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Anandamide- and Δ^9-Tetrahydrocannabinol-Evoked Arachidonic Acid Mobilization and Blockade by SR141716A [*N*-(Piperidin-1-yl)-5-(4-Chlorophenyl)-1-(2,4-Dichlorophenyl)-4-Methyl-1*H*-Pyrazole-3-Carboximide Hydrochloride]*

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ABSTRACT. The purpose of this study was to investigate whether anandamide induces cannabimimetic responses, mainly mobilization of arachidonic acid, in primary cultures of rat brain cortical astrocytes. Confluent monolayer cultures of astrocytes, prelabeled with [^{3}H]arachidonic acid, were incubated with anandamide or Δ^9-tetrahydrocannabinol (Δ^9-THC) in the presence or absence of thimerosal, a fatty acyl CoA transferase inhibitor and phenylmethylsulfonyl fluoride, an amidohydrolase inhibitor. Anandamide and Δ^9-THC induced a time- and concentration-dependent release of arachidonic acid in the presence, but not in the absence, of thimerosal. Anandamide- and Δ^9-THC-stimulated arachidonic acid release was pertussis toxin-sensitive, indicating a receptor/G-protein involvement. A novel and selective cannabinoid receptor antagonist, SR141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride], blocked the arachidonic acid release, suggesting a cannabinoid receptor-mediated pathway. In astrocytes, the magnitude of anandamide-induced arachidonic acid release was equal to that released by equimolar concentrations of Δ^9-THC. Furthermore, direct assay of amidohydrolase activity indicated that degradation of anandamide into arachidonic acid and ethanolamine was negligible in cortical astrocytes. Our results suggest that anandamide stimulates receptor-mediated release of arachidonic acid, and the receptor may be the cannabinoid receptor. Astrocytes, containing a cannabinoid receptor and lower or negligible amidohydrolase activity, may be an important brain cell model in which to study the cannabimimetic effects of anandamide at a cellular and molecular level. BIOCHEM PHARMACOL 51;5:669–676, 1996.

KEY WORDS. anandamide; astrocytes; arachidonic acid; cannabinoid receptor antagonist; Δ^9-tetrahydrocannabinol; amidohydrolase activity

The psychoactive marijuana plant-derived cannabinoid Δ^9-THC‡ and other synthetic analogs have been shown to stimulate arachidonic acid release and the subsequent generation of biologically active eicosanoids in peripheral tissues [1–5] and in the brain [6]. Δ^9-THC-induced arachidonic acid release is shown to be the result of a receptor/G-protein-mediated activation of phospholipases [7, 8].

Anandamide, an ethanolamine derivative of arachidonic acid, is a naturally occurring endogenous ligand for the brain cannabinoid receptor [9]. Anandamide shares many pharmacological and biochemical actions of Δ^9-THC such as hypothermia, hypoactivity, and analgesia [10, 11], inhibition of adenylyl cyclase activity [12], N-type calcium current [13], and hormone release [14]. Anandamide has been shown recently to activate cytoplasmic phospholipase A_2 (cPLA$_2$) and eicosanoid production via the mitogen-activated protein (MAP) kinase signal transduction pathway [15]. Thus far, no direct evidence is available to demonstrate that anandamide- or Δ^9-THC-induced arachidonic acid release is cannabinoid receptor mediated. Moreover, the actions of anandamide are difficult to study due to its rapid degradation to arachidonic acid and ethanolamine by an amidohydrolase activity [16]. We report herein the ability of anandamide to stimulate arachidonic acid mobilization in primary cultures of rat brain cortical astrocytes with a potency equal to that of Δ^9-THC. The arachidonic acid release is pertussis toxin-sensitive and blocked by SR141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride], a novel antagonist for the brain cannabinoid receptor [17]. Furthermore, direct measurement of amidohydrolase activity revealed that cortical astrocytes contain lower or neg-

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‡ *Abbreviations:* CB1, cannabinoid receptor subtype 1; DMEM, Dulbecco's modified Eagle's medium; PMSF, phenylmethylsulfonyl fluoride; RP-HPLC, reverse-phase high performance liquid chromatography; and Δ^9-THC, Δ^9-tetrahydrocannabinol.

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ligible activity compared with the activity in the liver, brain hippocampus, or brain cortex.

MATERIALS AND METHODS

Materials

DMEM and other supplies were obtained from GIBCO (Grand Island, NY). Fetal bovine serum was obtained from Hyclone (Logan, VT). [^{3}H]Arachidonic acid and [^{3}H]anandamide (arachidonyl 5,6,8,9,11,12,14,15-^{3}H) were obtained from New England Nuclear (Boston, MA). Thimerosal and PMSF were obtained from the Sigma Chemical Co. (St. Louis, MO). Unlabeled anandamide and Δ^9-tetrahydrocannabinol were obtained from Organix, Inc. (Cambridge, MA) and the National Institute on Drug Abuse (Bethesda, MD), respectively. Pertussis toxin was obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). All solvents were HPLC-grade and obtained from Burdick & Jackson Laboratories (Muskegon, MI).

