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Research Article

Anandamide induces cell death independently of cannabinoid receptors or vanilloid receptor 1: possible involvement of lipid rafts

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Abstract. Anandamide triggers various cellular activities by binding to cannabinboid (CB1/CB2) receptors or vanilloid receptor 1 (VR1). However, the role of these receptors in anandamide-induced apoptosis remains largely unknown. Here, we show that SR141716A, a specific inhibitor of cannabinoid receptor (CB1-R), did not block anandamide-induced cell death in endogenously CB1-R expressing cells. In addition, CB1-R-lacking Chinese hamster ovary (CHO) cells underwent cell death after anandamide treatment. SR144528, a specific inhibitor of CB2-R also failed to block anandamide-induced cell death in HL-60 cells. Capsazepine, a specific antagonist of VR1 could not prevent anandamide-induced cell death in constitutively and endogenously VR1 expressing PC12 cells. Moreover, anandamide noticeably triggered cell death in VR1-lacking human embryonic kidney (HEK) cells. In contrast, methyl-β cyclodextrin (MCD), a membrane cholesterol depletor, completely blocked anandamide-induced cell death in a variety of cells, including PC12, C6, Neuro-2a, CHO, HEK, SMC, Jurkat and HL-60 cells. MCD also blocked anandamide-induced superoxide generation, phosphatidyl serine exposure and p38 MAPK/JNK activation. Thus, our data imply a novel role for of membrane lipid rafts in anandamide-induced cell death.

Key words. Anandamide; cannabinoid receptor; vanilloid receptor 1; lipid raft; apoptosis.

Anandamide, an arachidonic acid derivative, chemically defined as arachidonoylethanolamide (AEA), has been isolated from porcine brain lipid extract by Devane et al. [1]. Anandamide activates brain cannabinoid receptors, and mimics the pharmacological effects of Δ^9-tetrahydrocannabinol (THC), the active principle of hashish and marijuana, and is termed endocannabinoid [1, 2]. Cellular effects of anandamide are thought be mediated through to binding to cannabinoid receptors. Two cannabinoid receptors, CB1-R and CB2-R, have been identified thus far. CB1-R is primarily expressed in the central nervous system (CNS), while CB2-R is expressed in peripheral tissues [1]. Recently, anandamide has been found to be a ligand for vanilloid receptor 1 (VR1) [3], the site of action of the pungent component of hot red peppers, capsaicin [4]. Expression of VR1 has been identified in sensory neurons and in selected areas of the CNS of rats and humans [5].

p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) belong to the family of

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serine/threonine protein kinases, and are activated in response to inflammatory cytokines [6], environmental [7] and oxidative stresses [8, 9], resulting in cell differentiation and apoptosis.

Plasma membranes have been demonstrated to be compartmentalized into lipid raft and non-raft microdomains [10], the raft domains being abundant in glycosphingolipids, saturated phospholipids and cholesterol. A subset of cellular proteins has been reported to be present in lipid rafts [10]. Lipid rafts have been implicated in cytoskeletal reorganization, protein sorting and membrane trafficking in many cell types [10]**.** The cholesterol-rich region can include or exclude proteins to varying degrees, and may serve as foci for the recruitment and concentration of signaling molecules at the plasma membrane, and thus have been implicated in signal transduction from cell surface receptors [11, 12]. Depletion of cholesterol in lipid rafts inhibits anti-tumor drug-induced apoptosis [13].

Anandamide has been reported to induce apoptosis in a number of cell types such as lymphocytes [14], pheochromocytoma [15] and neuroblastoma [16]. Recently, we reported that p38 MAPK and JNK relay anandamide-induced cell death signaling in PC12 cells [17]. However, the molecular mechanism(s) of anandamide-induced cell death at the receptor level is not yet defined. In the present study, we provide evidence that anandamide mediates cell death in a manner independent of CB-Rs or VR1. Methyl-β cyclodextrin (MCD), a membrane cholesterol depletor [18], abolishes anandamide-induced cell death, superoxide generation, phosphatidyl serine (PS) exposure, and p38 MAPK/JNK activation, suggesting the involvement of membrane lipid rafts in anandamide-induced cell death.

Materials and methods

Materials

Anandamide, capsaicin, 2-arachidonoylglycerol (2-AG), palmitoylethanolamide (PEA) and arachidonic acid (AA) were purchased from Calbiochem (San Diego, Calif.). MCD was purchased from Sigma (St. Loius, Mo.). SR141716A and SR144528 were kindly provided by Sanofi Researche (France) and Ono Phamaceutical Co. (Osaka, Japan), respectively.

Antibodies

MAPK assay kits (containing polyclonal antibody to p38, and JNK/SAPK, and polyclonal antibody to phospho-p38 MAPK, and phospho-JNK/SAPK) were purchased from Cell Signaling Techonology Inc. (Beverly, Mass.). Cannabinoid rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.).

Cell culture

Rat pheochromocytoma (PC12) cells were cultured in RPMI 1640 (Gibco BRL, Rockville, Md.) media supplemented with 10% horse serum (HS) and 5% fetal bovine serum (FBS) plus antibiotics at 37 °C in the presence of 95% air and 5% CO_2. Mouse neuroblastoma (Neuro-2a), rat glioblastoma C6, human vascular smooth muscle (HVSM) and human embryonic kidney (HEK) 293 cells were cultured in 10%-FBS-containing D-MEM medium. Human neuroblastoma (NB-1) cells were maintained in a combination of RPMI and DMEM (1:1) containing 10% HS and 10% FBS. Chinese hamster ovary (CHO) cells were maintained in 10%-FBS-containing α-MEM. Jurkat and HL-60 cells were cultured in 10%-FBS-containing RPMI medium with appropriate antibiotics.

