells are enhanced by cholesterol enrichment, which does not affect CB2R, TRPV1, or the enzymes that metabolize AEA or 2-AG. Taken together, these data suggest that type-1 cannabinoid receptors and endocannabinoid transporters are localized within lipid rafts, both in neuronal and immune cells. Incidentally, this is the first report showing MAGL and DAGL activity in cells of the immune system.

The cannabinoid receptor subtypes, CB1 and CB2, are encoded by different genes, exhibit 44% amino acid identity throughout the whole protein, and have been classified into the class A rhodopsin-like family of GPCR (1–3, 57). In the absence of crystal structures, studies have been conducted to understand the three-dimensional structure of CB receptors and their mechanisms of action by using computer molecular modeling and nuclear magnetic resonance (NMR) approaches. Central and peripheral CB1 and CB2 receptors are both activated by AEA or 2-AG, and trigger common signaling pathways mainly based on AC inhibition and MAPK activation (1–3, 57). Therefore, it would be of utmost importance to identify a possible differential regulation of CB1R and CB2R, also in view of the fact that these two receptor subtypes have been recognized.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>+Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB2R binding (fmol/mg protein)*</td>
<td>160 ± 20</td>
<td>150 ± 20 (94%)</td>
</tr>
<tr>
<td>TRPV1 binding (fmol/mg protein)*</td>
<td>80 ± 10</td>
<td>76 ± 10 (95%)</td>
</tr>
<tr>
<td>NAPE-PLD activity (pmol/min per mg protein)*</td>
<td>37 ± 4</td>
<td>40 ± 4 (108%)</td>
</tr>
<tr>
<td>AMT activity (pmol/min per mg protein)*</td>
<td>120 ± 10</td>
<td>215 ± 20 (179%)*</td>
</tr>
<tr>
<td>FAAH activity (pmol/min per mg protein)*</td>
<td>245 ± 22</td>
<td>230 ± 24 (94%)</td>
</tr>
<tr>
<td>DAGL activity (pmol/min per mg protein)*</td>
<td>22 ± 3</td>
<td>18 ± 2 (82%)</td>
</tr>
<tr>
<td>[3H]2-AG uptake (pmol/min per mg protein)*</td>
<td>60 ± 5</td>
<td>105 ± 10 (175%)*</td>
</tr>
<tr>
<td>MAGL activity (pmol/min per mg protein)*</td>
<td>82 ± 9</td>
<td>70 ± 8 (85%)</td>
</tr>
</tbody>
</table>

* Values in parentheses represent percentage of the control, set to 100.
* Substrate was 400 PM [3H]CP55,940.
* Substrate was 200 PM [3H]resiniferatoxin.
* Substrate was 100 PM [3H]NArPE.
* Substrate was 400 PM [3H]AEA.
* Substrate was 10 PM [3H]AEA.
* Substrate was 10 PM 3-1-stearyl-2-[14C]arachidonoyl-glycerol.
* Substrate was 400 PM [3H]2-AG.
* Substrate was 10 PM 2-oleoyl-[3H]glycerol.
* Denotes p < 0.01 compared with untreated control (p > 0.05 in all other cases).
as distinct drug discovery targets for numerous potential therapeutic applications. These include food intake, cancer, and immune suppression (22, 67, 68). Recently, we have shown that in rat C6 glioma cells CB1R are regulated by lipid rafts, so that raft perturbation by cholesterol depletion enhanced CB1R binding and signaling (34), whereas raft perturbation by cholesterol enrichment had the opposite effect (42). Shortly afterward, an independent report has shown that CB1R are localized within lipid rafts also in human MDA-MB231 cells, a breast cancer cell line (69). More notably, a solid-state NMR study has shown that AEA undergoes a fast lateral diffusion within the bilayer outer leaflet before making a productive interaction with CB1R (70), giving ground to the concept that the membrane environment is critical for CB1R binding and signaling. In the present study, the following lines of evidence support the major finding of this study, i.e., that CB2R, unlike CB1R, are not affected by perturbation of lipid rafts: 1) binding of [3H]CP55,940 to CB2R and kinetic constants of saturation curves of this binding were not affected by MCD, used in a concentration range that enhances CB1R in glioma cells (34) and in breast cancer cells (69); 2) AEA-induced stimulation of the binding of GTPγS to DAUDI cells was not enhanced by MCD at any dose, neither was 3) CB2R-dependent signaling through AC and MAPK; 4) the antiapoptotic effect of CB2R activation, exerted through the MAPK pathway, was not further enhanced by MCD; and 5) lipid rafts perturbation by membrane cholesterol enrichment was ineffective on CB2R. Remarkably, the effect of MCD on membrane properties of DAUDI cells (Table III) was superimposable on that observed in C6 cells (34), ruling out that MCD treatment was ineffective on leukemic cell membranes. In addition, transfection of the human immune U937 cell line with CB1R- or CB2R-expressing plasmids ruled out that the gross cellular environment could be responsible per se for the different effect of MCD on CB1 and CB2 receptors (Table I). Furthermore, the effect of MCD on the binding of CB1R, but not CB2R, in primary cells (Fig. 2) suggests that type-1 but not type-2 cannabinoid receptors are localized within lipid rafts also in normal cells.