Cell Culture

Primary cultures of rat cortical astrocytes were grown in 75 cm^2 flasks, and the purity of the astrocytes was assayed immunocytochemically by staining for glial fibrillary acidic protein as described previously [18].

Drug Solution Preparation

Stock solutions of Δ^9-THC, anandamide, and SR141716A (a gift from Dr. John A. Lowe) were prepared in absolute ethanol (1 μg/μL), and the final ethanol concentration in the incubation medium was always less than 0.15%. The stock solution (1 mg/mL) of PMSF was prepared in absolute ethanol; however, for complete solubilization the stock solution was maintained at 37° before addition to the incubation medium. The stock solution of thimerosal (404 μg/100 μL) was prepared in DMEM. All drug stock solutions were stored at −20°.

Cell Labeling and Stimulation

Confluent, 20- to 25-day-old, astrocyte monolayer cultures (5×10^6 cells/75 cm^2 flask, ≈500 μg protein) were incubated overnight with [^{3}H]arachidonic acid (0.5 μCi/mL; 3.7 TBq/mmol) in DMEM, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, in a sterile atmosphere of 95% O_2 and 5% CO_2. In some cases, pertussis toxin (100 ng/mL) was added during the last 2-hr labeling period with [^{3}H]arachidonic acid. The monolayer cultures were washed twice with DMEM to remove any unincorporated radioactivity and were preincubated for 10 min with fresh DMEM with or without thimerosal (100 μM) or PMSF (1 mM). Thimerosal is a known inhibitor of acyl CoA:acyl lysophospholipid transferase and inhibits reincorporation of released arachidonic acid to membrane phospholipids [19]. PMSF is known to inhibit amidohydrolase degradation of anandamide [16]. PMSF was added in combination with thimerosal only to those cells stimulated by anandamide. After preincubation, the medium was replaced by fresh DMEM, and cells were stimulated for 20 min at 37° with Δ^9-THC (1 μM) or anandamide (1 μM) with or without the inhibitors. Aliquots of the medium were taken at different time intervals for counting radioactivity by liquid scintillation spectrometry. After the incubation, radioactivity released into the medium was extracted as described previously [20]. The lipid residues were stored under N_2 at −70° until further analysis by HPLC.

Labeled cells were stimulated with various concentrations of Δ^9-THC or anandamide for 20 min at 37°. Pertussis toxin (100 ng/mL) treated or untreated labeled cells were incubated with or without anandamide or Δ^9-THC for 20 min. Control cells were incubated with either thimerosal, pertussis toxin, or thimerosal plus pertussis toxin. In some experiments, labeled cells were incubated with thimerosal (100 μM) and different concentrations of SR141716A, a novel antagonist selective for the brain cannabinoid receptor subtype 1 (CB1) [17], before incubating with either Δ^9-THC or anandamide. Control cells were incubated with vehicle (10 μL ethanol) or 10 μM SR141716A, or thimerosal plus SR141716A.

Anandamide Metabolism Studies

To examine whether anandamide undergoes metabolism by astrocyte amidohydrolase activity, intact cells were incubated with [^{3}H]anandamide (1 μM; 0.3 μCi/mL), labeled at the arachidonic acid moiety (5,6,8,9,11,12,14,15-^{3}H-; 0.2 μCi/mL; 8.2 TBq/mmol), for 20 min in the presence or absence of PMSF (1 mM). At the end of the incubation period, medium aliquots and cell lysate were counted for radioactivity. The medium-associated labeled lipids were extracted with 2 vol. of ethyl acetate. The organic layer was separated, and the lower aqueous phase was reextracted once with ethyl acetate. The organic layers were combined and evaporated to dryness under N_2; the lipid residues were resuspended in ethanol (200 μL) and stored under N_2 at −70° until further analysis by HPLC.

HPLC Analysis

Separation of radioactive products in the medium lipid extract was performed using a Waters Associates HPLC, equipped with a high pressure pump (model 510), a Waters Automated Gradient Controller, and a Waters Guard-pak precolumn module connected to a 150 mm × 4.6 mm (i.d.) Absorbosphere C_{18}, 5 μm column (Alltech Co., Deerfield, IL). The lipid residue (200–400 μL in ethanol) was loaded onto the column and eluted using an isocratic gradient of methanol:water:acetic acid (80:15:0.1, by vol.) for 30 min at a flow rate of 1 mL/min. Elution of radioactivity was monitored by diverting a 0.5-mL/min portion of the eluate to a flow-through Raytest radioactive detector-Ramona 90 (Fairfield, NJ). Similarly, the labeled arachidonic acid, released into the medium after treatment with anandamide or Δ^9-THC, was analyzed by reverse-

phase HPLC using the solvent gradient system as described previously [18].