For differentiation, PC12 cells were seeded onto collagen-coated dishes (Iwaki, Osaka, Japan) and differentiated by incubating the cells in the medium containing low serum (0.2% HS) plus nerve growth factor (NGF) 100 ng/ml for 7 days. Cells with neurites longer than double of the cell body were considered differentiated cells. All experiments were carried out in the presence of low serum (0.2%).

Cell viability

Cell viability was assessed by fluorescence-activated cell sorting (FACS) analysis. At the end of treatment, total cells (floating and adherent) were harvested and pelleted by centrifugation (200 g). Cells were fixed in 70% ethanol for 20 min at –20 °C. Cells were then incubated with propidium iodide (PI) (10 µg/ml) and RNase (5 µg/ml) for 20 min. Cell death was evaluated by counting PI-positive cells, using a FACS can analyzer (EPICS; Coulter; New York).

Preparation of cell lysates and Western blotting

Preparation of cell lysates and activation of MAPKs were undertaken as described by Sarker et al. [9].

RT-PCR for VR1

Expression of VR1 in PC12 cells was determined by RT-PCR. Total RNA was extracted from cells (5×10^6) using TRIzol-Reagent (Invitrogen, Burlington, Canada) according to the manufacturer's instructions. After treatment with RNase-free DNase, the purified total RNA (2 µg) was converted to cDNA by reverse transcriptase (Gibco BRL). The reaction mixture (50 µl) consisted of 100 ng of cDNA, 1 µM of each primer, 200 µmol of each deoxynucletide triphosphate, and 2.0 units of Taq polymerase (Takara Biomedicals, Osaka, Japan). The amplification profile consisted of an initial denaturation of 3 min at 95 °C and 30 cycles of 1 min at 95 °C, 1 min at 55 °C, and 2 min at 72 °C. A final extension of 3 min was carried out at 73 °C. The primers used were VR1 sense primer, 5′-TGTCAGCTCTGTTCTAAC, and VR1 antisense primer,

5′-CTTCACAATGGCCAGCTG. The expected size of the amplification product was 564 bp. β-Actin was used as the housekeeping gene. PCR products (20 µl) were electrophoresed on a 2% agarose gel and visualized by ethidium bromide fluorostaining. No PCR product was detected in the absence of cDNA or primers. DNA ladder of 1 Ab was run as marker.

Determination of PS externalization by FACS

Externalization of PS was determined as described previously by Sarker et al. [15].

Superoxide anion assay

The cytochrome c reduction method was used to measure intracellular superoxide by a slight modification of Pick et al. [19]. Briefly, cells (1×10^6/ml) were suspended in Hank's balanced salt solution containing cytochrome c (100 µM). One hundred microliters of cell suspension was incubated with anandamide (10 µM) in the presence or absence of the cholesterol depletor, MCD (1 mM) at 37 °C for 1 h. Superoxide production was measured by a UV-spectrophotometer (Ultrospec III; Pharmacia LKB) at 550 nm.

Results

CB-Rs do not appear to mediate anandamide-induced cell death

To determine whether anandamide-induced cell death is mediated by CB1-R, endogenously CB1-R expressing C6 and PC12 cells (fig. 1A) were pre-incubated with

