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The ALIAmide palmitoylethanolamide and cannabinoids, but not anandamide, are protective in a delayed postglutamate paradigm of excitotoxic death in cerebellar granule neurons

(*N*-methyl-D-aspartate/neurotoxicity/*N*-acylethanolamides/neuroprotection/receptor)

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ABSTRACT **The amino acid L-glutamate is a neurotransmitter that mediates fast neuronal excitation in a majority of synapses in the central nervous system. Glutamate stimulates both *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors. While activation of NMDA receptors has been implicated in a variety of neurophysiologic processes, excessive NMDA receptor stimulation (excitotoxicity) is thought to be primarily responsible for neuronal injury in a wide variety of acute neurological disorders including hypoxia–ischemia, seizures, and trauma. Very little is known about endogenous molecules and mechanisms capable of modulating excitotoxic neuronal death. Saturated *N*-acylethanolamides like palmitoylethanolamide accumulate in ischemic tissues and are synthesized by neurons upon excitatory amino acid receptor activation. Here we report that palmitoylethanolamide, but not the cognate *N*-acylamide anandamide (the ethanolamide of arachidonic acid), protects cultured mouse cerebellar granule cells against glutamate toxicity in a delayed postagonist paradigm. Palmitoylethanolamide reduced this injury in a concentration-dependent manner and was maximally effective when added 15-min postglutamate. Cannabinoids, which like palmitoylethanolamide are functionally active at the peripheral cannabinoid receptor CB2 on mast cells, also prevented neuron loss in this delayed postglutamate model. Furthermore, the neuroprotective effects of palmitoylethanolamide, as well as that of the active cannabinoids, were efficiently antagonized by the candidate central cannabinoid receptor (CB1) agonist anandamide. Analogous pharmacological behaviors have been observed for palmitoylethanolamide (ALIAmides) in downmodulating mast cell activation. Cerebellar granule cells expressed mRNA for CB1 and CB2 by *in situ* hybridization, while two cannabinoid binding sites were detected in cerebellar membranes. The results suggest that (*i*) non-CB1 cannabinoid receptors control, upon agonist binding, the downstream consequences of an excitotoxic stimulus; (*ii*) palmitoylethanolamide, unlike anandamide, behaves as an endogenous agonist for CB2-like receptors on granule cells; and (*iii*) activation of such receptors may serve to downmodulate deleterious cellular processes following pathological events or noxious stimuli in both the nervous and immune systems.**

Dicarboxylic amino acids form the most widespread excitatory transmitter network in the mammalian brain (1). Glutamate interactions with specific membrane receptors are responsible for many neurologic functions, including cognition, memory, movement, and sensation (2). In addition, the excitation produced by glutamate is important in influencing the developmental plasticity of synaptic connections in the nervous system (3, 4). Excitatory amino acids (EAAs) have also been implicated in neurotoxicity. Excessive activation of EAA receptors may be responsible for much of the neuronal damage associated with certain acute insults, including hypoxia–ischemia, hypoglycemia, epilepsy, and trauma (5, 6). Furthermore, exaggerated EAA receptor activity has been suggested by some to also underly chronic neurodegenerative disorders, including Huntington disease, Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis, and acquired immune deficiency syndrome–dementia complex (7–9). In many experimental systems, overstimulation of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor with protracted entry of Ca^{2+} into neurons appears to be a principal mechanism for subsequent damage (5). There is growing evidence for participation of non-NMDA receptors in EAA-mediated neurotoxicity as well, especially in cases of prolonged or chronic insult (10, 11).

N-acylethanolamides, like palmitoylethanolamide, and *N*-acylphosphatidylethanolamides accumulate in conditions involving degenerative changes to tissues (12), including brain (13) and cardiac (14) ischemia. Furthermore, EAAs can stimulate the synthesis of *N*-acylethanolamides and *N*-acylphosphatidylethanolamides in cultured central neurons (15, 16). The possibility that compounds of this type could defend against an excitotoxic insult may be entertained (16). Interestingly, it has recently been reported that mast cells, multifunctional immune cells implicated in immediate hypersensitivity and inflammatory reactions (17), express a peripheral-type cannabinoid receptor (designated CB2; ref. 18) that recognizes palmitoylethanolamide (ALIAmides) and downmodulates activation of these cells *in vitro* (19). The candidate endogenous agonist for the brain cannabinoid receptor CB1 (20, 21), arachidonylethanolamide (anandamide) (22, 23), binds to mast cell CB2 and actually antagonizes the functional effects of palmitoylethanolamide and several cannabinoids (19).

We now report that palmitoylethanolamide and 2-*O*-(β-D-glucopyranosyl)-*N*-palmitoylethanolamide, as well as some natural and synthetic cannabinoids but not anandamide, are efficacious in protecting cultured cerebellar granule cells from glutamate toxicity in a delayed postagonist paradigm without affecting EAA receptor function. Anandamide, however, antagonized these neuroprotective effects. Furthermore, granule cells expressed mRNA for both CB1 and CB2 and cerebellar membranes displayed two cannabinoid binding sites.

MATERIALS AND METHODS

Primary Neuron Cultures. Cultures containing granule neurons were prepared from dissociated cerebella of 7- to 8-day-old BALB/c mice (Modelli Biologici Sperimentali, Treviso, Italy) (24). Cells were plated in Eagle's basal medium

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Abbreviations: EAA, excitatory amino acid; NMDA, *N*-methyl-D-aspartate; DIV, days *in vitro*; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMSF, phenylmethylsulfonyl fluoride; KA, kainic acid.