Amidohydrolase Assay

Amidohydrolase activity was measured in various tissue homogenates using [^{3}H]anandamide (labeled at the arachidonyl moiety) as substrate by the procedure described previously [16, 21] with slight modifications. Crude homogenates of freshly isolated rat liver, brain cortex, hippocampus, or primary cultures of cortical astrocytes were hand-homogenized in cold buffer (100 mM Tris–HCl, 0.2 mM EDTA, and 1.15% KCl buffer, pH 7.5). The homogenate (1 mg protein/mL) was preincubated for 10 min at 37° with or without 1.0 mM PMSF and then incubated for 20 min with 10 μM [^{3}H]anandamide (0.2 μCi/mL). Buffer controls were incubated with BSA (1 mg/mL) for any nonenzymatic degradation of anandamide under the incubation conditions. After the incubation, the labeled lipids were extracted with 2 vol. of acidified ethyl acetate. The lipid residues were loaded to TLC plates (20 × 20 cm), developed by using the organic layer of ethyl acetate: hexane:acetic acid:water (100:50:20:100, by vol.), and the lipid bands were visualized by brief exposure to iodine vapor. The lipid bands, comigrated with anandamide and arachidonic acid, were scraped and counted for radioactivity. The formation of radioactive arachidonic acid was calculated as percent recovered radioactivity for the measurement of the amidohydrolase activity.

Effect of Thimerosal on CP 55,940 Binding Assay

Radioligand binding of [^{3}H]CP 55,940 (10 nM) to brain membrane fraction was done using the procedure described by Compton *et al.* [22] in the presence or absence of 100 μM thimerosal.

Statistical Data Analysis

Values are means ± SEM for at least 3 experiments unless otherwise indicated. One-factor analysis of variance was used for statistical comparisons. Fisher's PLSD post-hoc analysis was used for pairwise comparisons between treatment groups. Differences were considered to be significant at $P \leq 0.05$.

RESULTS

Anandamide and Δ^9-THC Stimulation of [^{3}H]Arachidonic Acid Release

Both anandamide and Δ^9-THC stimulated [^{3}H]arachidonic acid release in the presence of the acyl CoA transferase inhibitor thimerosal (100 μM). Figure 1A shows the nonspecific release of radioactivity in control cells incubated for 20 min with either vehicle (10 μL ethanol), PMSF (1 mM) or