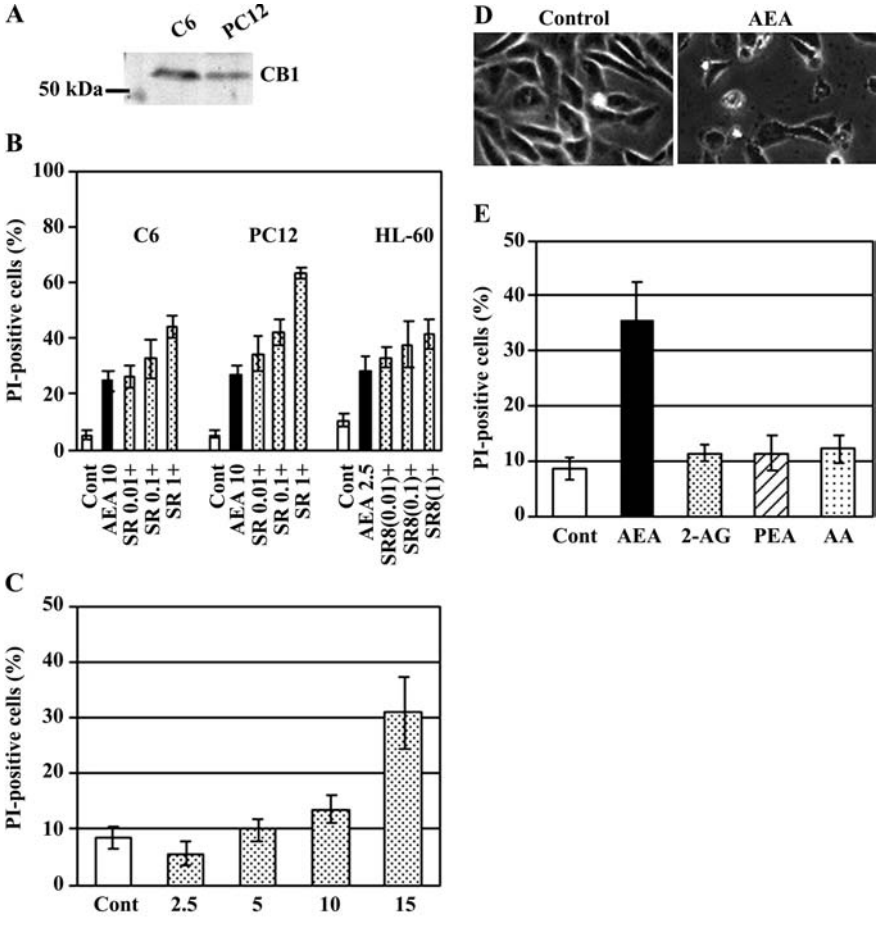

Figure 1. CB1 activation is not required in anandamide-induced cell death. (*A*) Expression of CB1-R in C6 and PC12 cells determined by Western blotting. (*B*) After 24 h plating, cells were pre-treated with micromolar concentrations of SR141716A (SR) for 30 min, and then treated with 10 µM anandamide (AEA), or with solvent ethanol (0.1%) as a control for 24 h. After the treatment, cells were harvested, and viable cells were counted by a PI-dye exclusion method by FACS-can analyzer as described in Materials and methods. (*C*) CHO cells were plated onto 60-mm dishes (5×10^5 cells/dish). After 24 h plating, cells were exposed to micromolar concentrations of anandamide for 24 h. Viable cells were counted by the PI-dye exclusion method. (*D*) Photomicrographs of CHO cells treated with anandamide (15 µM) for 24 h. Results are given as mean ±SD of the three independent experiments in triplicate. (*E*) Comparison of anandamide (AEA)-induced cell death with 2-arachidonoylglycerol (2-AG), palmitoylethanolamide (PEA), and arachidonic acid (AA). PC12 cells were exposed to AEA (10 µM), 2-AG (20 µM), PEA (20 µM) or AA (20 µM) for 24 h. Viable cells were counted by the PI-dye exclusion method. Values are given as mean ±SD of three separate experiments.

SR141716A (0–10 µM), a potent and specific antagonist of CB1-R [20], followed by exposure to 10 µM anandamide. There was no inhibition of anandamide-induced cell death in the presence of SR141716A. Figure 2B shows that pre-treatment of the cells with SR141716A resulted in an increase in, rather than an inhibition of, cell death in both cell lines. SR141716A also failed to affect anandamide-induced cell death in CB1-R-expressing Neuro-2a cells (data not shown). We then investigated whether CB2-R mediated anandamide-induced cell death. As PC12 cells do not express CB2-R, HL-60 cells, which express CB2-R (data not shown), were pre-treated with SR144528. Like SR141716A, SR144528, a specific and potent inhibitor of CB2-R, failed to block anandamide-induced HL-60 cell death (fig. 1B, right panel). SR141716A or SR144528 alone up to 10 µM did not induce any cytotoxic effects; however, substantial cell death was observed when they were applied in combination (at levels greater than 5 µM) with 10 µM anandamide (data not shown).

We then investigated whether anandamide could induce cell death in CHO cells, which do not express CB1-R [21]. Anandamide, at a concentration of 10 µM, slightly induced cell death, while at 15 µM, cell death increased dramatically (fig. 1C). Anandamide (15 µM) triggered 31 ± 6.5% cell death at 24 h versus 8.6 ± 2% in the control (without anandamide). Cytotoxic effects of anandamide on CHO cells were also confirmed by morphological changes such as rounded cells and the phase becoming bright (fig. 1D).

The potential role of anandamide in triggering cell death was then compared with the second endogenous cannabinoid 2-AG, as well as with PEA, and AA. A 10-µM concentration of anandamide induced some degree of cell death, but none of the other agents, at concentrations similar to or higher than that of anandamide, induced cell death in PC12 cells (fig. 1E), suggesting a distinct signaling pathway in anandamide-induced cell death.

VR1 does not appear to mediate anandamide-induced cell death

Our next approach was to investigate whether VR1 mediates anandamide-induced cell death, using the VR1 antagonist, capsazepine, the agonist, capsaicin, and cells expressing or lacking VR1. PC12 cells, which express VR1 (as determined by RT-PCR; fig. 2A), were incubated with

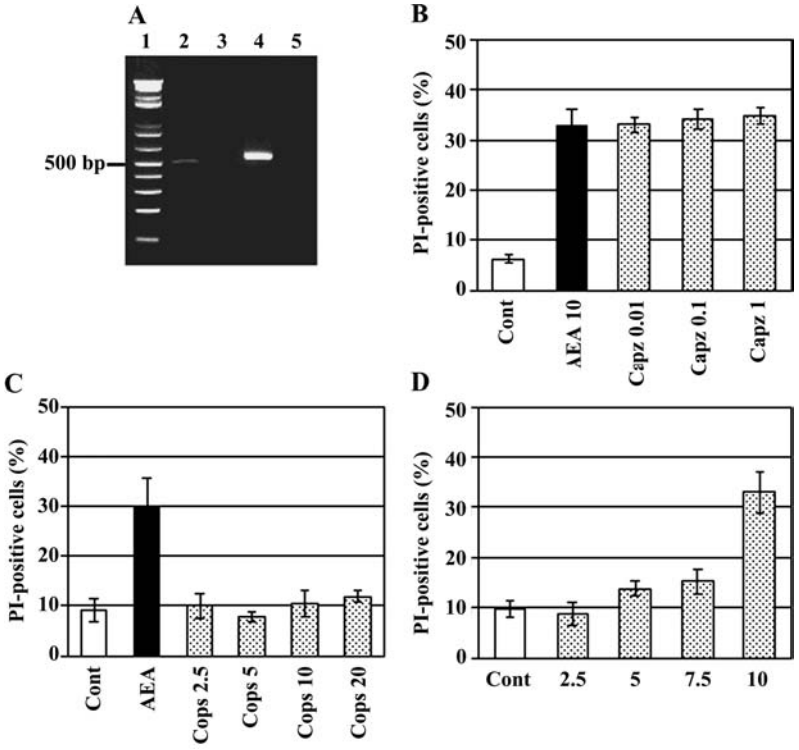

Figure 2. Anandamide-induced cell death appears to be independent of VR1 activation. (*A*) expression of vanilloid receptor in PC12 cells determined by RT-PCR. Lane 1, marker; lane 2, PC12 cells; lane 3, H_2O; lane 4, β-actin; lane 5, HEK 293 cells. (*B*) PC12 cells plated onto 60-mm dishes (5×10^5 cells/dish) pre-treated with micromolar concentrations of capsazepine (Capz) for 30 min, followed by anandamide (10 µM) exposure. Viable cells were counted by the PI-staining method. (*C*) PC12 cells were treated with micromolar concentrations of capsaicin (Caps). Viable cells were counted after 24 h of treatment by the PI-staining method. (*D*) HEK 293 cells were exposed to micromolar concentrations of anandamide for 24 h. Cell death was evaluated by the PI-staining method. (*B–D*) Results are given as the mean ± SD of three independent experiments in triplicate.

