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Stimulation of cannabinoid receptor agonist 2-arachidonylglycerol by chronic ethanol and its modulation by specific neuromodulators in cerebellar granule neurons

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Abstract

In an earlier study, we reported that chronic ethanol (EtOH) stimulates the formation of anandamide in human SK-N-SH cells. In the present study, we investigated the effect of chronic EtOH on the formation of yet another cannabinoid receptor (CB1) agonist, 2-arachidonylglycerol (2-AG), in cerebellar granule neurons (CGNs). The formation of 2-[^{3}H]AG without any stimulation was more pronounced in the older cultures than in younger cultures. Exposure of CGNs to EtOH led to a significant increase in the level of 2-[^{3}H]AG ($P < 0.05$). Incubation with the anandamidehydrolase inhibitor phenylmethylsulfonyl fluoride and EtOH did result in an additive increase in 2-[^{3}H]AG, but did not with *E*-6-(bromomethylene)tetrahydro-3-(1-naphthelenyl)-2*H*-pyran-2-one. The formation of 2-[^{3}H]AG was enhanced by ionomycin in both the control and EtOH-exposed CGNs, and the ionomycin-stimulated 2-[^{3}H]AG synthesis was inhibited by the intracellular chelating agent 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid. Further, glutamate increased the formation of 2-[^{3}H]AG only in control CGNs. MK-801 inhibited the EtOH-induced 2-[^{3}H]AG synthesis, suggesting the participation of intracellular Ca^{2+} in EtOH-induced 2-[^{3}H]AG synthesis. The dopamine receptor (D2) agonist did not modify the 2-AG synthesis in either the control or EtOH-exposed CGNs. However, the D2 receptor antagonist inhibited the EtOH-induced formation of 2-[^{3}H]AG. The EtOH-induced 2-[^{3}H]AG formation was inhibited by SR141716A and pertussis toxin, suggesting the CB1 receptor- and Gi/o-protein-mediated regulation of 2-AG. The observed increase in 2-AG level in CGNs is possibly a mechanism for neuronal adaptation to the continuous presence of EtOH. These findings indicate that some of the pharmacological actions of EtOH may involve alterations in the endocannabinoid signaling system.

Keywords: Cerebellar granule neuron; Ethanol; 2-Arachidonylglycerol; Endocannabinoid

Abbreviations: CGN, cerebellar granule neuron; DIV, days in vitro; EtOH, ethanol; CB1, cannabinoid receptor; 2-AG, 2-arachidonylglycerol; AnNH, anandamide; AA, arachidonic acid; PTX, pertussis toxin; Δ^9-THC, Δ^9-tetrahydrocannabinol; PMSF, phenylmethylsulfonyl fluoride; PLA_2, phospholipase A_2; N-ArPE, *N*-arachidonylphosphatidylethanolamine; NAE, *N*-acylethanolamine; BTNP, *E*-6-(bromomethylene)tetrahydro-3-(1-naphthelenyl)-2*H*-pyran-2-one; TMS, trimethylsilyl ether; 7-H-DPAT, (±)-2-dipropylamino-7-hydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid

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1. Introduction

Chronic ethanol (EtOH) has been shown to affect neurobiological and neurodegenerative processes in the brain. Although the molecular mechanisms by which EtOH produces its pharmacological effects have not been established, recent evidence suggests that many components of intracellular signal transduction pathways, including several receptors, ligand-gated ion channels, and enzymes, are targets of EtOH action in the central nervous system (CNS) [1–4].

Cannabinoid receptors (CB1) belong to G-protein-coupled receptors and are known to share G-proteins and/or effector molecules with other G-protein-coupled receptors [5]. This produces biological responses by regulation of enzymes and/or ion channel activity through selective activation of signal transducing G-proteins [6]. The only endogenous cannabinoid substances isolated and characterized so far that are capable of mimicking the pharmacological actions of Δ^9-tetrahydrocannabinol (Δ^9-THC), the active ingredient of marijuana, and other synthetic agonists [7] are anandamide (AnNH) and 2-arachidonylglycerol (2-AG) [8–10]. Both AnNH and 2-AG have been shown to bind specifically to CB1 receptors in the brain [8,9,11]. Although recent evidence indicates the involvement of a CB1 receptor signal transduction system in the pharmacological actions of EtOH including voluntary EtOH consumption [3,4,12–18], the exact molecular mechanism remains to be established. Recently, we demonstrated that chronic EtOH increases the formation of the CB1 receptor agonist AnNH through selective activation of arachidonic acid-specific phospholipase A_2 (PLA_2) in human SK-N-SH cells and mouse brain [14,19,20]. In the present study, we examined whether or not chronic EtOH treatment has any effect on the formation of yet another CB1 receptor agonist, 2-AG, using rat cerebellar granule neurons (CGNs). We found that chronic EtOH treatment increased the synthesis of 2-AG in CGNs. The results also indicate that calcium ions, CB1 receptors, and Gi/o-proteins may regulate 2-AG levels in CGNs.