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supplemented with 10% fetal calf serum (BIOSPA, Wedel, Germany), 25 mM KCl, 2 mM glutamine, and gentamicin (50 μg/ml) on 35-mm-diameter dishes (Falcon) coated with poly-(L-lysine) (10 μg/ml) (M_r, 68,000), 2.5×10^6 cells per dish. Cytosine β-D-arabinofuranoside (10 μM) was added to the culture medium 18–20 h after plating to halt nonneuron growth. The cultures were used at 8–10 days *in vitro* (DIV), and contain ≥95% glutamatergic granule neurons (25).

Induction of Glutamate Neurotoxicity. Neurotoxicity was induced essentially as described (24). Medium from 8- to 10-day-old granule cell cultures was removed and saved. Culture dishes were washed twice with Mg^{2+}-free Locke's solution, and the cells were then incubated with 500 μM glutamate in Mg^{2+}-free Locke's solution for 5 min (23–25°C). The glutamate-containing solution was then removed by aspiration, and the dishes were washed twice with complete Locke's solution and then returned to the incubator in their original medium for a further 24 h. Acute glutamate exposure under these conditions consistently resulted in a 50–65% loss in neuron numbers 24 h later, at which time cell survival was routinely evaluated. Drug treatment protocols are described in the appropriate figure or table legend as introduced. Stock solutions of cannabinoids and *N*-acylethanolamides were made in dimethyl sulfoxide, while anandamide was dissolved in ethanol. The final concentration of solvent in the culture never exceeded 0.2%, except in the case of palmitoylethanolamide, which necessitated a higher concentration (1%). These concentrations of solvent were found to have no effect on the response of the granule cells to glutamate.

Quantitation of Neurotoxicity. Glutamate neurotoxicity was gross enough to be evident morphologically when viewed under a phase-contrast microscope. Neuronal survival was quantified using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which yields a blue formazan product in living cells but not in dead cells or their lytic debris (26). The reaction product, solubilized in dimethyl sulfoxide, is easily measured with an ELISA plate reader and is directly proportional to the number of neurons present (24, 27, 28). The MTT technique is equivalent to lactate dehydrogenase release in the measurement of excitotoxin-mediated neuronal death *in vitro* (29).

***In Situ* Hybridization.** BALB/c mouse (4-week-old) brain was snap-frozen in 2-methylbutane, 12-μm coronal sections cut with a Jung model CM 3000 cryostat and thaw-mounted on polylysine-coated glass slides. All sections were then fixed in 4% paraformaldehyde, dehydrated in a graded series of ethanols, incubated 5 min in chloroform, and air-dried. Cerebellar granule cells growing on polylysine-coated cover glasses at 8 DIV were fixed with 4% paraformaldehyde, washed twice with phosphate-buffered saline, and permeabilized in 70% ethanol for 48 h at 4°C. The cells were then dehydrated using a higher graded series of ethanols and air-dried. Detection of CB1- and CB2-specific mRNA made use of the following oligonucleotides prepared with a Beckman model Oligo 1000 DNA synthesizer: CB1, 5′-GGT GAC GAT CCT CTT ATA GGC CAG AGG CCT TGT AAT GGA TAT GTA-3′ (21); CB2, 5′-GGT GAC GAG AGC TTT GTA GGT AGG TGG GTA GCA CAG ACA TAG GTA-3′ (18). A random sequence was used as control. Oligonucleotides were 3′ end-labeled with dATP[α-^{35}S] (1136 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) by terminal deoxynucleotidyltransferase (Pharmacia) to a specific activity of $\approx 10^9$ cpm/μg. All cover glasses were hybridized in standard solutions (30) with 1.5×10^7 dpm/ml overnight in a humidified chamber at 42°C. The cover glasses were then washed once with 1× SSC/0.1% SDS (30 min, 55°C), twice with 1× SSC (15 min, 55°C), and once with 0.1× SSC (30 min, 25°C), followed by a 2-min rinse in autoclaved water and dehydration with a graded series of ethanols. The air-dried cover glasses were then dipped in K.5 photoemulsion (Ilford) (diluted 1:1 with water), exposed for 5 weeks at 4°C, developed with Phenisol (Ilford), fixed with Hypam (Ilford), and counterstained with cresyl violet.

Radioligand Binding Assays. Cerebella from 20-day-old BALB/c mice were removed, cleaned of meninges, and stored at −80°C for up to 1 month. Membrane preparation and binding assays were carried out following published procedures (19, 31), with modification. Groups of five (frozen) cerebella were homogenized in 20 ml of binding buffer (3 mM $MgCl_2$/1 mM EDTA/50 mM Tris, pH 7.4) containing 0.32 M sucrose. The homogenate was centrifuged at $2000 \times g$ for 10 min at 4°C, and the resulting supernatant was centrifuged at $15{,}000 \times g$ for 15 min at 4°C. The pellet (P2) was resuspended in 20 ml of binding buffer containing 0.1% fatty acid-free bovine serum albumin, and the last centrifugation step was repeated. The final pellet was gently rinsed with distilled water and then resuspended in 1 ml of binding buffer (0.8–1.1 mg per ml of protein). Membranes were kept on ice and used within 1 h. Binding experiments were performed in silicon-treated tubes. [^{3}H]WIN 55,212–2 (45.5 Ci/mmol; 22 μM in ethanol; New England Nuclear) and nonradioactive ligands were serially diluted in dimethyl sulfoxide and were added at the desired concentration to a final volume of 500 μl of binding buffer. The final concentration of dimethyl sulfoxide was always 1%. Binding was initiated by adding 30 μg (protein) of membranes and the tubes were incubated for 55 min at 30°C. Binding was terminated by transferring the reaction mixture to an Eppendorf tube, followed by addition of fatty acid-free bovine serum albumin to 0.1% and centrifugation at $39{,}000 \times g$ for 10 min at 20°C. The supernatants were collected and counted to determine the concentration of free ligand, while the pellets were suspended in 1% Triton X-100/ethanol (1:1; vol/vol) and radioactivity was assayed by liquid scintillation counting. Nonradioactive WIN 55,212–2 (1 μM) was used to displace specific binding.