capsazepine (0–1 μM) before anandamide (10 μM) treatment. Capsazepine failed to prevent cell death when anandamide exposure continued for 24 h (fig. 2B). Similar results were obtained with Neuro-2a and NB-1 cells (data not shown). Capsazepine alone at a concentration up to 10 μM did not exert a cytotoxic effect; however, capsazepine (≥2.5 μM) in combination with 10 μM anandamide did induce cytotoxic effects in PC12 cells.

To determine whether capsaicin can mimic anandamide-induced cell death, PC12 cells were treated with a dose of capsaicin similar to or higher than anandamide. Concentrations of capsaicin similar to those of anandamide induced no cell death, and even a 20 μM concentration did not affect the viability of PC12 cells (fig. 2C).

To confirm that anandamide can induce cell death without VR1 activation, HEK 293 cells, which do not express VR1 [22; K. P. Sarker and I. Maruyama, unpublished results], were incubated with increasing concentrations of anandamide. A dose of anandamide as low as 5 μM induced cell death slightly, and cell death increased to 32.93 ± 4.1% when cells were exposed to 10 μM anandamide (fig. 2D). Morphological changes reflecting

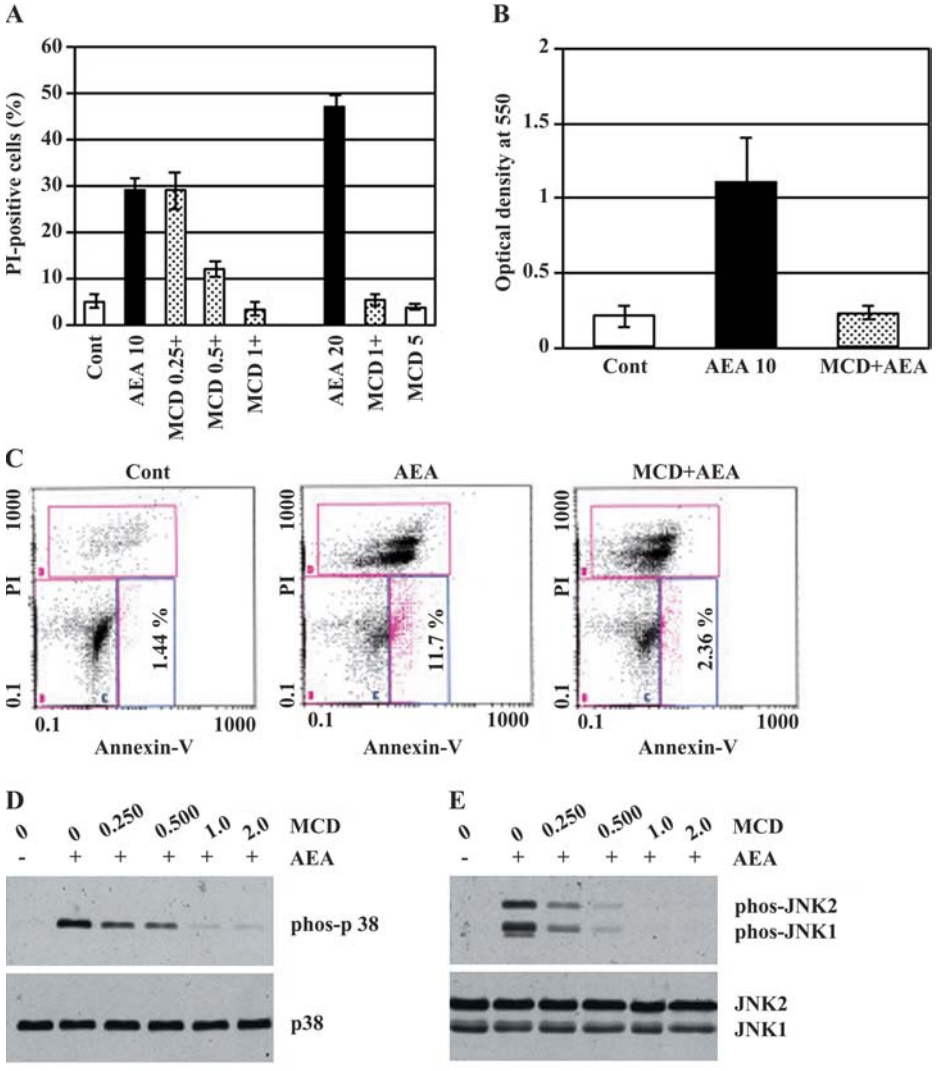

Figure 3. Effects of MCD on anandamide-induced cell death. (*A*) MCD dose-dependently inhibits anandamide-induced cell death. Following 18 h plating, cells were pre-treated with the indicated concentrations of MCD for 30 min and then exposed to 10 or 20 μM anandamide for 24 h. Viable cells were counted by the PI-dye exclusion method. (*B–E*) MCD potently inhibits anandamide-induced superoxide production, PS exposure and stress-activated protein kinase p38MAPK/JNK activation. (*B*) A PC12 cell suspension in Hank's balanced salt solution was pre-incubated with MCD for 15 min, followed by addition of 10 μM anandamide for 1 h. Superoxide anion was measured as described in Materials and methods. (*C*) PC12 cells were pre-incubated with MCD for 30 min and then treated with 10 μM anandamide for 24 h. After 2 h of treatment, PS exposure was determined using annexin-V-FITC. The panel is representative of three experiments. (*D, E*) Inhibition of p38 MAPK and JNK activation by MCD. After 18 h of plating, cells were pre-treated with 1 mM MCD 30 min prior to the addition of 10 μM anandamide (AEA) or ethanol (0.1%). Activation of MAPKs was determined by immunoblotting using phospho-specific antibodies to p38 MAPK or JNK. The blots are representative of three independent experiments.

signs of cell death, such as rounding of cells, and brightness of the cell body, were noted in anandamide-treated cells (data not shown), consistent with the above results.