2. Materials and methods

2.1. Materials

All culture plastic supplies were purchased from Falcon Labware (VWR Scientific, Pascataway, NJ). Basal medium Eagle's, heat-inactivated fetal calf serum (FBS), streptomycin, and penicillin solutions were from Sigma (St. Louis, MO). Liquid scintillation cocktail (Insta-Fluor) was purchased from Packard (Meriden, CT). Arachidonic acid (AA) was obtained from Avanti Polar Lipids (AL). 2-Arachidonylglycerol was purchased from Deva Biotech (Hartboro, PA). (±)-2-Dipropylamino-7-hydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide (7-H-DPAT), haloperidol, and MK-801 were from RBI (Natick, MA). *E*-6-(Bromomethylene)tetrahydro-3-(1-naphthelenyl)-2*H*-pyran-2-one (BTNP) was from Biomol (Plymouth Meeting, PA). SR141716A was a kind gift from Sanofi Pharmaceuticals (Montpellier, France). [^{3}H]Arachidonic acid (200 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). All other chemicals were obtained from Sigma (St. Louis, MO).

2.2. Primary cultures of cerebellar granule neurons

Cerebellar granule neuronal primary cultures were prepared from dissociated cerebella of 8-day-old Sprague-Dawley rat pups, as described previously [21]. Briefly, cells were counted, and approx. 8×10^6 cells were plated on a polylysine-coated 100 mm culture dish with 8 ml of BEM (basal medium Eagle's, Sigma) supplemented with 10% fetal calf serum (FCS), 25 mM KCl, 200 mM glutamine, 10 000 units penicillin, and 10 mg streptomycin. Cells were maintained at 37°C in a 95% air/5% CO_2 incubator. Cytosine-β-D-arabinofuranoside (10 μM) (Sigma) was added to the culture medium 18–22 h after plating. After 7 days in vitro (DIV), the cells were used for experiments. In some experiments 2 DIV CGNs were used.

2.3. Incubations

The cells were incubated with [^{3}H]arachidonic acid (AA) (1 μCi/ml) in BEM (without FCS) for 5 h. The medium was monitored for uptake and it was found

that 85% of the total AA was incorporated into the cells within 5 h of incubation. The cells were then washed three times with 0.1% BSA:BEM to remove essentially all the free AA. The cells were then replenished with the old conditioned medium (5 ml) and then subjected to EtOH exposure. The culture conditions were similar to the ones described by others and us for chronic EtOH investigations [14,22]. EtOH levels (50, 100, 150 mM) were maintained by adding appropriate concentrations of absolute alcohol. All dishes were flushed with 5% CO_2-95% air and sealed with parafilm before incubation. The medium was then supplemented at 24 and 48 h with absolute EtOH (4 μl/5 ml for 50 mM; 25 μl/5 ml for 100 mM; 30 μl/5 ml for 150 mM). EtOH level in the medium at 72 h was determined by an enzymatic method [23] (50 ± 1.0 mM; 100 ± 6.0 mM; 150 ± 17.0 mM, $n = 6$). Appropriate control cultures were similarly maintained in a medium containing no EtOH. The cultures were incubated in BEM containing drugs at the indicated concentrations. Ionomycin (1–10 μM), phenylmethylsulfonyl fluoride (PMSF) (10 μM), and SR141716A (1–10 μM) were added from stock solutions in dimethyl sulfoxide (DMSO). Final concentration of DMSO never exceeded 0.2%, and this concentration had no effect on cell viability. For treatment with various agents, culture plates were co-treated with PMSF (50 μM) or BTNP (10 μM) or glutamate (10 μM) or MK-801 (10 μM) or 7-H-DPAT (1 μM) or haloperidol (1 μM) or SR141716A (1 μM) or pertussis toxin (PTX) (100 ng/ml) and with or without EtOH. For Ca^{2+} ionophore ionomycin treatment, neurons, with or without being first exposed to EtOH (100 mM, 72 h), were exposed to ionomycin (1–10 μM) with or without 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM) (30 μM) for 10 min. Cells were examined for viability by trypan blue exclusion and counted. The incubations were terminated by collecting the medium. The cells were scraped and then suspended in 10 mM Tris-HCl buffer pH 7.4 containing protease inhibitor cocktail (Sigma). Data (medium and cells) are expressed as the (mean ± S.E.M.) dpm/mg cellular protein or % of control.