Materials. Tissue culture media and supplements and cannabidiol were obtained from Sigma. WIN 55,212–2 and Δ^8-THC were from Research Biochemicals (Natick, MA). Palmitoylethanolamide, 2-*O*-(β-D-glucopyranosyl)-*N*-palmitoylethanolamide (glucosylpalmitoylethanolamide), and anandamide were synthesized by standard chemical techniques, with purity of >99.5% as assessed by HPLC. All other reagents, unless specified otherwise, were from Sigma.

RESULTS

Palmitoylethanolamide- and Cannabinoid-Mediated Protection from Glutamate Neurotoxicity. Although glutamate is a neuroexcitant agonist at NMDA, quisqualate, and kainate postsynaptic receptors (32), its neurotoxicity is predominantly mediated by NMDA receptors alone (33). Given that palmitoylethanolamide accumulates in ischemic brain (13) and its production is induced in central nervous system neurons by EAA receptor activation (15, 16), it was of interest to examine possible modulatory effects of the *N*-acylethanolamide against NMDA receptor-mediated neurotoxicity. Most studies on the neuroprotective efficacy of glutamate antagonists or other drugs have used pre- and cotreatment protocols. The present experimental setting was chosen, as others have done (34, 35), to allow for conditions that provide potentially rescuable neurons in a delayed postagonist paradigm.

Palmitoylethanolamide (100 μM) or MK-801 (10 μM) was added during or at various times after conclusion of a 5-min glutamate exposure. Neuronal cell loss was quantitatively monitored by the MTT reaction, which is widely used to assess neuron numbers in culture (24, 27, 28) and is considered equivalent to lactate dehydrogenase release (29). Microscopic observation of the cultures confirmed the results obtained by MTT (data not shown). The potent noncompetitive NMDA antagonist MK-801 (36) while maximally efficacious when present during glutamate exposure rapidly decayed with a 15-min postagonist delay (Fig. 1) (see also ref. 34). In contrast,

palmitoylethanolamide achieved maximal efficacy when added 15 min postglutamate (Fig. 1). Neuroprotection produced by the delayed, postagonist addition of palmitoylethanolamide was also concentration dependent (Table 1) and related to the duration of drug exposure, being maximal when present for 20 min or more. Addition of a glucose residue to palmitoylethanolamide (glucosylpalmitoylethanolamide) increased its activity as a neuroprotectant (Table 1), perhaps due to improved solubility. The potent non-NMDA receptor antagonist CNQX (11), Ca^{2+} channel blockers (diltiazem, nifedipine, ω-conotoxin, diphenylhydantoin, flunarizine), free radical scavengers, and monosialogangliosides were also tested in this delayed postagonist rescue paradigm and found to produce little or no reduction in neuronal injury (data not shown).

Given that cannabimimetics display functional effects analogous to palmitoylethanolamide on mast cells (19), cannabinoid compounds were also explored for possible protection against glutamate neurotoxicity. The synthetic cannabinoid nabilone (10 μM) and Δ^8-THC (10 μM), like saturated *N*-acylethanolamides, were maximally effective with a delayed, 15-min postagonist application (Fig. 1); exposure times of 5–10 min proved to be optimally efficacious. The respective EC_{50} values are given in Table 1. In contrast, the nonpsychoactive cannabinoid cannabidiol, which has weak affinity for CB1 and CB2 receptors (18, 20), was inactive (Table 1).

Anandamide Antagonizes the Neuroprotective Effects of Saturated *N*-Acylethanolamides and Cannabinoids. Anandamide, a candidate agonist for the brain cannabinoid receptor (22, 23), produces many of the behavioral and physiological responses of cannabinoids attributed to activation of the CB1 receptor (37, 38). Unlike the active cannabinoids tested (Table 1), a 10-min anandamide exposure of up to 100 μM (in the presence of 150 μM phenylmethylsulfonyl fluoride; PMSF) failed to protect granule cells from glutamate toxicity. When added together with saturated *N*-acylethanolamides or cannabinoids, anandamide (10 μM) actually antagonized their delayed postagonist neuroprotective effects, shifting the concentration–response curve for the drug (Table 1). The anandamide congener homo-γ-linolenylethanolamide (C20:3), which binds the CB1 receptor more weakly than anandamide (23), antagonized neither CB2 receptor function in mast cells nor the rescue effects of saturated *N*-acylethanolamides and nabilone against glutamate neurotoxicity when used up to 25 μM (data not shown), indicating a degree of specificity in the observed behaviors of anandamide.