MCD abolishes anandamide-induced cell death

Plasma membranes are enriched in cholesterol, and depletion of cellular cholesterol can disrupt cellular functions [11]. To explore the role of membrane cholesterol-rich regions/lipid rafts in anandamide-induced apoptosis, we disrupted membrane cholesterol by incubating cells with MCD, which specifically removes cholesterol from the plasma membrane [18]. PC12 cells pre-incubated with MCD were exposed to 10 or 20 µM anandamide for 24 h. MCD was observed to dose-dependently block anandamide-induced cell death. As shown in figure 3A**,** 1 mM MCD completely abolished anandamide-induced cell death. Surprisingly, complete inhibition of cell death was observed in cells exposed to even 20 µM anandamide (fig. 3A, right panel). MCD alone, at concentrations up to 5 mM, appeared to have no cytotoxic effect on PC12 cells.

Anandamide triggers superoxide generation and PS exposure in PC12 cells [15]. To determine whether MCD blocks the anandamide-induced superoxide generation, PC12 cells were pre-treated with 1 mM MCD followed by the addition of anandamide (10 µM). Figure 3B shows that MCD markedly inhibited anandamide-induced superoxide generation. PS is believed to be a membrane 'flag mark' of apoptosis, appearing on outer membranes during early stages of apoptosis, and helping macrophage or neighboring cells to engulf apoptotic cells [23]. We further investigated whether MCD can block anandamide-induced PS externalization. An almost complete inhibition of anandamide-induced PS exposure was observed with MCD (fig. 3C). Recently, we showed that anandamide fails to trigger substantial cell death when cells were pre-treated with an inhibitor of p38 MAPK or overexpressed with mutants of JNK, suggesting that p38 MAPK and JNK mediate anandamide-induced PC12 cell death [17]. We then examined whether MCD blocks the activation of anandamide-induced stress-activated protein kinase p38 MAPK and JNK. MCD drastically reduced anandamide-induced activation of p38 MAPK and JNK (fig. 3D, E)**.** Cytochrome c release and caspase-3 activation have been found to occur in anandamide-induced apoptosis of PC12 cells [15, 17]. MCD was found to block anandamide-induced cytochrome c release, mitochondrial dysfunction, and caspase-3 activation, consistent with the above results (data not shown).

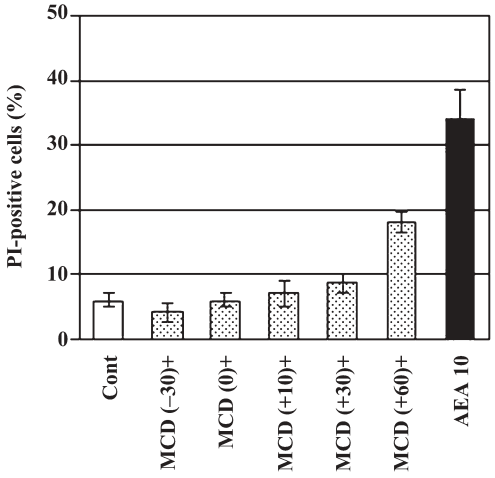

Figure 4. Timely MCD treatment is required for protection from anandamide-induced cell death. PC12 cells were treated with 1 mM MCD 30 min prior to, at the same time, or at the indicated times following exposure to 10 µM anandamide. Cell viability was determined 24 h after anandamide treatment. Values are the mean ± SD of three independent experiments.

We then addressed whether inhibition of anandamide-induced apoptosis by MCD is confined to PC12 cells. A number of cell types such as C6, Neuro-2a, HEK 293, CHO, HVSM cells, Jurkat, HL-60 and nerve growth factor (NGF)-differentiated PC12 (d-PC12) cells were incubated with MCD, followed by anandamide (2.5–10 µM) exposure. As shown in table 1, MCD concentrations ranging from 500 µM to 1 mM produced a marked inhibitory effect on anandamide-induced cell death, demonstrating

Table 1. MCD blocks anandamide-induced cell death regardless of cell type.

Cell types	AEA con. (µM)	Cell death percentage (%)			MCD conc. (mM)
		AEA	Cont	MCD+AEA	
HL-60	2.5	33.56 ± 4.4	8.30 ± 1.7	12.1 ± 3.4	0.5
C6	10	24.86 ± 2.8	6.56 ± 2.2	7.16 ± 1.8	1
Neuro-2a	10	41.33 ± 3.2	13.6 ± 1.38	15.23 ± 1.9	1
HEK	10	28.73 ± 1.9	8.1 ± 1.8	10.0 ± 2.0	1
CHO	15	32.16 ± 3.6	8.83 ± 0.51	11.1 ± 3.5	1
SMC	5.0	31.23 ± 4.8	12.2 ± 0.5	14.03 ± 2.1	1
Jurkat	2.5	28.96 ± 7.6	10.03 ± 2.68	12.83 ± 2.3	0.5
d-PC12	10	35.83 ± 5.8	11.76 ± 1.9	10.16 ± 3.5	1

After 18 h of plating, cells were pre-treated with the indicated concentration of MCD for 30 min and then exposed to anandamide for 24 h. Cell viability was counted by the PI-dye exclusion method as described in Materials and methods.

that anandamide triggers apoptosis in a variety of cells via a general mechanism at the plasma membrane level, where membrane lipid raft acts as platforms in controlling anandamide-induced cell death.