2.4. Extraction and chromatography

Lipids from cells and medium were extracted using a mixture of chloroform/methanol (2:1, v/v) [24], 2 μg of unlabeled 2-AG was included as a carrier. Butylated hydroxytoluene (BHT) (0.05%) was added to prevent lipid peroxidation. The dried extracts were redissolved in a mixture of chloroform/methanol (2:1, v/v), spotted onto TLC plates (silica gel 60, 250 μm thickness), and developed with one of the following solvent systems: the organic phase of a mixture of isooctane/ethyl acetate/water/acetic acid (50:110:100:30, v/v) (solvent system A), which allows the separation of 2-AG ($R_F = 0.53$) from *N*-arachidonylphosphatidylethanolamine (N-ArPE) ($R_F = 0.14$), AnNH ($R_F = 0.43$) and AA ($R_F = 0.78$); chloroform/methanol/acetic acid (85:15:1, v/v) (solvent system B), which allows separation of N-ArPE ($R_F = 0.35$) from AnNH ($R_F = 0.7$) and AA+2-AG ($R_F = 0.9$); diethyl ether/petroleum ether/NH_4OH (50:50:1, v/v) (solvent system C), which allows separation of AA ($R_F = 0.0$) and 2-AG ($R_F = 0.2$). The band corresponding to authentic 2-AG was scraped off the plate into scintillation vials containing 1 ml of chloroform/methanol (2:1). The radioactivity was determined using 10 ml of scintillation liquid (Insta-Fluor, Packard). In a separate experiment, the lipids were extracted from the medium and the cells (12 plates) and were subjected to TLC purification. The identity of 2-AG was confirmed by scraping the band corresponding to authentic 2-AG and gas chromatography-MS (GC-MS) analysis of trimethylsilyl ether (TMS) derivatives. In brief, the isolated 2-AG fraction was treated with 60 μl of TMS reagent (Alltech Associates, Deerfield, IL) at 80°C for 1 h [25,26]. The TMS derivative was then injected directly into the GC-MS. The GC-MS was performed on a Hewlett Packard 5988 mass spectrometer equipped with 5890 series GC and a 7673 automatic injector. The capillary column (HP5MS, 30 m × 0.25 mm i.d.) was temperature-programmed from 90 to 270°C at 10°C/min. The mass spectrometer was operated in the electron impact (EI) (70 eV) mode and the electron impact mass spectra of the TMS derivative of both the synthetic and tissue-derived 2-AG were compared.

2.5. Protein determination

Protein concentration of the cell homogenates was

determined by the Lowry procedure using bovine serum albumin as the standard [27].

2.6. Statistical analysis

Statistical analysis was performed by Student's *t*-test and one-way analysis of variance (ANOVA) followed by Dunnett's test using the Graphpad Prism version 2.01 software program (Graphpad Software, San Diego, CA). Differences were considered to be significant if $P < 0.05$. Data are presented as means ± S.E.M. from at least three separate experiments run in triplicate.

3. Results

The identity of 2-AG extracted from the cells and medium was confirmed by comparison of R_F values with those of the authentic 2-AG after separation in three different solvent systems. Further characterization of 2-AG was achieved by GC-MS analysis of the TMS derivative of TLC-purified 2-AG. Diagnostic ions for 2-AG included *m/z* 522 (M^+, molecular ion), 507 $[M-15]^+$, loss of methyl radical, 451 $[M-71]^+$, loss of pentyl radical and 432 $[M-90]^+$, loss of trimethyl silenol (data not shown).

It is observed that the formation of 2-[^{3}H]AG in unstimulated CGNs in culture was found to be age-dependent (DIV) (Fig. 1). The formation of 2-[^{3}H]AG was more pronounced in the older cultures (7–8 DIV) than in younger cultures (3–5 DIV). Therefore, the 7 DIV cultures were employed for further studies.

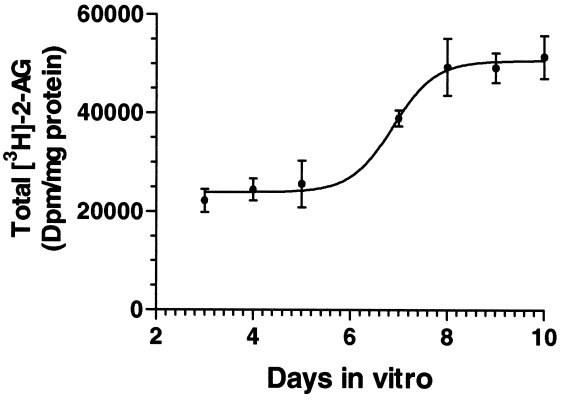

Fig. 1. Formation of 2-[^{3}H]AG in CG neurons as a function of days in vitro. Cells were labeled with [^{3}H]AA (1 μCi/ml) at 2 DIV in BME (without FCS) for 5 h and then incubated as described in Section 2. The 2-[^{3}H]AG was extracted from medium and cells and separated on TLC using solvent system A and the results are expressed as dpm/mg cellular protein. Each value represents the mean ± S.E.M. ($n = 6$).