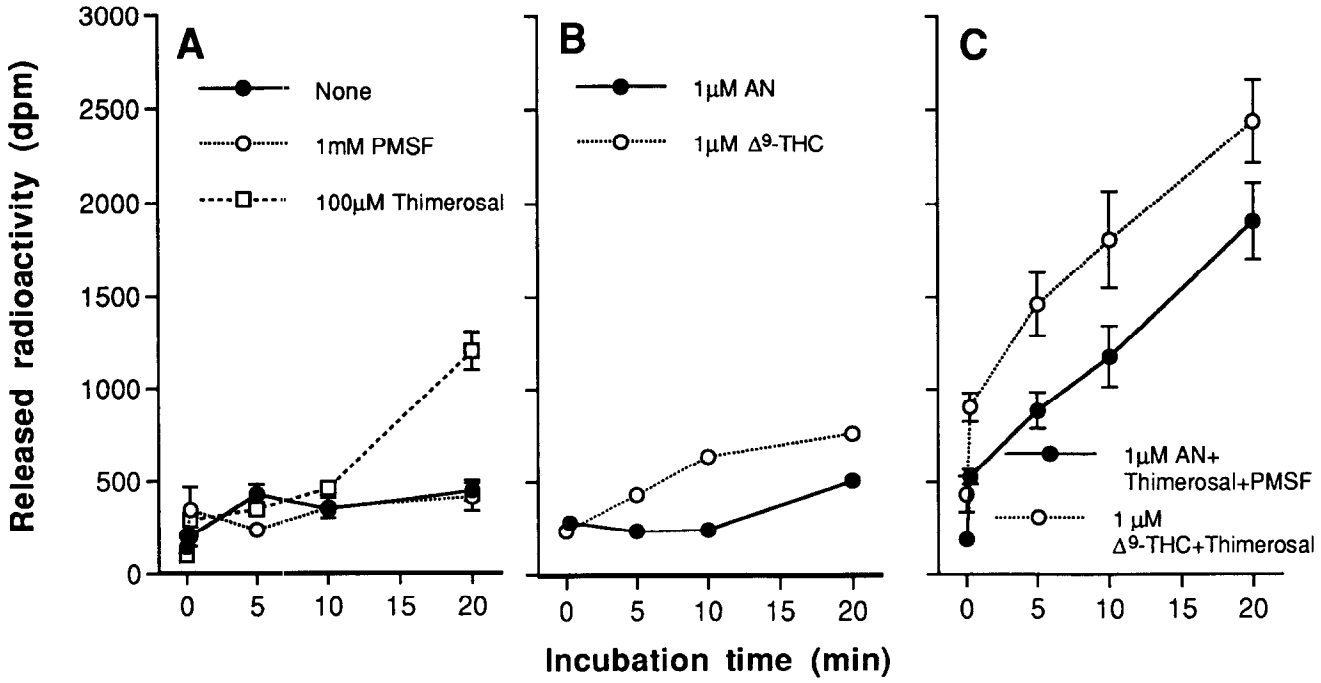

FIG. 1. Time course of Δ^9-THC- and anandamide-evoked arachidonic acid mobilization in primary cultures of astrocytes. Confluent monolayers of rat brain cortical astrocytes (5 × 10^6 cells) were prelabeled overnight with [^{3}H]arachidonic acid (0.5 μCi/mL; 100 Ci/mmol). The washed cells were preincubated for 10 min in serum-free DMEM with or without thimerosal or PMSF or thimerosal + PMSF. After preincubation, cells were stimulated for up to 20 min with various agents, and the released radioactivity in the medium was counted by liquid scintillation spectrometry. Panel A shows the nonspecific release of arachidonic acid when astrocytes were treated with ethanol vehicle, 100 μM thimerosal, or 1 mM PMSF. Values are means ± SEM, N = 3 independent experiments. Panel B shows arachidonic acid release by Δ^9-THC or anandamide in the absence of thimerosal or PMSF. Panel C shows arachidonic acid release by Δ^9-THC and anandamide in thimerosal- or thimerosal + PMSF-pretreated astrocytes. Values are means ± SEM, N = 6 independent experiments.

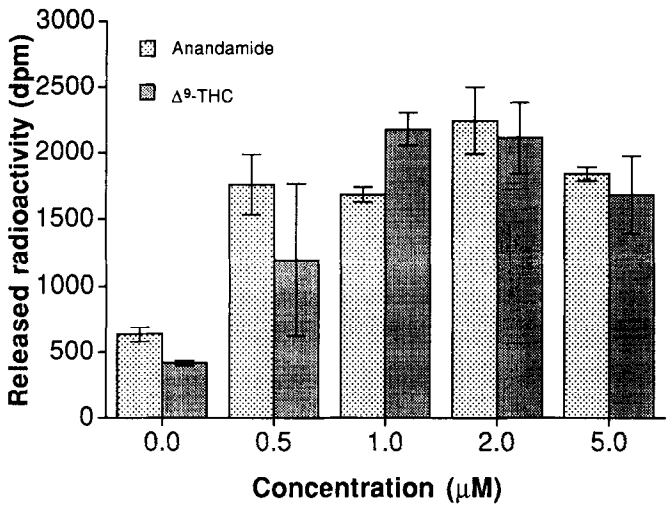

FIG. 2. Effect of various concentrations of anandamide and Δ^9-THC on arachidonic acid release from astrocytes. The [^{3}H]arachidonic acid-labeled astrocytes were stimulated with various concentrations of Δ^9-THC or anandamide in the presence of thimerosal or thimerosal + PMSF, respectively. Control cells were incubated with only thimerosal or thimerosal + PMSF. Values are means ± SEM, N = 3 independent experiments.

thimerosal (100 μM). While the basal level of medium radioactivity remained unchanged in vehicle control and PMSF-treated cells, a higher basal level was observed for thimerosal-treated cells, especially at 20 min. Addition of PMSF in combination with thimerosal did not have any additive effect on the release of arachidonic acid and was omitted from Fig. 1A. Trypan blue dye uptake studies revealed that incubation of cells with 1 mM PMSF or 100 μM thimerosal produced no cell death and no lifting of the cell monolayer. In the absence of PMSF and thimerosal, anandamide and Δ^9-THC induced only a small release of radioactivity (Fig. 1B). In contrast, 1 μM Δ^9-THC induced a time-dependent release of radioactivity from cells pretreated with 100 μM thimerosal (Fig. 1C), and it was 2-fold higher than the thimerosal controls. Similarly, 1 μM anandamide released arachidonic acid from astrocytes in the presence of thimerosal and PMSF; however, the response was slightly lower than that of Δ^9-THC. Greater than 85% of released radioactivity was eluted as a major HPLC peak at the retention time of arachidonic acid, and a small portion of the radioactivity (<15%) was eluted as minor peaks that were not identified in this study (data not shown). To determine whether thimerosal affects arachidonic or Δ^9-THC binding to the cannabinoid receptor and arachidonic acid release, we examined the effect of 100 μM thimerosal on radioligand receptor binding of [^{3}H]CP 55,940 to brain synaptosomal membranes. We found that the [^{3}H]CP 55,940 binding was decreased by less than 5% at this concentration of thimerosal.