To determine whether time is crucial to achieving the inhibitory effects of MCD on anandamide-induced cell death, PC12 cells were either incubated with MCD prior to or following anandamide exposure. As shown in figure 4, pre-incubation or treatment with 1 mM MCD within 10 min following the addition of 10 µM anandamide almost completly inhibited anandamide-induced cell death. A marked inhibition of cell death was also observed when MCD was added 30 min after anandamide exposure. However, MCD showed no marked inhibitory effect when treatment was delayed for 60 min or more following anandamide exposure. Similar results were also obtained with other cell lines (data not shown).

To rule out the possibility that the protecting effect of MCD against anandamide-induced cell death was mediated by simple binding to anandamide, PC12 cells were incubated with 1 mM MCD and washed before addition of anandamide into the fresh medium. Consistent with the results presented above, a notable inhibition of anandamide-induced cell death was observed when cells were incubated with anandamide after washing MCD from the cells. Figure 5 shows that anandamide-induced cell death dropped sharply from 34.43 ± 5.4% to 13.9 ± 3.13% after removal of MCD from the culture medium.

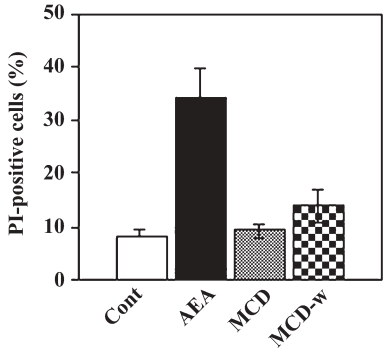

Figure 5. Effects of removal of MCD before addition of anandamide. PC12 cells were incubated with 1 mM MCD for 30 min. After this treatment, 10 µM anandamide was added without washing (MCD) or after washing MCD (MCD-w) from the cells. Cell viability was determined 24 h after anandamide exposure. Values are the mean ± SD of three independent experiments.

Model 1: proposed signaling pathway of anandamide-induced apoptosis

Based on our findings, we propose that (1) an anandamide membrane transporter or carrier is present in the

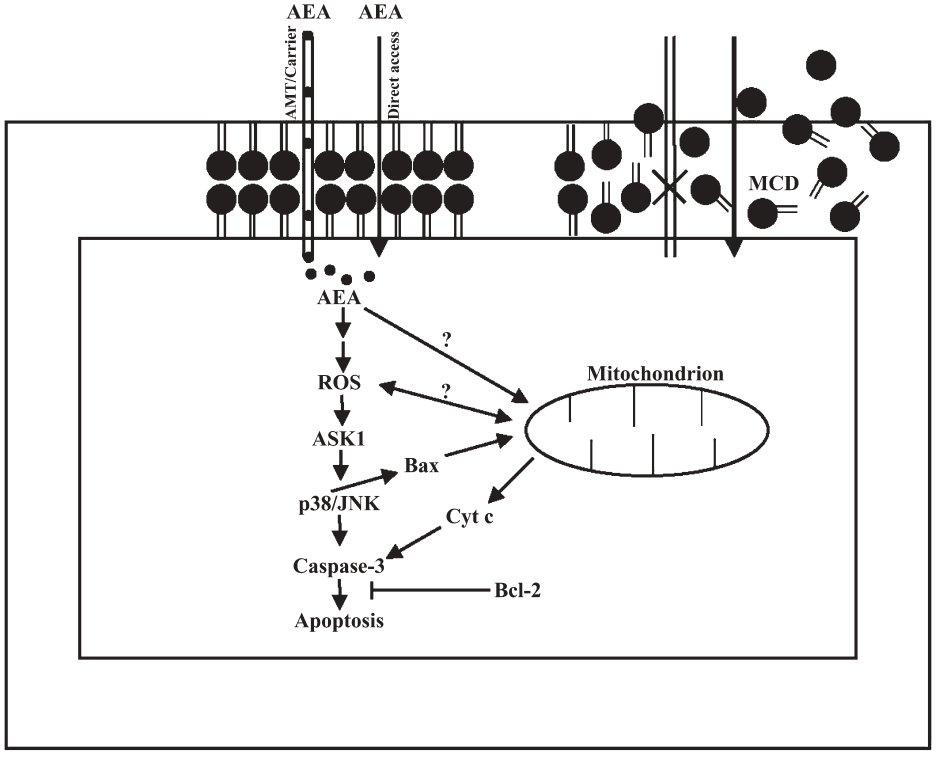

Figure 6. Proposed mechanism of the anandamide-induced apoptotic cell death signaling pathway. Anandamide may enter the cells through an anandamide membrane transporter or carrier located in lipid rafts. Anandamide may cross the membrane through cholesterol-rich lipid rafts. Accumulation of anandamide may lead to an increase in intracellular reactive oxygen species, which, in turn, triggers the apoptosis-inducing signaling cascade. Disruption of lipid rafts prevents anandamide-induced apoptosis.

cholesterol-rich lipid rafts, which entraps extracellular anandamide in a manner dependent on intact membrane cholesterol and (2) anandamide has greater access to cells through cholesterol-rich lipid rafts, and depletion of cholesterol results in inhibition of anandamide access, blocking subsequent apoptosis-inducing signaling cascades (fig. 6).

Discussion

In the present report, we provide evidence for the first time that anandamide-induced cell death is mediated through membrane lipid rafts, not via a putative CB-R, or VR1 or membrane transporter. CB1-R has been shown to be involved in anandamide-induced proliferation of rat C6 cells [24]. Anandamide from 10 to 15 µM induces cell growth and MAPK activation in JB6+ cells without using CB-Rs [25]. CB1-R has been suggested not to be involved in anandamide-induced apoptosis [16]. Results presented in this study are in line with the latter reports, as the selective inhibitors of CB-Rs, were found to not inhibit anandamide-induced apoptosis and anandamide-induced cell death in CHO cells, which have been widely used in studies of heterologous transfection of CB-Rs [26].