The molecular basis of the different sensitivity of CB1 and CB2 receptors to raft integrity might be complex, and need a thorough analysis of the lipid environment of the receptors, along with the characterization of the three dimensional structures of the two receptor subtypes in the context of membrane bilayers. The present study suggests that CB2R, unlike CB1R, does not interact with lipid rafts, a conclusion further supported by preliminary fractionation studies showing that CB1R in C6 cells, but not CB2R in DAUDI cells, colocalize with the raft marker caveolin-1 (M. Barri, M. Ranalli, A. Finazzi-Agrò, and M. Maccarrone, manuscript in preparation). In this context, it seems noteworthy that in the membrane outer leaflet AEA takes an extended conformation that enables it to interact with a hydrophobic groove formed by helices 3 and 6 of CB1R, where its terminal carbon is positioned close to a key cysteine residue (Cys47) in helix 6 (70). This interaction is essential for receptor activation (70), suggesting that differences in folding between the two CB receptor subtypes might lead to different activity and regulation. In the same line, a recent study using combined high resolution NMR and computer modeling has shown that CB1 and CB2 receptors have indeed conformational properties and salt bridge differences in the so-called juxtapembrane segment (or helix 8), which is critical for their activity and regulation and, more notably, is under the influence of the surrounding chemical environment (71). Therefore, it is tempting to speculate that lipid rafts might regulate CB1 receptor by interacting with specific regions of its three-dimensional structure, like helices 3 and 6 (70) or helix 8 (71). The lack of these interactions could make CB2R insensitive to lipid rafts perturbation.

This study has also extended for the first time the role of lipid rafts perturbation to the metabolism of 2-AG, in both immune and neuronal cells. This seems of major interest, because DAGL is the only enzyme that synthesizes 2-AG (18), whereas MAGL is the main enzyme that hydrolizes it (19), with a minor contribution of FAAH (16). Critical activities of 2-AG independent of those of AEA are emerging both in the CNS (72) and in the periphery (73).

Thus, a better understanding of DAGL and MAGL regulation, and of their role in maintaining the endocannabinoid tone in vivo, can be of utmost importance, as it has been the case for NAPE-PLD (12) and FAAH (16). Critical activities of 2-AG independent of those of AEA have been developed recently, and have identified this enzyme as a novel target for drug design (74). In this line, selective inhibitors of MAGL have been developed recently, and have identified this enzyme as a novel target for drug design (74). In the present study, we show that neither DAGL, a membrane-bound enzyme, nor MAGL, a cytosolic enzyme, were affected by perturbation of lipid rafts integrity in immune or neuronal cells. However, cholesterol depletion of enrichment, respectively reduced or enhanced the uptake of 2-AG by DAUDI cells or C6 cells, in much the same way as they modulated AEA transport by AMT. This observation, along with the similar Km values of AEA or 2-AG uptake, favors the hypothesis that 2-AG might be transported by the same AMT that takes up AEA (13, 20, 21). The inhibition of AMT by 2-AG, and that of 2-AG uptake by AEA, seem to strengthen this hypothesis. In this context, it should be recalled that AMT or other endocannabinoid transporter(s) have not been identified yet, and there is controversy about their existence (75). While it is clear that AEA uptake has the features of a facilitated transport, and there may be indeed a site through which it diffuses (76), the molecular identity of, and the gene encoding for, an AEA membrane transporter still remain elusive (14, 15, 77). At any rate, the data demonstrate that lipid rafts integrity modulates endocannabinoid transport across cell membranes. In addition, the observation that raft disruption reduced Vmax, without affecting Km of AEA or 2-AG transport by DAUDI cells, seems to favor the “endoctyic hypothesis” of McFarland et al. (36), who proposed that the AEA transporter may be internalized upon MCD treatment, thus reducing the number (and hence the Vmax) of transporter molecules on the cell surface. However, until the existence and identity of the putative AMT is known and specific Abs have been

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### Table V. Effect of MCD or cholesterol treatment on 2-AG metabolism in C6 cells

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>+2.5 mM MCD</th>
<th>+Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAGL activity (pmol/min per mg protein)</td>
<td>65 ± 8 (100%)</td>
<td>75 ± 7 (115%)</td>
<td>52 ± 7 (80%)</td>
</tr>
<tr>
<td>MAGL activity (pmol/min per mg protein)</td>
<td>40 ± 5 (100%)</td>
<td>25 ± 4 (62%)*</td>
<td>75 ± 7 (187%)*</td>
</tr>
</tbody>
</table>

*Denotes p < 0.01 compared with untreated control (p > 0.05 in all other cases).


Disclosures

The authors have no financial conflict of interest.

References