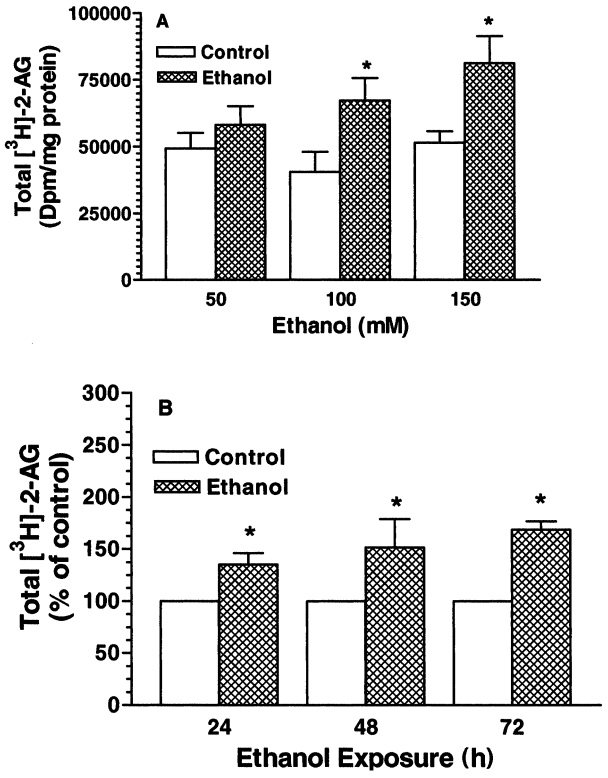

Fig. 2. Formation of 2-[^{3}H]AG in CG neurons following exposure to various concentrations of EtOH (A) and for various times (B). Cells (7 DIV) were labeled with [^{3}H]AA (1 μCi/ml) in BME (without FCS) for 5 h and then exposed to various concentrations of EtOH for 72 h or for various times at 100 mM EtOH as described in Section 2. The 2-[^{3}H]AG was extracted from medium and cells and separated on TLC using solvent system A. Values for basal control are similar to those in Fig. 1 and in panel A. Each value represents the mean ± S.E.M. ($n = 9$). *$P < 0.05$ (Student's *t*-test), compared with control.

Neurons (CGNs) were exposed to various concentrations of EtOH (50, 100, and 150 mM for 72 h) and also 100 mM EtOH for various times (24, 48 and 72 h). The cell viability was unaffected irrespective of the treatment conditions employed as determined by the trypan blue exclusion method (data not shown).

We investigated the effect of EtOH on 2-[^{3}H]AG formation using CGNs prelabeled with [^{3}H]AA. Treatment with EtOH caused a time-dependent increase in the formation of 2-[^{3}H]AG (Fig. 2A,B). The

results also indicated that EtOH treatment of CGNs did not have any significant effect on the level of 2-[^{3}H]AG at 50 mM for 72 h ($P > 0.05$), but produced a statistically significant increase at 100 and 150 mM for 72 h ($P < 0.05$). Exposure of CGNs to EtOH (100 mM) gradually increased the level of 2-[^{3}H]AG, reaching a statistically significant level after 24, 48, and 72 h ($P < 0.05$). Most of the synthesized 2-[^{3}H]AG was released from the cells and found in the medium (85%) at 72 h (data not shown). The data are shown as the total (medium plus cells) (Fig. 2A,B).

In order to determine the possible mechanism of EtOH-induced formation of 2-[^{3}H]AG, CGNs were treated with various neuromodulators that are suggested to modulate the endocannabinoid system [28,29].

A higher level of 2-[^{3}H]AG (2.45 fold; $P < 0.05$) was observed when CGNs were co-treated with EtOH (100 mM; 72 h) and PMSF (50 μM), an amidohydrolase inhibitor known to inhibit 2-[^{3}H]AG hydrolysis [30] (Table 1), whereas bromoenol lactone, BTNP (10 μM), a potent inhibitor of amidohydrolase, had no effect ($P > 0.05$). Incubation of cells with PMSF (50 μM) or BTNP (10 μM) produced no cell death and no lifting of the cell monolayer.