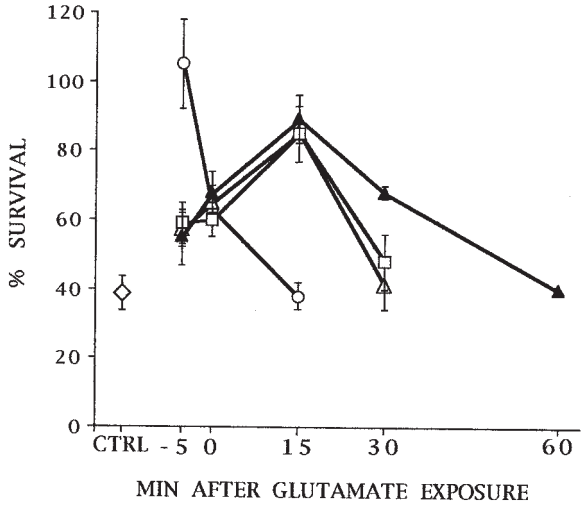

FIG. 1. Time course of rescue from glutamate neurotoxicity: palmitoylethanolamide and cannabinoids vs. MK-801. Sister cultures were exposed to 500 μM glutamate for 5 min and then rescued by addition of 10 μM MK-801 (○), 10 μM nabilone (△), 10 μM Δ^8-THC (□), or 100 μM palmitoylethanolamide (▲) at the indicated time in minutes after washout of the glutamate. ◇, Glutamate alone. PMSF (150 μM) was included in all incubations. Except for those cultures in which the drug was present only during glutamate exposure (−5 on the time axis), the duration of late addition drug treatment was as follows: MK-801, 30 min; palmitoylethanolamide, 30 min; cannabinoids, 10 min. Neuronal survival by MTT was measured 24 h later. Values are means ± SD (three experiments).

Table 1. Palmitoylethanolamide and cannabinoids concentration-dependently rescue cerebellar granule cells from glutamate toxicity with late addition: Antagonism by anandamide

Compound	EC_{50}, μM	
	Control	+ Anandamide
Palmitoylethanolamide	54.6 ± 15.3	>150
Glucosyl-PEA	12.1 ± 1.8	33.4 ± 3.5*
Nabilone	3.9 ± 1.1	15.5 ± 0.6*
Δ^8-THC	2.8 ± 0.7	16.9 ± 5.1*
11OH-Δ^9-THC	0.88 ± 0.28	ND
WIN 55,212–2	24.5 ± 4.1	ND
Cannabidiol	>100	ND
Anandamide	>100	ND

Cannabinoids were added to the cultures for a 10-min period and palmitoylethanolamide or glucosylpalmitoylethanolamide (glucosyl-PEA) was added for a 30-min period, all starting 15 min postglutamate. All incubations contained 150 μM PMSF to inhibit anandamide metabolism. Neuron survival was assessed 24 h later. EC_{50} is the concentration reducing by 50% the cell death caused by glutamate. Values are means ± SD from at least three experiments. Anandamide was used at 10 μM. ND, not determined.
*$P < 0.01$ vs. control.

When granule cell incubation with anandamide (50 μM) was prolonged beyond 1 h in the presence of 150 μM PMSF, an inhibitor of anandamide amidase (39), neuron survival was reduced to 30% ± 12% ($n = 6$) of control 24 h later. The potent cannabimimetic compound HU-210 reportedly is cytotoxic for neurons *in vitro*, an effect attributed to its interaction with the CB1 receptor (40). Active cannabinoids, but not cannabidiol, were also cytotoxic to granule cells when exposure to high (≥25 μM) concentrations exceeded 60 min: 50 μM nabilone or Δ^8-THC decreased survival by 80–85% after 24 h. Incubation of granule neurons with 100 μM palmitoylethanolamide or its glucosyl derivative for 24 h did not affect cell vitality.

Palmitoylethanolamide and Cannabinoids Do Not Antagonize EAA Receptor Function in Intact Neurons. The delayed postglutamate neuroprotection afforded by saturated *N*-acylethanolamides and cannabinoids (Table 1) made unlikely a direct effect at the EAA receptor level, as confirmed here. The delayed component of kainic acid (KA)-mediated neuronal death involving free radical production especially evident under conditions that eliminate the occurrence of acute ion-dependent neuronal swelling and cell lysis (40) was investigated. When mature granule cells were treated with 500 μM KA for 30 min in a Na^+-free buffer, the 75% loss seen 2 h later was, as expected (41), largely prevented by CNQX or the antioxidant butylated hydroxytoluene (Table 2). In contrast, neither palmitoylethanolamide nor cannabinoids were neuroprotective when present during and after KA exposure in these conditions (Table 2). Palmitoylethanolamide and CNQX, but not butylated hydroxytoluene, were cytoprotective for granule cells incubated with 500 μM KA in Na^+-containing buffer, where rapid lysis of ≈50% of the neurons occurs during the 30-min drug exposure period (42). Thus, a KA receptor antagonist (CNQX) was able to reduce both components of KA receptor-triggered neuronal injury while the *N*-acylethanolamide affected only the excitotoxic branch, indicating that the latter operates independently of the EAA receptor.

Cerebellar granule cell development *in vitro* is strictly dependent on Ca^{2+} influx, mediated by either voltage- or NMDA receptor-gated channels (43, 44). Predictably, MK-801 negated

Table 2. Palmitoylethanolamide and cannabinoids do not interfere with KA receptor activity in cerebellar granule cells in the absence of Na^+

Culture conditions	% survival
KA	25.6 ± 3.1
KA + CNQX	87.3 ± 11.1
KA + BHT	84.6 ± 10.0
KA + PEA	35.0 ± 4.2
KA + nabilone	36.2 ± 4.9

Nine-day-old cultures were incubated for 30 min (27°C) in Na^+-free Locke's solution containing 500 μM KA and one of the following: 10 μM CNQX, 100 μM butylated hydroxytoluene (BHT), 100 μM palmitoylethanolamide (PEA), 10 μM nabilone, or 3 μM 11OH-Δ^9-THC. Cultures were then washed free of KA and incubated 2 h more (27°C) in Na^+-free Locke's solution with the indicated compound. PMSF (150 μM) was present during all drug incubations. Cell survival was quantified at the end of the 2 h. Values are means ± SD (three experiments).

the trophic effect of NMDA for granule cells cultured 7 days in medium containing a reduced concentration (5 mM) of KCl. In contrast, the presence of either glucosylpalmitoylethanolamide or 11OH-Δ^9-THC throughout the same 7-day period (in place of MK-801) did not interfere with the NMDA trophic action (Table 3). The latter cannabimimetic was chosen because of its minimal long-term cytotoxicity. Palmitoylethanolamide was not tested here, because of the deleterious influence of long-term cell exposure to high solvent concentrations.