Anandamide has been found to mimic the functions of capsaicin by binding to VR1 [3, 27]. However, a study by Szolcsanyi [28] has demonstrated that anandamide can elicit cellular effects independent of VR1 activation. In this study, we provide evidence that anandamide can induce apoptosis independent of VR1 activation. Capsazepine, an antagonist of VR1 [21] has been shown to prevent anandamide-induced cellular function [3]. Anandamide-induced apoptosis has been shown to be partially blocked by capsazepine [16]. However, in the present study, we observed that capsazepine does not block anandamide-induced PC12 cell death. The reason for this discrepancy is not clear at present; however, it may be due to the different experimental systems as well as different cell types. We also observed that capsaicin, a potent agonist of VR1 [29], did not trigger apoptosis at the same dose as anandamide. Although the dose of anandamide that induces apoptosis varies with cell type, the concentration of anandamide inducing apoptosis in VR1-expressing PC12 cells also affected viability in VR1-lacking HEK 293 cells. These results also support the data obtained with capsazepine. Thus, anandamide may induce apoptosis through different pathways in different cells where VR1 may or may not be required.

Evidence has been mounting in recent years that rafts in the plasma membrane of mammalian cells, which are enriched with cholesterol and glycosphingolipids, play an important role in delivering a number of intracellular signals [30–32]. Depletion of membrane cholesterol results in a reduction in amyloid beta toxicity in hippocampal neurons and Alzheimer's disease [33, 34]. Of great interest has been to determine whether depletion of membrane cholesterol can modulate anandamide-induced cell death. We found evidence that MCD, which specifically removes cholesterol from the plasma membrane [18], strikingly inhibits anandamide-induced superoxide generation, PS exposure, p38 MAPK/JNK activation and apoptotic features in anandamide-treated cells. Jarho et al. [35] have shown that anandamide may bind to MCD, thus a possibility arises that MCD entraps anandamide resulting in an inhibition of anandamide-induced cell death. However, a noticeable inhibitory effect of MCD on anandamide-induced cell death remains after removal of MCD from cells prior to the addition of anandamide, suggesting that this is not the case. The inhibition of anandamide-induced cell death by MCD was found to be twofold. First, MCD potently inhibited anandamide-induced cell death in a variety of cells (with or without CB1,2-R/VR1), suggesting a general phenomenon in anandamide-induced cell death. Second, inhibition of anandamide-induced cell death by MCD newly suggests a key role for membrane lipid rafts in the field of anandamide-induced apoptosis. Our findings are in accordance with previous reports suggesting that disruption of membrane lipid rafts by incubation with MCD results in inhibition of lipid-induced apoptosis [13]. Therefore, future studies must determine whether there is an anandamide membrane transporter/carrier in lipid rafts, which functions in a manner dependent on intact membrane cholesterol, or whether anandamide has access to the cholesterol-rich lipid rafts, and disruption of lipid rafts results in an inhibition of intracellular anandamide accumulation and of the subsequent apoptosis-inducing signaling cascade.

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1 Devane W. A., Hanus L., Breuer A., Pertwee, R. G., Stevenson L. A., Griffin G. et al. (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science **258:** 1946–1949
2 Fride E. and Mechoulam R. (1993) Pharmacological activity of the cannabinoid receptor agonist, anandamide, a brain constituent. Eur. J. Pharmacol. **231:** 313–314
3 Zygmunt P. M., Petersson J., Andersson D. A., Chuang H., Sorgard M., Di-Marzo V. et al. (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. Nature **400:** 452–457
4 Szallasi A. and Blumberg P. M. (1999) Vanilloid (capsaicin) receptors and mechanisms. Pharmacol Rev. **51:** 159–212
5 Mezey E., Toth Z. E., Cortright D. N., Arzubi M. K., Krause J. E., Elde R. et al. (2000) Distribution of mRNA for vanilloid re-