Table 1 shows the effect of glutamate and MK-801, an NMDA receptor antagonist, on EtOH-induced formation of 2-[^{3}H]AG in CGNs. It was found that glutamate enhances the formation of 2-[^{3}H]AG only in control cells (1.6-fold; $P < 0.05$) and has no additive effect when CGNs were co-treated with EtOH (100 mM, 72 h) ($P > 0.05$). However, when CGNs were co-treated with MK-801 (10 μM) and EtOH (100 mM) for 72 h, a significant inhibition of EtOH-induced formation of 2-[^{3}H]AG (27%; $P < 0.05$) was observed, and this inhibition was not observed in control cells ($P > 0.05$) (Table 1). Further, when CGNs were co-treated (72 h) with dopamine receptor (D2) agonist (7-H-DPAT, 1 μM) and

Table 1
Effect of various neuromodulators on the formation of 2-[^{3}H]AG in CGNs following co-exposure to 100 mM EtOH for 72 h

Neuromodulators	Control	Ethanol
Basal	100.00 ± 14.50	165.70 ± 20.73* (a)
PMSF (50 μM)	245.90 ± 59.93* (c)	242.10 ± 14.10* (c, b)
BTNP (10 μM)	125.20 ± 16.06	156.90 ± 13.80* (a, c)
Glutamate (10 μM)	159.80 ± 11.68* (c)	166.10 ± 19.10* (c)
MK-801 (10 μM)	133.60 ± 10.86	120.40 ± 01.10* (b)
7-H-DPAT (1 μM)	109.60 ± 18.23	158.10 ± 19.23* (c)
Haloperidol (1 μM)	125.10 ± 11.73	107.90 ± 15.10* (b)

Cells were labeled with [^{3}H]AA (1 μCi/ml) in BME (without FCS) for 5 h and then co-treated with PMSF (50 μM), BTNP (10 μM), glutamate (10 μM), MK-801 (10 μM), 7-H-DPAT (1 μM), haloperidol (1 μM) and with or without EtOH as described in Section 2. The 2-[^{3}H]AG was extracted from medium and cells and separated on TLC, system A. Values for basal control are similar to those in Figs. 1 and 2. Each value represents the mean ± S.E.M. ($n = 9$). *$P < 0.05$ (ANOVA); (a), versus respective control; (b), versus basal EtOH; (c), versus basal control.

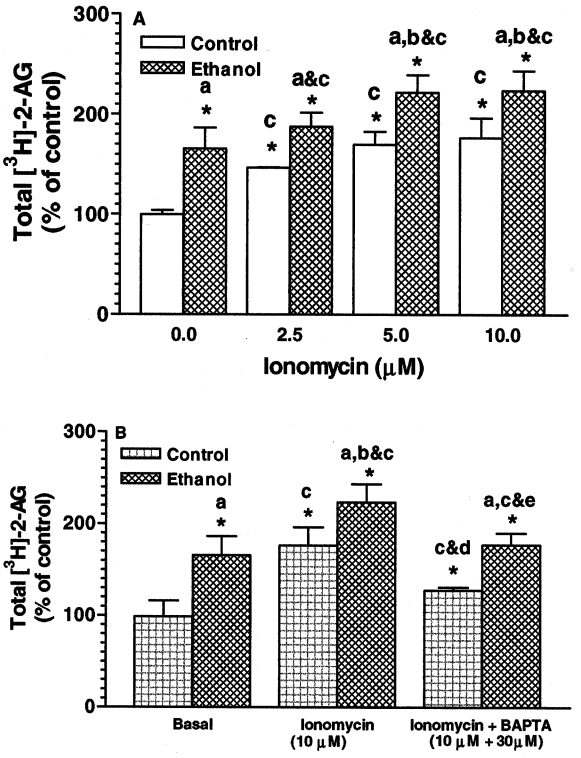

Fig. 3. Effect of ionomycin (A) on chronic EtOH-induced formation of 2-[^{3}H]AG or effect of BAPTA-AM (B) on the ionomycin-induced formation of 2-[^{3}H]AG in chronic EtOH-exposed CGNs. Cells were labeled with [^{3}H]AA (1 μCi/ml) in BME (without FCS) for 5 h and exposed to EtOH (100 mM, 72 h) and then treated with or without various concentrations of ionomycin or ionomycin (10 μM) with or without BAPTA-AM (30 μM) for 10 min as described in Section 2. The 2-[^{3}H]AG was extracted from medium and cells and separated on TLC, system A. Values for basal control are similar to those in Figs. 1 and 2. Each value represents the mean ± S.E.M. ($n = 9$). *$P < 0.05$ (ANOVA); a, versus respective control; b, versus basal EtOH; c, versus basal control; d, versus ionomycin control; e, versus ionomycin EtOH.