Cerebellar Granule Cells and Cerebellum Express the Genes Encoding Cannabinoid Receptors CB1 and CB2. The observed effects of cannabinoids and palmitoylethanolamide in downmodulating EAA neurotoxicity, and their antagonism by anandamide, prompted the question of whether cerebellar granule cells express cannabinoid receptor gene transcripts. *In situ* hybridization of 8 DIV granule cell monolayers using CB1- and CB2-specific oligonucleotide probes revealed the presence of mRNA for both receptor types in neuronal perikarya (Fig. 2 *A–C*). The CB1 and CB2 mRNAs were detectable in the majority of granule cells examined, suggesting the same neuron to be capable of simultaneously expressing the two known cannabinoid receptor subtypes. It was not possible, however, to ascertain the relative abundance of the two mRNAs. Sections of mouse cerebellum, when hybridized with CB1- and CB2-specific probes, also revealed the presence of the corresponding mRNAs in the granule cell layer (Fig. 2 *A′–C′*). The Purkinje cell layer, while producing a positive hybridization signal for CB2, was negative for CB1, indicating the ability of this *in situ* procedure to discriminate between different subsets of neurons in the same tissue section.

Cerebellum Contains Two Cannabinoid Binding Sites. Radioligand binding experiments demonstrated specific binding

Table 3. Glucosylpalmitoylethanolamide and cannabinoids do not interfere with NMDA receptor function in developing cerebellar granule cells

Culture conditions	% survival
K25	100
K5 plus	
None	31.6 ± 3.8
NMDA	69.6 ± 6.1
NMDA + MK-801	33.3 ± 4.2
NMDA + glucosyl-PEA	69.5 ± 1.5
NMDA + 11OH-Δ^9-THC	70.3 ± 2.9

Cultures were incubated from days 1–8 in medium containing 25 mM KCl (K25) or 5 mM KCl (K5) and the indicated additions: NMDA, 150 μM; MK-801, 10 μM; glucosylpalmitoylethanolamide (glucosyl-PEA), 50 μM; 11OH-Δ^9-THC, 3 μM. The last two compounds were resupplied daily. Cell survival was quantified at the end of the eighth day. Values are means ± SD (three experiments).

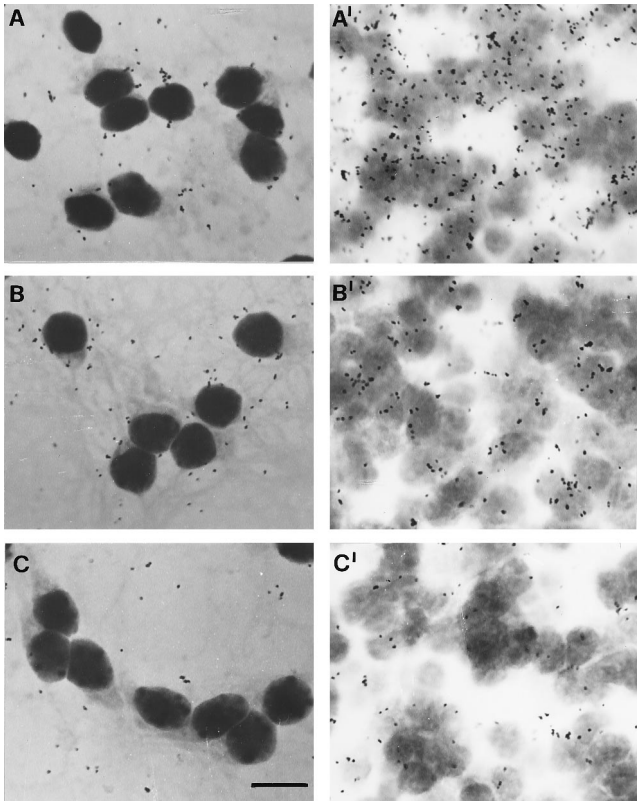

FIG. 2. *In situ* hybridization of cannabinoid receptor mRNA in cultured mouse cerebellar granule cells (*A–C*) and 4-week cerebellum (*A′–C′*). (*A* and *A′*) CB1. (*B* and *B′*) CB2. (*C* and *C′*) Random. (Bar = 10 μm.)

of [^{3}H]WIN 55,212–2 to membranes from mouse cerebellum, with two apparent binding sites. The Scatchard (Rosenthal) plot is shown in Fig. 3. Values for K_{d1} of 1.6 ± 1.0 nM and K_{d2} of 11.0 ± 1.5 nM, with corresponding B_{max} of 0.7 ± 0.1 and 3.2 ± 0.3 pmol per mg of protein, respectively, were obtained by analyzing the data according to Rosenthal (45) and using the FIG. P computer program for a two binding site fitting (BIOSOFT, Cambridge, U.K.). Prior studies of ligand binding to cannabinoid receptors in adult rat brain have described a single