When arachidonic acid-labeled astrocytes were treated with increasing concentrations of anandamide or Δ^9-THC, a 3- to 4-fold stimulation of arachidonic acid release, over the control, was observed (Fig. 2). It should be noted that at higher concentrations (>2 μM) anandamide and Δ^9-THC released equal amounts of arachidonic acid from astrocytes.

Amidohydrolase Metabolism of Anandamide

We next examined whether [^{3}H]anandamide is degraded by astrocyte amidohydrolase activity. After a 20-min incubation of [^{3}H]anandamide with astrocytes, in the presence or absence

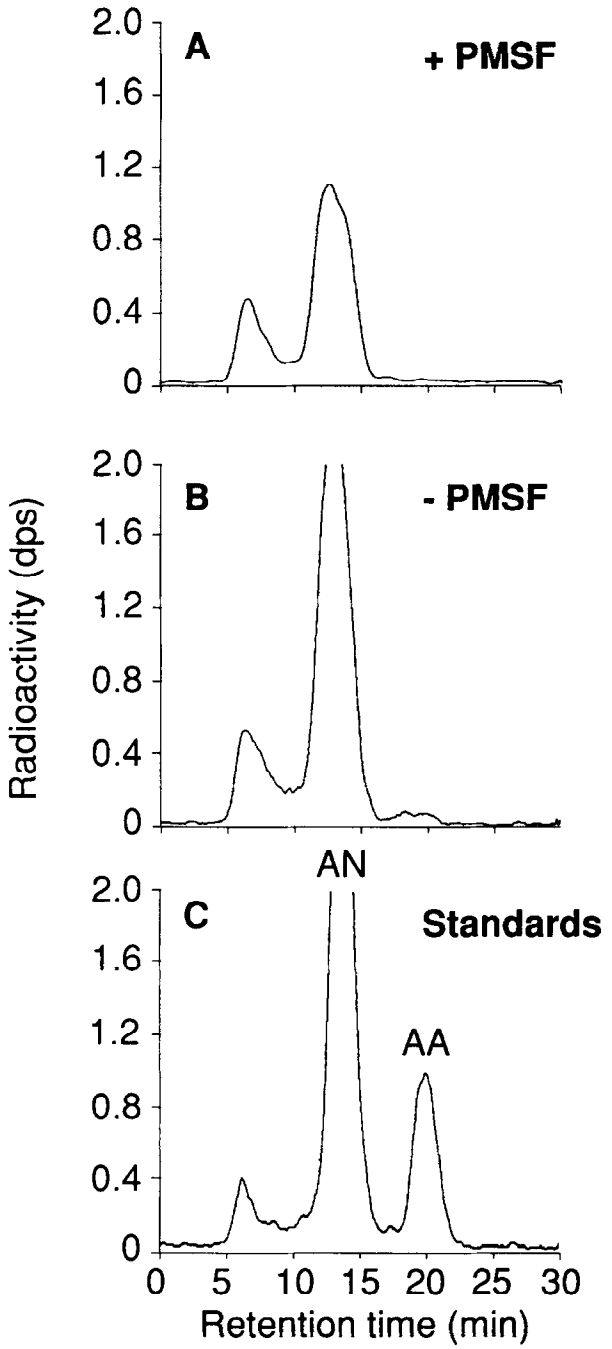

FIG. 3. RP-HPLC elution profiles of [^{3}H]anandamide recovered in the medium after incubation with astrocytes. Confluent astrocyte monolayer cultures were incubated for 20 min with 1 μM anandamide ([arachidonyl 5,6,8,9,11,12,14,15-^{3}H], 0.25 μCi/mL) in the presence (panel A) or in the absence (panel B) of PMSF in serum-free DMEM. After incubation, the medium radioactivity was extracted and analyzed by RP-HPLC as described in Materials and Methods. Panel C shows the elution profiles of authentic standards of radioactive anandamide and arachidonic acid.

of PMSF, more than 90% of the added radioactivity was recovered in the medium and less than 10% was recovered in the cells; the latter was not analyzed further in this study. The medium radioactivity was eluted as a single major peak at the RP-HPLC retention time of authentic standard anandamide (Fig. 3, A and B), indicating that under the experimental condition [^{3}H]anandamide was not metabolized to [^{3}H]arachidonic acid. To determine whether the lack of anandamide hydrolysis in cultured astrocytes is due to the absence of amidohydrolase activity *per se*, we assayed the amidohydrolase activity in the homogenate of cortical astrocytes and compared it with that of other tissues where amidohydrolase activity has been reported previously [21]. Our results show that formation of arachidonic acid was in the order liver >> brain hippocampus > brain cortex > cortical astrocytes (Table 1).