EtOH (100 mM), no significant effect on the EtOH-induced formation of 2-AG was observed ($P > 0.05$) (Table 1). However, the treatment with dopamine receptor antagonist (haloperidol, 1 μM) and EtOH (100 mM) inhibited the EtOH-induced formation of 2-AG significantly (35%; $P < 0.05$) (Table 1).

When chronic EtOH-exposed CGNs (100 mM; 72 h) were treated with various concentrations of ionomycin (0–10 μM) for 10 min, in addition having dose-dependent stimulatory action on control CGNs, ionomycin was found to be potent in enhancing EtOH-induced formation of 2-[^{3}H]AG (Fig. 3A) at 2.5 μM, 5 μM and there was no significant difference between 5 μM and 10 μM ionomycin. Furthermore, when chronic EtOH-exposed CGNs were co-treated with ionomycin (10 μM) and BAPTA-AM (10 μM), an intracellular Ca^{2+} chelator, for 10 min, the result was an inhibition of ionomycin-stimulated 2-[^{3}H]AG formation both in control and EtOH-exposed cells (Fig. 3B).

We studied the effect of the CB1 receptor antagonist SR141716A and pertussis toxin to determine further whether EtOH-induced formation of 2-[^{3}H]AG in CGNs is receptor/G-protein mediated. Co-treatment of CGNs with various concentrations of CB1

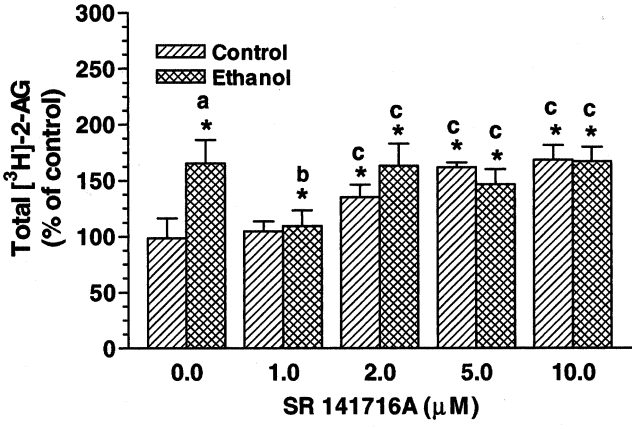

Fig. 4. Effect of CB1 receptor antagonist SR141716A on the formation of 2-[^{3}H]AG in CGNs following exposure to 100 mM EtOH for 72 h. Cells were labeled with [^{3}H]AA (1 μCi/ml) in BME (without FCS) for 5 h and then treated with various concentrations of SR141716A (1–10 μM) and with or without EtOH as described in Section 2. The 2-[^{3}H]AG was extracted from medium and cells and separated on TLC, system A. Values for basal control are similar to those in Figs. 1 and 2. Each value represents the mean ± S.E.M. ($n = 9$). *$P < 0.05$ (ANOVA); a, versus respective control; b, versus basal EtOH; c, versus basal control.

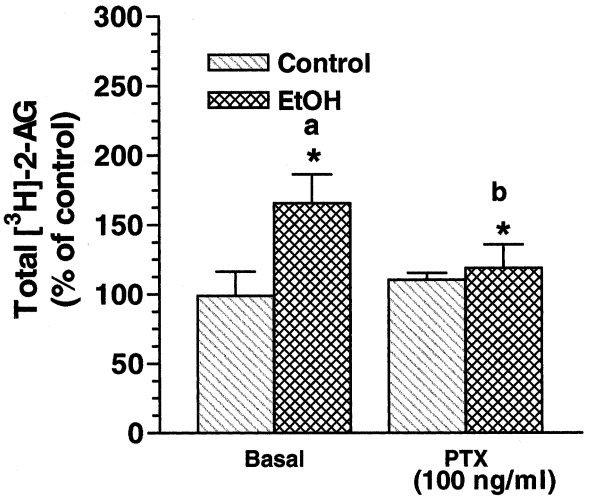

Fig. 5. Effect of PTX on the formation of 2-[^{3}H]AG in CGNs following exposure to 100 mM EtOH for 72 h. Cells were labeled with [^{3}H]AA (1 μCi/ml) in BME (without FCS) for 5 h and then co-treated with PTX (100 ng/ml) and with or without EtOH as described in Section 2. The 2-[^{3}H]AG was extracted from medium and cells and separated on TLC, system A. Values for basal control are similar to those in Figs. 1 and 2. Each value represents the mean ± S.E.M. ($n = 9$). *$P < 0.05$ (ANOVA); a, versus respective control; b, versus basal EtOH.