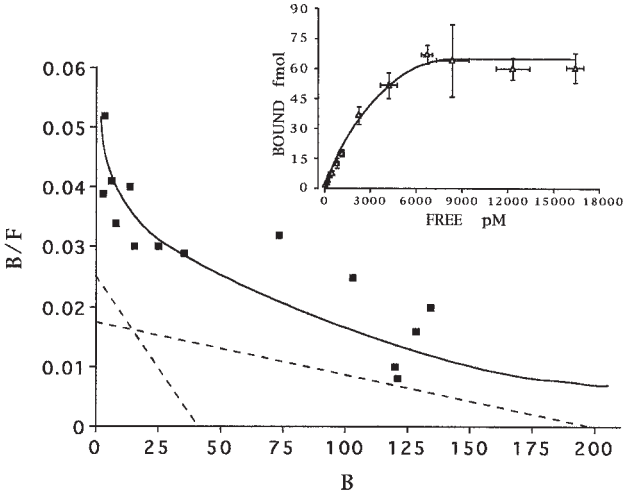

FIG. 3. Scatchard plot of specific [^{3}H]WIN 55,212–2 binding to cerebellar membranes. Specific binding was defined as the difference between binding that occurred in the presence and absence of 1 μM nonradioactive ligand. (*Inset*) Saturation isotherm. Data are means ± SD (seven experiments). B, bound (pM); F, free.

binding site (31, 44). In these latter experiments, however, steps were taken (preincubation and washing) that were reported necessary for observing a homogeneous binding site (31), or that may have masked the presence of a higher affinity site by the use of albumin (46). In initial trials anandamide fully displaced bound radioligand, while palmitoylethanolamide seemed to be only partially effective (data not shown). Given that WIN 55,212–2 has similar affinities for CB1 and CB2 (18, 47), it is difficult to assign a site to palmitoylethanolamide, although it is reported to not bind CB1 (22, 23).

DISCUSSION

The experiments described here assign a functional correlate to the naturally occurring saturated *N*-acylamide, palmitoylethanolamide, in central neurons. We have demonstrated that (*i*) palmitoylethanolamide and cannabinoids, but not the unsaturated *N*-acylamide anandamide, downmodulate in a delayed postagonist manner the toxic consequences of EAA receptor activation in cultured cerebellar granule cells; (*ii*) anandamide antagonizes the neuroprotection afforded by palmitoylethanolamide and cannabinoids; (*iii*) granule cells express the genes for CB1 and CB2; and (*iv*) [^{3}H]WIN 55,212–2 binding to cerebellar membranes displays two different binding affinities, suggesting the presence of at least two binding sites. Because palmitoylethanolamide, but not anandamide, appears to behave as an endogenous agonist for the CB2 receptor on mast cells and downregulates their activation *in vitro* (19), the present findings suggest that a CB2-like receptor may also exert a negative regulatory effect on postglutamate receptor events following excessive excitatory stimulation.

Palmitoylethanolamide, unlike anandamide, is reported to not bind the CB1 receptor (22, 23). Anandamide, by binding to brain CB1 (22, 23), produces many of the behavioral and physiological responses of cannabinoids (47). The psychotropic effects of cannabinoids are presumably mediated via activation of the brain CB1 receptor (31), which is a typical member of the G-protein-coupled superfamily of receptors (20, 21). Cultured granule cells and cerebellum expressed mRNA for both CB1 and CB2 by *in situ* hybridization but other receptor variants could exist. The binding data with cerebellar membranes are consistent with the presence of two binding sites with affinities close to those published for CB1 and CB2 (18, 47). Although CB2 receptor identification until now has been limited to peripheral tissues (18, 19, 48), a detailed study of its expression or that of other cannabinoid receptor forms in different brain areas as a function of species/development is lacking. G-protein-linked cannabinoid receptors have been described to be present in rat cerebellar granule cells *in vitro* (49), although receptor subtypes were not evaluated. Neuronal expression of CB2 could be regulated by culture conditions, or *in vivo* by injury, as in the case of some EAA receptor subtypes *in vitro* in cerebellar granule cells (50) and after cerebral ischemia (51). Palmitoylethanolamide, but not anandamide, was neuroprotective for granule cells; anandamide actually antagonized the protective action of the former. We recently reported that anandamide binds to the CB2 receptor on mast cells and antagonizes the ability of palmitoylethanolamide and cannabinoids to inhibit mediator release (19). Anandamide also inhibited specific [^{3}H]WIN 55,212–2 binding to mast cell membranes (19), suggesting it to behave as a functional antagonist at least for CB2 on mast cells. Such opposite behavior is typical of differences in the agonistic ability of receptor ligands in cerebellar granule cells and, in analogy with mast cells (19), suggests that the two *N*-acylethanolamides have differing roles for different cannabinoid receptors. Interestingly, cannabinoid receptor activation may inhibit the presynaptic release of glutamate via an inhibitory G protein in hippocampal neurons (52), suggesting a presynaptic CB1 location, thus further distinguishing the present delayed postagonist rescue effects from a CB1-mediated process. It is tempting to speculate that saturated (ALIAmides) and unsaturated (anandamides) long-chain fatty acid ethanolamides may be cannabinoid receptor type-specific endogenous agonists.

The ability of glucosylpalmitoylethanolamide to prevent excitotoxic neuronal injury in a delayed postagonist setting, and with greater efficacy than the parent molecule, may be attributable to one or several possibilities. Although modified solubility may be a factor, metabolism or transformation of the glucosyl derivative to palmitoylethanolamide or a related *N*-acylamide needs to be considered. This avenue is currently being explored. While the glucosylacylamide exhibited a pharmacological profile not unlike that of the starting compound, it is presently not possible to draw conclusions as to whether or not the molecule *per se* is recognized by cannabinoid receptors.