Effect of Pertussis Toxin on Arachidonic Acid Release

To determine whether anandamide- or Δ^9-THC-induced arachidonic acid release in astrocytes is receptor/G-protein mediated, we studied the effect of pertussis toxin. Exposure of cells to pertussis toxin (100 ng/mL) during the last 2-hr incubation period with [^{3}H]arachidonic acid did not alter the cellular uptake of [^{3}H]arachidonic acid. Furthermore, pretreatment of cells for 20 min with pertussis toxin (100 ng/mL) did not stimulate any release of radioactivity (Fig. 4). Our results show that pertussis toxin inhibited >90% of the anandamide-induced release of radioactivity, indicating a receptor/G-protein involvement. Similar results were obtained in the case of Δ^9-THC, and the inhibition was >50%.

Effect of the Cannabinoid Receptor Antagonist SR141716A on Arachidonic Acid Release

To determine whether the receptor involved in anandamide- or Δ^9-THC-induced arachidonic acid release is a cannabinoid receptor, we pretreated astrocytes with SR141716A. As shown in Fig. 5, anandamide-induced arachidonic acid release was blocked by SR141716A, which was statistically significant ($P < 0.05$) at higher concentrations. Similar results were obtained with Δ^9-THC, indicating that the arachidonic acid release response may be cannabinoid receptor mediated. Incubation of astrocytes for 20 min with SR141716A (10 μM) did not stimulate any arachidonic acid release nor did it cause any cell toxicity as determined by trypan blue dye uptake.

DISCUSSION

Our results are consistent with a recent study suggesting that anandamide-induced arachidonic acid release is a receptor/G-protein-mediated cellular response [15]. However, it remains unclear whether the cannabinoid receptor mediates this response. In the present study, we report that anandamide- and Δ^9-THC-induced arachidonic acid release from primary cultures of rat brain cortical astrocytes and SR141716A blocks this response. In our study, we could not detect a net release of arachidonic acid when astrocytes were stimulated with 1 μM anandamide or Δ^9-THC in the absence of thimerosal, and we believe that it may be due to rapid reincorporation of released arachidonic acid into phospholipids [23]. In our study, the arachidonic acid release was detectable even at concentrations as low as 1 μM anandamide and Δ^9-THC in the presence of thimerosal. However, higher concentrations of Δ^9-THC have been shown to release arachidonic acid in the absence of acylating enzyme inhibitors in other cell types [24]. Unlike the other sulfhydryl compounds that have been shown previously [25] to interfere with the cannabinoid receptor binding, the presence of thimerosal, in our hands, did not affect the radioligand binding of [^{3}H]CP 55,940.

It is intriguing to note that anandamide-induced arachidonic acid release response in astrocytes was equal in magnitude to the release response evoked by equimolar concentrations of Δ^9-THC. Generally, anandamide-evoked *in vivo* cannabimimetic responses are shorter in duration and lower in magnitude than those evoked by cannabinoids [10]. The milder response of anandamide, however, is believed to be due

TABLE 1. Amidohydrolase hydrolysis of [^{3}H]anandamide to [^{3}H]arachidonic acid by various tissue homogenates in the presence or absence of PMSF

Sample	Total recovered cpm	% Anandamide remaining	% Arachidonate formed
Buffer control	33,510 ± 1,740	96 ± 8.5	4 ± 0.3
Liver	40,770 ± 1,270	23 ± 3.9	77 ± 0.8
Liver + PMSF	39,320 ± 810	97 ± 2.5	3 ± 0.3
Hippocampus	34,620 ± 470	69 ± 1.1	31 ± 0.3
Hippocampus + PMSF	37,310 ± 220	98 ± 0.8	2 ± 0.2
Brain cortex*	35,400	79	21
Brain cortex + PMSF*	36,800	97	3
Cortical astrocytes	37,910 ± 1,850	94 ± 4.2	6 ± 1.1
Cortical astrocytes + PMSF	38,340 ± 780	98 ± 1.8	2 ± 0.3

Rat liver, brain cortex, hippocampus, or primary cultures of cortical astrocytes were homogenized in 100 mM Tris–HCl, 0.2 mM EDTA and 1.15% KCl buffer, pH 7.5. The homogenates (1 mg protein/mL) were preincubated for 10 min with or without PMSF (1.0 mM) and then incubated for 20 min with 10 μM [^{3}M]anandamide (0.2 μCi/mL) as described in Materials and Methods. Buffer controls were supplemented with 1 mg/mL BSA. Values are means ± SEM for two independent experiments done in duplicate unless otherwise indicated. * Values are means of duplicates of a single experiment.