receptor antagonist SR141716A (0–10 μM) and EtOH (100 mM) for 72 h blocked (100%; $P < 0.05$) the EtOH-induced formation of 2-[^{3}H]AG at lower concentration (1 μM) but at higher concentrations it increased the formation of 2-[^{3}H]AG in a dose-dependent manner in both control and EtOH-treated cells ($P < 0.05$) (Fig. 4). Co-exposure of CGNs to pertussis toxin (100 ng/ml) and EtOH (100 mM) for 72 h resulted in an inhibition of EtOH-induced formation of 2-[^{3}H]AG (100%) (Fig. 5).

4. Discussion

Recently, a second phospholipid-derived messenger, 2-AG, has been identified and reported to display properties of an endogenous cannabinoid receptor agonist [9,10,31]. The CGNs could start synthesis of 2-AG as early as 3 DIV and further increase with the age of the culture. Although the physiological roles of 2-AG in normal brain cells and tissue is not clear, a substantial amount of 2-AG has been detected in normal rat brain and cortical neurons [32,33]. 2-AG may have a role in lipid remodeling

leading to increases in cellular diglyceride levels that in turn are important receptor-coupled signaling molecules. To our knowledge, this study represents the first examination of chronic EtOH-stimulated 2-[^{3}H]AG formation in CGNs. Previously, we demonstrated that chronic EtOH exposure increased the synthesis of CB1 receptor agonist AnNH and its precursor, N-ArPE in SK-N-SH cells. It was shown that chronic EtOH downregulates the CB1 receptor number and function [3,4] in mouse brain. Distinct differences in the CB1 receptor binding in the brain of two inbred strains of mice shown to differ in their preference for EtOH have also been demonstrated [17]. Furthermore, the inhibition of EtOH intake by the CB1 receptor antagonist SR141716A in rats [13] and mice [12] has been reported. These results, taken together, point to an important role for the endocannabinoid signaling system in the pharmacological actions of EtOH.

Similar to AnNH synthesis in SK-N-SH cells [14], EtOH treatment of CGNs led to the enhanced formation of 2-[^{3}H]AG in a dose- and time-dependent manner. This increase in endogenous cannabinoids, 2-AG and AnNH [14] levels following exposure to chronic EtOH may be responsible for the downregulation of CB1 receptor function in EtOH-tolerant mice observed in our previous studies [3,4]. Recently, such a phenomenon was also found in some brain regions of Δ^9-tetrahydrocannabinol-tolerant rats [34]. Although the levels of AnNH and other *N*-acylethanolamines (NAEs) are considerably low in normal tissues, their levels have been found to increase significantly during cell injury, tissue degeneration, and during the post mortem period [25,35–37]. The brain levels of 2-AG were shown to be 170 times greater than those of AnNH [10]. Interestingly the levels of 2-AG could be enhanced by electrical high frequency stimulation in hippocampal slices [10]. The exact mechanism by which 2-AG is synthesized has not been clearly established. The available evidence suggests that three main biochemical pathways seem to exist in neurons for the formation of 2-AG through the sequential action of a PI-specific phospholipase C, *sn*-1-diacylglycerol lipase, and/or calcium-dependent transacylation and phospholipase C [10,28,29,38].

The majority of the EtOH-stimulated 2-AG was found to be accumulated in the medium (85%), suggesting that a significant portion of biosynthesized 2-AG is released outside neurons. These results are comparable to analogous data obtained by stimulation of 2-AG formation by ionomycin in neuroblastoma cells [38]. The amidohydrolase enzyme inhibitor PMSF was also shown to inhibit the 2-AG hydrolysis in neurons and was also effective in enhancing the accumulation of 2-AG in both the control and EtOH-exposed CGNs. Conversely, 2-AG accumulation was not affected by the more selective anandamide amidohydrolase inhibitor BTNP. These results are in agreement with the previous observation, where BTNP (5 μM) had no effect on 2-AG accumulation or metabolism in human astrocytoma cells [39]. Therefore, the possibility remains for the existence of a specific lipase for 2-AG hydrolysis, which may be responsible for the physiological inactivation of 2-AG in neurons and this should be investigated in future studies.