The observed neuroprotective effects of palmitoylethanolamide and cannabinoids probably did not result from interference with EAA receptor function. These compounds, in contrast to NMDA antagonists, were only modestly protective when added concurrently with glutamate, but they became more efficacious with increasing delay of postagonist introduction. Saturated *N*-acylethanolamides and cannabinoids, however, reduced neither neuronal injury caused by kainate receptor-induced oxidative stress nor neuronal survival promoted by NMDA receptor stimulation in immature cerebellar granule cells. As expected, the latter two processes were sensitive to kainate and NMDA receptor-specific antagonists, respectively. Moreover, palmitoylethanolamide and cannabinoids prevented neurotoxicity triggered by NMDA or by kainate under conditions favoring excitotoxicity over free radical toxicity. The nonpsychotropic cannabinoid HU-211 attenuated NMDA receptor-mediated toxicity to cultured cortical neurons, apparently by binding to NMDA receptors (40). HU-211 was most effective when coapplied with EAA agonist (40). Antagonists of NMDA and kainate/AMPA receptors, Ca^{2+} channel blockers, free radical scavengers, inhibitors of protein and RNA synthesis, phosphatases and nitric oxide synthase, and monosialogangliosides all failed to show a delayed postagonist neuroprotective action (unpublished observations). The nonspecific endonuclease inhibitor ATA attenuated glutamate neurotoxicity in cotreatment but not when added 15-min postglutamate, in difference to a recent report of delayed ATA protection for cortical neurons (35). The *N*-acylamide and cannabinoid protective effects in the delayed postagonist paradigm required only a limited exposure window, suggesting these compounds to interfere with one or more downstream consequences of excitotoxic glutamate receptor overaction.

Given its clinical relevance, much effort has been directed to devising pharmacological means of preventing or reducing the neuropathological consequences of exaggerated EAA receptor stimulation, mainly by antagonizing receptor activation, blocking receptor- and voltage-gated ion channels, inhibiting EAA release, or by interfering with postreceptor processes (53–55). An alternative strategy to mitigate excitotoxic neuronal death would be to identify natural pathways whose activation downregulates the deleterious outcome of toxic or noxious stimuli. In this respect, it is of more than passing interest that EAAs themselves can induce brain neurons *in vitro* to produce palmitoylethanolamide and other *N*-acylethanolamides (15, 16). Conceivably, certain of these molecules might play a role in modulating cellular defense mechanisms by acting at non-CB1 cannabinoid receptors, much as they do in the case of mast cells (19). By providing the neuron with exogenous palmitoylethanolamide (or semisynthetic saturated *N*-acylethanolamides), one might be making available quantities of its physiological modulator sufficient to restore cellular homeostasis in the face of an excitotoxic challenge. This pharmacologic approach has recently found application in the ability of palmitoylethanolamide to control

mast cell activation by a local autacoid antiinflammatory mechanism, hence the term ALIA (19, 56, 57). Palmitoylethanolamide, in reality, appears to exert a more broad local autacoid antiinjury function, thus the acronym autacoid local injury antagonism for ALIA. Therapeutic implications of this mechanism include the development of innovative neuroprotective drugs for central nervous system injury.