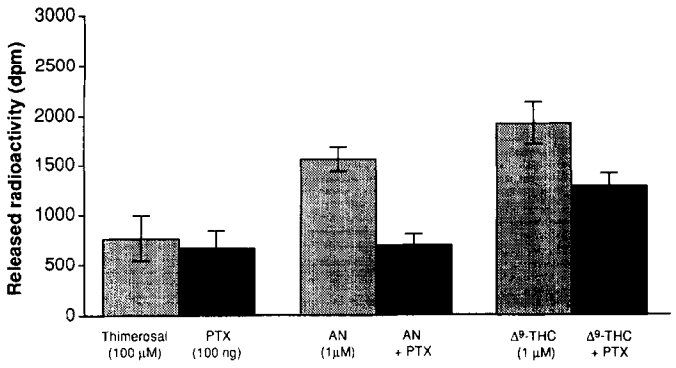

FIG. 4. Effect of pertussis toxin (PTX) on anandamide- or Δ^9-THC-induced arachidonic acid release. Astrocyte monolayer cultures were treated with PTX (100 ng/mL) during the last 2-hr period of labeling with [^{3}H]arachidonic acid. The washed cells were preincubated with thimerosal or PTX for 10 min and then stimulated with either Δ^9-THC or anandamide for 20 min. Controls received only thimerosal or PTX. Values are means ± SEM for triplicates of two experiments.

to its degradation by amidohydrolase to arachidonic acid and ethanolamine [16, 26]. In our study, the inability of astrocytes to metabolize radiolabeled anandamide suggests that astrocytes may lack amidohydrolase activity. The lack of degradation of anandamide may have facilitated the action of anandamide to evoke arachidonic acid release with a potency equal to that of Δ^9-THC in astrocytes. In astrocytes, anandamide-induced arachidonic acid release is comparable to that evoked by an equivalent concentration of anandamide in fetal lung fibroblasts [15]. However, it is not known whether anandamide is metabolized to arachidonic acid and ethanolamine in the latter cell type.

Furthermore, the direct measurement of amidohydrolase activity in astrocyte homogenate clearly suggests that these cells contain low or negligible activity when compared with the activity in the homogenates of liver, brain hippocampus, or brain cortical tissues. Our results are consistent with a previous report [21] showing maximum amidohydrolase activity in the liver followed by the brain. In the brain, the globus pallidus is shown to contain the highest amidohydrolase activity followed by the hippocampus; the brain cortex contains only moderate activity. Therefore, the lower amidohydrolase activity observed in the brain cortical astrocytes may be due to the regional heterogeneity in the brain. To test this speculation, one may have to culture astrocytes from different brain regions, such as hippocampus, that contains higher amidohydrolase activity. From our study, it is not clear whether cortical astrocytes lack amidohydrolase activity *per se* or whether these cells simply lost their amidohydrolase activity under the culture conditions. However, glioma cells and neuroblastoma cells