ceptor subtype 1 (VR1), and VR1-like immunoreactivity, in the central nervous system of the rat and human. Proc. Natl. Acad. Sci. USA **97:** 3655–3660
6 Raingeaud J., Gupta S., Rogers J. S., Dickens M., Han J., Ulevitch R. J. et al. (1995) Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J. Biol. Chem. **270:** 7420–7426
7 Hibi M., Lin A., Smeal T., Minden A. and Karin M. (1993) Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev. **7:** 2135–2148
8 Callsen D. and Brune B. (1999) Role of mitogen-activated protein kinases in S-nitrosoglutathione-induced macrophage apoptosis. Biochemistry **38:** 2279–2286
9 Sarker K. P., Nakata M., Kitajima I., Nakajima T. and Maruyama I. (2000b) Inhibition of caspase-3 activation by SB 203580, p38 mitogen-activated protein kinase inhibitor in nitric oxide-induced apoptosis of PC-12 cells. J. Mol. Neurosci. **15:** 243–250
10 Simons K. and Ikonen E. (1997) Functional rafts in cell membranes. Nature **387:** 569–572
11 Simons K. and Toomre D. (2000) Lipid rafts and signal transduction. Nat. Rev. Mol. Cell Biol. **1:** 31–39
12 Moran M. and Miceli MC. (1998) Engagement of GPI-linked CD48 contributes to TCR signals and cytoskeletal reorganization: a role for lipid rafts in T cell activation. Immunity **9:** 787–796
13 Gajate C. and Mollinedo F. (2001) The antitumor ether lipid ET-18-OCH(3) induces apoptosis through translocation and capping of Fas/CD95 into membrane rafts in human leukemic cells. Blood **98:** 3860–3863
14 Schwarz H., Blanco F. J. and Lotz M. (1994) Anadamide, an endogenous cannabinoid receptor agonist inhibits lymphocyte proliferation and induces apoptosis. J. Neuroimmunol. **55:** 107–115
15 Sarker K. P., Obara S., Natkata M., Kitajima I. and Maruyama I. (2000) Anandamide induces apoptosis of PC-12 cells: involvement of superoxide and caspase-3. FEBS Lett. **472:** 34–44
16 Maccarrone M., Lorenzon T., Bari M., Melino G. and Finazzi-Agro A. (2000) Anandamide induces apoptosis in human cells via vanilloid receptors: evidence for a protective role of cannabinoid receptors. J. Biol. Chem. **275:** 31938–31945
17 Sarker K. P., Biswas K. K., Yamakuchi M., Lee K.-Y., Hahiguchi T., Kracht M. et al. (2003) ASK1-p38 MAPK/ JNK signaling cascade mediates anandamide-induced PC12 cell death. J. Neurochem. **85:** 50–61
18 Neufeld E. B., Cooney A. D., Pitha J., Dawidowicz E. A., Dwyer N. K., Pentchev P. G. et al. (1996) Intracellular trafficking of cholesterol monitored with a cyclodextrin. J. Biol. Chem. **271:** 21604–21613
19 Pick E. and Mizel D. (1981) Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. J. Immunol. Methods **46:** 211–226
20 Rinaldi-Carmona M., Barth F., Heaulme M., Shire D., Calandra B., Congy C. et al. (1994) SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. FEBS Lett. **350:** 240–244
21 Bevan S., Hothi S., Hughes G., James I. F., Rang H. P., Shah K. et al. (1992) Capsazepine: a competitive antagonist of the sensory neuron excitant capsaicin. Br. J. Pharmacol. **107:** 544–552
22 De Petrocellis L., Bisogno T., Maccarrone M., Davis J. B., Finazzi-Agro A. and Di Marzo V. (2001) The activity of anandamide at vanilloid VR1 receptors requires facilitated transport across the cell membrane and is limited by intracellular metabolism. J. Biol. Chem. **276:** 12856–12863
23 Connor J., Pak C. C. and Schroit A. J. (1994) Exposure of phosphatidylserine in the outer leaflet of human red blood cells: relationship to cell density, cell age, and clearance by mononuclear cells. J. Biol. Chem. **269:** 2399–2404
24 Jacobsson S. O., Wallin T. and Fowler C. J. (20001) Inhibition of rat C6-glioma cell proliferation by endogenous and synthetic cannabinoid: relative involvement of cannabinoid and vanilloid receptors. J. Pharmacol. Exp. Ther. **299:** 951–959
25 Berdyshev E. V., Schmid P. C., Krebsbach R. J., Hillard C. J., Huang C., Chen N. et al. (2001) Cannabinoid-receptor-independent cell signalling by N-acylethanolamines. Biochem. J. **360:** 67–75
26 Rinaldi-Carmona M., Le Duigou A., Oustric D., Barth F., Bouaboula M., Carayon P. et al. (1998) Modulation of CB1 cannabinoid receptor functions after a long-term exposure to agonist or inverse agonist in the Chinese hamster ovary cell expression system. J. Pharmacol. Exp. Ther. **287:** 1038–1047
27 Smart D., Gunthorpe M. J., Jerman J. C., Nasir S., Gray J., Muir A. I. et al. (2000) The endogenous lipid anandamide is a full agonist at the human vanilloid receptor1 (hVR1). Br. J. Pharmacol. **129:** 227–230
28 Szolcsanyi J. (2000) Anandamide and the question of its functional role for activation of capsaicin receptors. Trends Pharmacol. Sci. **21:** 203–204
29 Szallasi A., Jonassohn M., Biro T., Blumberg P. M. and Sterner O. (1996) The stimulation of capsaicin-sensitive neurones in a vanilloid receptor-mediated fashion by pungent terpenoids possessing an unsaturated 1,4-dialdehyde moiety. Br. J. Pharmacol. **119:** 283–290
30 Kasahara K., Watanabe K., Takeuchi K., Kaneko H., Oohira A., Yamamoto T. et al. (2000) Involvement of gangliosides in glycosylphosphatidylinositol anchored neuronal cell adhesion molecule TAG-1 signaling in lipid rafts. J. Biol. Chem. **275:** 34701–34709
31 Yamauchi S., Tokita Y., Aono S., Matsui F., Shuo T., Ito H. et al. (2002) Phosphorylation of neuroglycan C, a brain-specific transmembrane chondroitin sulfate proteoglycan, and its localization in the lipid rafts. J. Biol. Chem. **277:** 20583–20590
32 Sehgal P. B., Guo G. G., Shah M., Kumar V. and Patel K. (2002) Cytokine signaling: STATS in plasma membrane rafts. J. Biol. Chem. **277:** 12067–12074
33 Simons M., Keller P., De Strooper B., Beyreuther K., Dotti C. G. and Simons K. (1998) Cholesterol depletion inhibits the generation of beta-amyloid in hippocampal neurons. Proc. Natl. Acad. Sci. USA **95:** 6460–6464
34 Fassbender K., Simons M., Bergmann C., Stroick M., Lutjohann D., Keller P. et al. (2001) Simvastatin strongly reduces levels of Alzheimer's disease beta-amyloid peptides Abeta 42 and Abeta 40 in vitro and in vivo. Proc. Natl. Acad. Sci USA **98:** 5856–5861
35 Jarho P., Urtti A., Jarvinen K., Pate D. W. and Jarvinen T. (1996) Hydroxypropyl-beta-cyclodextrin increases aqueous solubility and stability of anandamide. Life Sci. **58:** 181–185