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1. Cotman, C. W., Monaghan, D. T., Offersen, O. P. & Storm-Mathisen, J. (1987) *Trends Neurosci.* **10,** 273–280.
2. Gasic, G. P. & Hollmann, M. (1992) *Annu. Rev. Physiol.* **54,** 507–536.
3. Collingridge, G. & Bliss, T. V. P. (1987) *Trends Neurosci.* **10,** 288–294.
4. Lipton, S. A. & Kater, S. B. (1989) *Trends Neurosci.* **12,** 265–270.
5. Choi, D. W. (1988) *Neuron* **1,** 623–634.
6. Choi, D. W. & Rothman, S. M. (1990) *Annu. Rev. Neurosci.* **13,** 171–182.
7. DiFiglia, M. (1990) *Trends Neurosci.* **13,** 286–289.
8. Meldrum, B. & Garthwaite, J. (1990) *Trends Pharmacol. Sci.* **11,** 379–387.
9. Lipton, S. A. & Rosenberg, P. A. (1994) *N. Engl. J. Med.* **330,** 613–622.
10. Koh, J. Y., Goldberg, M. P., Hartley, D. M. & Choi, D. W. (1990) *J. Neurosci.* **10,** 693–705.
11. Sheardown, M. J., Nielsen, E. O., Hansen, A. J., Jacobsen, P. & Honóre, T. (1990) *Science* **247,** 571–574.
12. Schmid, H. H. O., Schmid, P. C. & Natarajan, V. (1990) *Prog. Lipid Res.* **29,** 1–43.
13. Natarajan, V., Schmid, P. C. & Schmid, H. H. O. (1986) *Biochim. Biophys. Acta* **878,** 32–41.
14. Epps, D. E., Natarajan, V., Schmid, P. C. & Schmid, H. H. O. (1980) *Biochim. Biophys. Acta* **618,** 420–430.
15. Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J.-C. & Piomelli, D. (1994) *Nature (London)* **372,** 686–691.
16. Hansen, H. A., Lauritzen, L., Strand, A. M., Moesgaard, B. & Frandsen, A. (1995) *Biochim. Biophys. Acta* **1258,** 303–308.
17. Hollister, L. E. (1986) *Pharmacol. Rev.* **38,** 1–20.
18. Munro, S., Thomas, K. L. & Abu-Shaar, M. (1993) *Nature (London)* **365,** 61–65.
19. Facci, L., Dal Toso, R., Romanello, S., Buriani, A., Skaper, S. D. & Leon, A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3376–3380.
20. Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C. & Bonner, T. I. (1990) *Nature (London)* **346,** 561–564.
21. Gérard, C. M., Mollereau, C., Vassart, G. & Parmentier, M. (1991) *Biochem. J.* **279,** 129–134.
22. Devane, W. A., Hanuš, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A. & Mechoulam, R. (1992) *Science* **258,** 1946–1949.
23. Felder, C. C., Briley, E. M., Axelrod, J., Simpson, J. T., Mackie, K. & Devane, W. A. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 7656–7660.
24. Skaper, S. D., Facci, L., Milani, D., Leon, A. & Toffano, G. (1990) in *Methods in Neurosciences*, ed. Conn, P. M. (Academic, San Diego), Vol. 2, pp. 17–33.
25. Levi, G., Aloisi, F., Ciotti, M. T. & Gallo, V. (1984) *Brain Res.* **290,** 77–86.
26. Mossmann, T. (1983) *J. Immunol. Methods* **65,** 55–63.
27. Manthorpe, M., Fagnani, R., Skaper, S. D. & Varon, S. (1986) *Dev. Brain Res.* **25,** 191–198.
28. Ohsawa, F., Widmer, H. R., Knusel, B., Denton, T. L. & Hefti, F. (1993) *Neuroscience* **57,** 67–77.
29. Patel, J., Zinkand, W. C., Thompson, C., Keith, R. & Salama, A. (1990) *J. Neurochem.* **54,** 849–854.
30. Cosi, C., Spoerri, P. E., Comelli, C., Guidolin, D. & Skaper, S. D. (1993) *NeuroReport* **4,** 527–530.
31. Devane, W. A., Dysarz, F. A., III, Johnson, M. R., Melvin, L. S. & Howlett, A. C. (1988) *Mol. Pharmacol.* **34,** 605–613.
32. Watkins, J. C. & Olverman, H. J. (1987) *Trends Neurosci.* **10,** 265–272.
33. Choi, D. W., Koh, J.-Y. & Peters, S. (1988) *J. Neurosci.* **8,** 185–196.
34. Hartley, D. M. & Choi, D. W. (1989) *J. Pharmacol. Exp. Ther.* **250,** 752–758.
35. Csernansky, C. A., Canzoniero, L. M. T., Sensi, S. L., Yu, S. P. & Choi, D. W. (1994) *J. Neurosci. Res.* **38,** 101–108.
36. Wong, E. H., Kemp, J. A., Priestley, T., Knight, A. R., Woodruff, G. N. & Iversen, L. L. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 7104–7108.
37. Crawley, J. N., Corwin, R. L., Robinson, J. K., Felder, C. C., Devane, W. A. & Axelrod, J. (1993) *Pharmacol. Biochem. Behav.* **46,** 967–972.
38. Smith, P. B., Compton, D. R., Welch, S. P., Razdan, R. K., Mechoulam, R. & Martin, B. R. (1994) *J. Pharmacol. Exp. Ther.* **270,** 219–227.
39. Deutsch, D. G. & Chin, S. (1993) *Biochem. Pharmacol.* **46,** 791–796.
40. Nadler, V., Mechoulam, R. & Sokolovsky, M. (1993) *Neurosci. Lett.* **162,** 43–45.
41. Puttfarcken, P. S., Getz, R. L. & Coyle, J. T. (1993) *Brain Res.* **624,** 223–232.
42. Kato, K., Puttfarcken, P. S., Lyons, W. E. & Coyle, J. T. (1991) *J. Pharmacol. Exp. Ther.* **256,** 402–411.
43. Gallo, V., Kingsbury, A., Balázs, R. & Jørgensen, O. S. (1987) *J. Neurosci.* **7,** 2203–2213.
44. Balázs, R., Jorgensen, O. S. & Hack, N. (1988) *Neuroscience* **27,** 437–451.
45. Rosenthal, H. E. (1967) *Anal. Biochem.* **20,** 525–532.
46. Hillard, C. J., Edgemond, W. S. & Campbell, W. B. (1995) *J. Neurochem.* **64,** 677–683.
47. Howlett, A. C. (1995) *Annu. Rev. Pharmacol. Toxicol.* **35,** 607–634.
48. Lynn, A. B. & Herkenham, M. (1994) *J. Pharmacol. Exp. Ther.* **268,** 1612–1623.
49. Pacheco, M. A., Ward, S. J. & Childers, S. R. (1993) *Brain Res.* **603,** 102–110.
50. Aronica, E., Dell'Albani, P., Condorelli, D. F., Nicoletti, F., Hack, N. & Balázs, R. (1993) *Mol. Pharmacol.* **44,** 981–989.
51. Paschen, W., Schmitt, J. & Uto, A. (1995) *Soc. Neurosci. Abstr.* **21,** 765.
52. Shen, M., Piser, T. M. & Thayer, S. A. (1995) *Soc. Neurosci. Abstr.* **21,** 1569.
53. Lipton, S. A. (1993) *Trends Neurosci.* **16,** 527–532.
54. Choi, D. W. (1990) *Cerebrovasc. Brain Metab. Rev.* **2,** 105–147.
55. Mercanti, D., Galli, C., Liguori, M., Ciotti, M. T., Gullà, P. & Calissano, P. (1992) *Eur. J. Neurosci.* **4,** 733–744.
56. Aloe, L., Leon, A. & Levi-Montalcini, R. (1993) *Agents Actions* **39,** C145–C147.
57. Mazzari, S., Canella, R., Petrelli, L., Marcolongo, G. & Leon, A. (1996) *Eur. J. Pharmacol.*, in press.