We have extended these observations with various neuromodulators in order to elucidate the possible mechanism by which EtOH might increase the formation of 2-AG in CGNs. Treatment of chronic EtOH-exposed CGNs with increasing doses of ionomycin (2.5–10 μM) for 10 min led to an increase in the formation of 2-AG. Further, we assessed whether ionomycin-induced 2-AG formation was Ca^{2+}-dependent, and therefore stimulated the chronic EtOH-exposed cells with ionomycin (10 μM) in the presence or absence of the intracellular Ca^{2+} chelating agent BAPTA-AM (30 μM). The BAPTA-AM inhibited significantly the effect of ionomycin (10 μM) on EtOH-induced formation of 2-AG (Fig. 3A,B). A similar ionophore-induced AnNH and N-ArPE biosynthesis has been shown in various neuronal cells [14,32,40,41]. It has been shown that chronic EtOH exposure leads to an increase in intracellular Ca^{2+} levels in mouse brain synaptosomes [42], rat cerebellar macroneurons [43], PC12 cells [44], and cerebral vascular smooth muscle cells [45,46]. Based on these data, it is suggested that, in CGNs, the chronic EtOH-induced formation of 2-AG might be triggered by an increase in cytoplasmic Ca^{2+} concentration.

It is interesting to note that the endocannabinoid signal transduction system may be modulated by various other neurotransmitter systems [47], which are shown to be involved in chronic EtOH action [2]. In

order to establish possible interactions between the EtOH-induced formation of 2-AG and other neurotransmitter systems, the effect of NMDA receptor agonist and antagonist and D2 receptor agonist and antagonist on the EtOH-induced formation of 2-AG in CGNs was investigated. Glutamate, which is also known to stimulate the formation of AnNH in cortical neurons [32], stimulated the formation of 2-AG in CGNs. However, glutamate did not cause further enhancement of the EtOH-induced formation of 2-AG, suggesting EtOH might have caused a saturation of 2-AG levels that could not be increased further by glutamate. However, the EtOH-induced formation of 2-AG could be inhibited by the NMDA receptor antagonist MK-801. Interestingly, the CGNs exhibited 100% increase in $[Ca^{2+}]_i$ after 4 days of exposure to EtOH [48], suggesting that the glutamate-induced increase in intracellular Ca^{2+} may be responsible for the formation of 2-AG by CGNs. This also suggests that chronic EtOH-induced activation of the NMDA receptor may trigger an increase in cytoplasmic Ca^{2+} concentration, which in turn may be responsible for the enhanced synthesis of 2-AG. The D2 receptor agonist did not enhance the formation of 2-AG either in control or in EtOH-exposed CGNs, whereas co-treatment of CGNs with D2 receptor antagonist inhibited the EtOH-induced formation of 2-AG, suggesting the interaction of D2 receptor system or direct action of this compound on the EtOH-induced formation of 2-AG. The D2 receptor activation by the agonist quinpirole led to eightfold stimulation of AnNH release in striatum without affecting the 2-AG release, and this stimulation was inhibited by the antagonist raclopride [49]. These results suggest the possible differences in the activation and/or inhibition of D2 receptors in various regions of the brain, which may influence the formation of endocannabinoids. Further studies are necessary to establish this phenomenon.

Previously, it has been shown that cerebellum and cerebellar granular cells contain a high density of CB1 receptors and receptor-activated GTP binding proteins [50,51]. Our results with SR141716A and PTX suggest that the level of 2-AG in chronic EtOH-treated CGNs may be regulated by CB1 receptors and Gi/o-proteins, as observed for AnNH biosynthesis [14]. However, EtOH-induced formation of 2-AG was inhibited by SR141716A only at lower concentration. At higher concentration SR141716A was found to have a stimulatory effect in CGNs which are not exposed to EtOH, suggesting its dual nature of action. SR141716A probably acts through the CB1 receptor at lower concentration and at higher concentration it may be acting independent of this receptor or as an inverse agonist. It is entirely possible that high concentrations of SR141716A may result in non-specific effects which may not necessarily be related to CB1 receptor blockade. In fact, SR141716A has been reported to exhibit inverse agonism in vivo in causing hyperalgesia in a mouse model of thermal pain [52]. CB1 receptor antagonist SR141716A was shown to block voluntary EtOH intake in rats (EtOH preferring) [13] and mice [12] and CB1 receptor agonist CP,55,940 was shown to increase the motivation for beer in rats [15], suggesting that the endocannabinoid system may constitute part of a common brain pathway mediating reinforcement of drugs of abuse including alcohol. These results suggest a possible role for these endogenously formed cannabinoid agonists and a signal transduction system in alcohol tolerance and dependence and warrant further investigation.

In summary, we have found that CB1 receptor agonist 2-AG synthesis was stimulated by chronic EtOH in primary cultures of cerebellar granule neurons, probably as a neuroadaptation mechanism in response to the continuous presence of EtOH. The EtOH-induced formation of 2-AG is found to be regulated by CB1 receptors and by various neuromodulators.

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