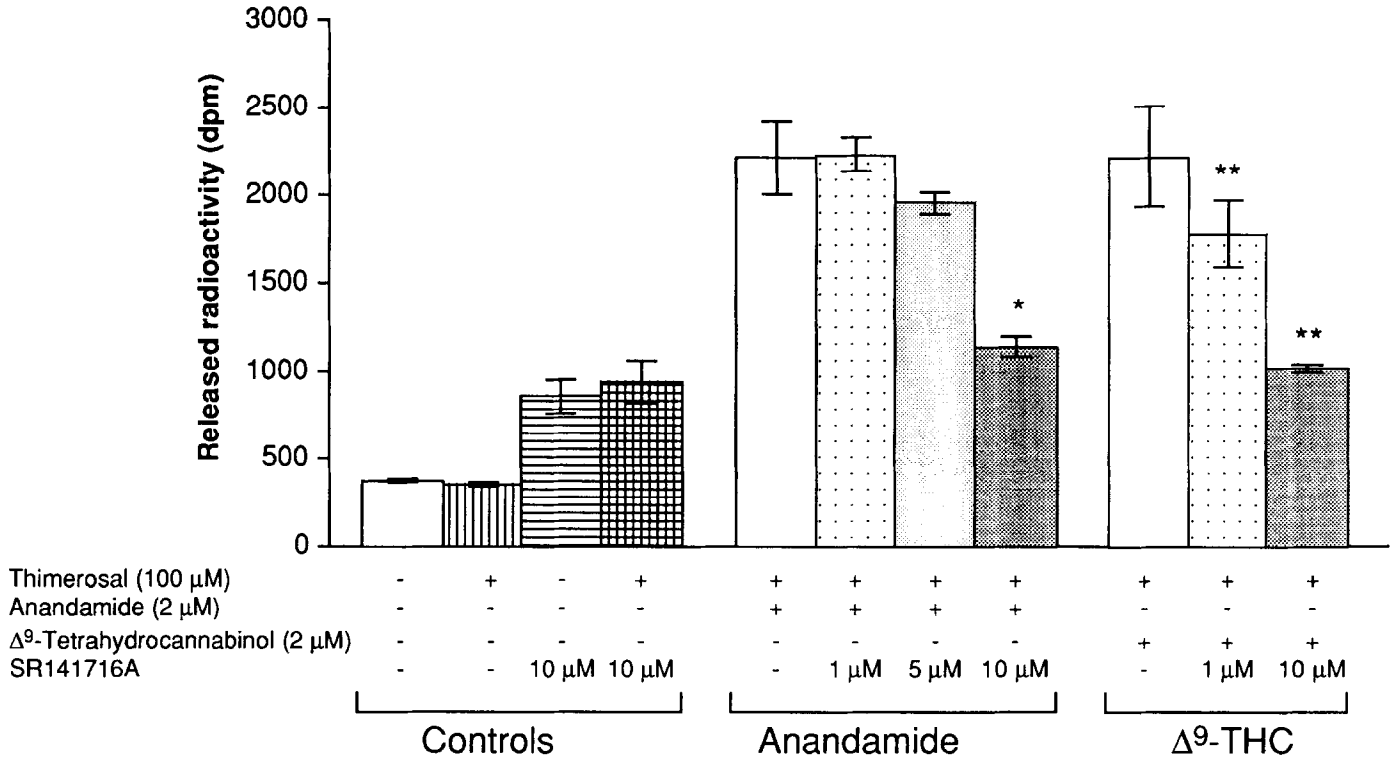

FIG. 5. Effect of the cannabinoid receptor antagonist SR141716A on anandamide- and Δ^9-THC-induced arachidonic acid release. [^{3}H]Arachidonic acid-labeled astrocyte monolayer cultures were preincubated for 10 min with thimerosal and various concentrations of SR141716A at 37°. Cells were then stimulated with 2 μM anandamide or Δ^9-THC in a fresh medium containing thimerosal with or without SR141716A. After a 20-min incubation, aliquots of the medium were counted for radioactivity. Controls received either ethanol (10 μM), thimerosal (100 μM) or SR141716A (10 μM). Values are means ± SEM, N = 3 independent experiments. Key: (*) $P < 0.05$ vs anandamide control, and (**) $P < 0.05$ vs Δ^9-THC control as determined by Fisher's PLSD post-hoc analysis for pairwise comparisons between treatment groups.

rapidly hydrolyze anandamide to arachidonic acid and ethanolamine [16]. Furthermore, a recent study claims that brain cortical astrocytes metabolize anandamide as rapidly as cortical neuronal cells [27]. Therefore, it is not clear whether the observed discrepancies are due to the presence of other contaminating glial cells or to differences in culture conditions used in different laboratories to grow these cells.

Our results with SR141716A suggest that astrocytes may contain the CB1, which is shown to be localized in the brain [28]. This conclusion is based on a recent report on the localization of CB1 receptor subtype in an astrocytoma cell line and in the primary cultures of astrocytes [29]. However, indirect evidence from previous studies [30–32], using radioligand binding and *in situ* hybridization histochemistry, suggest that the brain cannabinoid receptors are distributed mainly in neurons of various brain regions. Nevertheless, distribution of cannabinoid receptors in astrocytes may have profound functional significance in the brain, since astrocytes play active roles in regulating both neuronal and vascular cell function [33]. Further characterization of cannabinoid receptor signal transduction pathways in astrocytes needs to be done to understand the role of cannabinoid receptors in astrocyte functions. Our study is the first to establish a link between the cannabinoid receptors in astrocyte signaling to release arachidonic acid, the substrate for various eicosanoids that may affect neuronal as well as cerebrovascular activity.

Our previous studies show that astrocytes are capable of metabolizing exogenous arachidonic acid via cyclooxygenase, lipoxygenase, and cytochrome P450 epoxygenase pathways [18]. Among the epoxygenase products, 14,15-epoxyeicosatrienoic acid (14,15-EET) is a major product, and interaction of this eicosanoid with glycerolipids and/or protein kinase C may lead to functional consequences within astrocytes [20]. Anandamide, being capable of stimulating arachidonic acid release, may be a natural stimulant for the production of EETs and other second messengers that may modulate astrocyte function via protein kinase C. It is not clear from our study whether anandamide- and THC-induced arachidonic acid release is via activation of the MAP kinase signal transduction pathway as described in other cell types [15]. Further studies are needed to identify the generation of eicosanoid second messengers in response to anandamide or Δ^9-THC stimulation of astrocytes. Although no direct evidence for the occurrence of anandamide as a cellular constituent in astrocytes is documented, the recent identification of a novel class of anandamide-containing ethanolamine phospholipids in cultured neurons [27] leads to further speculation that anandamide, released from neuronal cells, may stimulate arachidonic acid release in astrocytes. Therefore, anandamide may play a neurotransmitter role in neuronal-glial signaling in the brain.

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